学位論文
Doctor's Thesis

Functional Analysis of Ephrin-B2/EphB4 in Angiogenesis
(Ephrin-B2/EphB4 シグナルによる血管新生の制御)

Zhang Xiu-Qin
張　秀琴

指　導　教　官
須田　年生　教授
熊本大学大学院医学研究科・造血発生学

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著者名：張　秀琴

Xiu-Qin Zhang

指導教官: 熊本大学大学院医学研究科・造血発生分野教授　須田年生

審査員名：１）熊本大学発生医学研究センター・細胞識別分野教授　栗原祐基

２）熊本大学医学研究科・神経分化学講座教授　田中英明

３）熊本大学医学部・循環器内科学講座教授　小川久雄

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Xiu-Qin Zhang

Supervisor: Toshio Suda
Professor
Department of Cell Differentiation, IMEG
Kumamoto University

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-Contents-

1.要旨

レセプター型チロシンキナーゼのファミリーである Eph レセプターおよびそのリガンドである ephrin は脊椎動物と無脊椎動物において多くの発生過程を調節している。Ephs と ephrins は血管の発生を調節し、神経軸索、神経堤細胞を適切な領域に誘導し、脳や体節のパターンを決定する作用を有する。また異なる細胞区画同士間で細胞の境界を明確にする。今まで、14 個の Eph レセプターと 8 個の ephrin リガンドが同定されている。この中で、ephrin-B2 は膜結合型蛋白で動脈の血管内皮細胞に発現し、その特異的レセプターである EphB4 は静脈の血管内皮細胞に発現することが示されている。両者ともに脈管形成と血管新生に重要な役割を果たしている。さらに、このレセプター、リガンドの相補的な両方向へのシグナルを介して動静脈血管の間の境界を維持すると考えられている。しかし、これらの分子の脈管形成と血管新生における役割はまだ明確にされていない。このような問題を解明するために、私は血管構築の過程である脈管形成と血管新生の両者が解析可能な培養系を用い検討した。ヘマンジオブラストが存在すると考えられている傍大動脈臓側中胚葉 (P-Sp)をストローマ細胞上で培養した。また、生体内における分子の発現の解析も行った。この in vitro の培養系を用いて、ストローマ細胞上に発現している ephrin-B2 が P-Sp からの血管網の形成と ephrin-B2 陽性の内皮細胞の増殖、またα-smooth muscle actin (α-SMA)陽性細胞のリクルートメントと増殖を促進することを明らかにした。ストローマ細胞上に発現している EphB4 は P-Sp からの血管網の形成と ephrin-B2 陽性の内皮細胞の増殖、またα-smooth muscle actin (α-SMA)陽性細胞の

リクルートメントと増殖を抑制することも示した。Ephrin-B2 遺伝子を LacZ 遺伝子に置換したヘテロマウスの胎児と成体マウスを用いた LacZ 染色と免疫染色により、ephrin-B2 が動脈の内皮細胞だけではなくて、動脈の平滑筋にも発現していることが分かった。以上の結果から、ephrin-B2 と EphB4 が動脈と静脈の内皮細胞、また内皮と内皮の周囲細胞の相互反応を調節して、各自の特徴を持つ動脈と静脈の形成に重要な役割を果たしていることが示唆された。

1. Summary

Tyrosine kinase receptor Ephs and their ligand ephrins mediate numerous developmental processes in both invertebrates and vertebrate. These processes include the developmental vascular assembly, direct axonal guidance, bundling in the developing brain, control cell migration and adhesion, and help patterning the embryo. The Eph family of receptor tyrosine kinases (RTKs) has 14 distinct members, and 9 ephrin ligands. Among those receptors and ligands, the ephrin-B2 is a transmembrane ligand that is specifically expressed on arterial endothelial cells (ECs) and surrounding cells, and interacts with multiple EphB class receptors. Conversely, EphB4, a specific receptor for ephrin-B2 is expressed on venous endothelial cells, and both ephrin-B2 and EphB4 plays essential roles in vascular development. The bi-directional signals between EphB4 and ephrin-B2 are thought to be specific for the interaction between arteries and veins, to regulate cell mixing and the making of particular boundaries. But the molecular mechanism of EphB4 and ephrin-B2 signals during vasculogenesis and angiogenesis remain unclear. To address this issue, I performed manipulative functional studies on these proteins in an *in vitro* stromal cell (OP9 cells) and a P-Sp (para-aortic splanchnopleura) co-culture endothelial cell assay system and *in vivo* expression analysis. Using this *in vitro* co-culture system, I found that the stromal cells expressing ephrin-B2 promoted vascular network formation and ephrin-B2-positive (ephrin-B2$^+$) endothelial cell proliferation, and that they also induced the recruitment and proliferation of α-smooth muscle actin (α-SMA)-positive cells from P-Sp explants. Stromal cells expressing EphB4 inhibited vascular network formation, ephrin-B2$^+$ EC proliferation and α-SMA-positive cell recruitment and proliferation from P-Sp explants. Also *in vivo* LacZ and immumohistochemical staining using ephrin-B2$^{LacZ/+}$

heterozygous mutant embryos and adult mice (ephrin-B2$^{LacZ/+}$ mice in which β-galactosidase (LacZ) expression is under the transcriptional control of an ephrin-B2 promoter) indicated that the ephrin-B2 was expressed not only on the arterial ECs, but also on arterial smooth muscle cells (SMCs). Thus, these data suggest that ephrin-B2 and EphB4 mediate reciprocal interactions between arterial and venous ECs and surrounding cells to form each characteristic vessel.

2. List of publications

1. 尾池雄一、張　秀琴、須田年生。Eph/ephrinシグナルと脈管形成・血管新生。治療学　34 (4): 23-28, 2000.

2. Zhang X-Q, Takakura N, Oike Y, Inada T, Gale N, Yancopoulos G, Suda T: Stromal cells expressing ephrin-B2 promote the growth and sprouting of ephrin-B2+ endothelial cells. Blood 98(4): 1028-1037, 2001.

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4. Abbreviation

RTKs: Receptor tyrosine kinases

EC: Endothelial cells

P-Sp: Para-aortic splanchnopleura

α-SMA: α-Smooth muscle actin

SMC: Smooth muscle cell

VEGF: Vascular endothelial cell growth factors

NP-1: Neuropilin-1

Ang1: Angiopoietin-1

Ang2: Angiopoietin-2

PDGF: Platelet-derived growth factor

TGF-β: Transforming growth factor-beta

GPI: Glycosyl phosphatidylinositol

ACE: Adrenal-cortex-derived microvascular endothelial

α-MEM: α-Modified minimum essential media

FCS: Fetal calf serum

GFP: Green fluorescent protein

LacZ: β-Galactosidase

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

BSA: Bovine serum albumin

2ME: 2-Mercaptoethanol

SCF: Stem cell factor

Epo: Erythropoietin

PCR: Polymerase chain reaction

EGF: Epidermal growth factor

ET-1: Endothelin-1

HBEGF: Heparin binding EGF-like factor

PECAM-1: Platelet endothelial cell adhesion molecule-1

MoAb: Monoclonal antibody

FACS: Fluorescence-active cell sorting

RT-PCR: Reverse transcription-polymerase chain reaction

5. Introduction and Purpose

The regulation of blood vessel formation and growth is currently an exciting and important area in the study of developmental and vascular biology, and in investigations of a variety of pathologies, particularly in diabetic retinopathy and tumor growth. During embryogenesis, the cardiovascular system is the first organ system to form on about 8 days of gestation as the heart begin to beat, vascular development consists of two processes, vasculogenesis and angiogenesis (Introductions Fig 1). Vasculogenesis, whereby individual endothelial cell (EC) precursors called angioblasts that derived from mesoderm differentiate and proliferate to form the initial vascular plexus (Risau 1995; Hanahan 1997; Risau 1997), tissues that are vascularized by this process are generally of endodermal origin and include lung, pancreas, and spleen as well as the heart tube and dorsal aorta (Pardanaud et al. 1989). And angiogenesis, where the initial vascular plexus forms mature vessels by sprouting, branching, pruning, differential growth of EC, and recruitment of supporting cells, such as pericytes for small capillaries, and smooth muscle cells (SMCs) for larger vessels, myocardial cells for the heart (Risau 1995; Folkman and D'Amore 1996; Risau 1997). Tissue of ectodermal and mesodermal derivation such as brain and kidney are thought to be vascularized primarily via angiogenesis. Information regarding the regulation of angiogenesis and vasculogenesis was largely descriptive and came from classic embryologic studies. In the past several years, identification of a number of defined molecules, in combination with the ability to genetically manipulate the mouse genome, has begun to provide insight into the molecular mechanisms that regulate vessel development. To date, three growth factor systems, vascular endothelial cell growth factor (VEGF), angiopoietins (Ang) and ephrins, have been identified as critical factors for angiogenesis (Risau 1995; Hanahan

1996; Folkman and D'Amore 1996; Beck and D'Amore 1997; Gale and Yancopoulos 1999; Robinson and Stringer 2001) (Introductions Fig 2).

5.1 The VEGFs and their receptor family

Vascular endothelial cell growth factor (VEGF) had been characterized as a heparin binding angiogenic growth factor displaying highly specificity for ECs (Gospodarowicz et al. 1989; Ferrara et al. 1989). Five VEGF isoforms (VEGF-A through VEGF-D) are generated from a single VEGF gene as a result of alternative mRNA splicing, which differ in their expression patterns, biochemical and biological properties such as their ability to bind cell-surface heparin-sulfate proteoglycans. The expression of VEGF is responsive to hypoxia, activated oncogenes, and a variety of cytokines (Brogi et al. 1994; Pertovaara et al. 1994). VEGF induces EC proliferation, promotes cell migration, and inhibits apoptosis by binding to their receptors VEGFR-2/Flk-1, VEGFR-1/Flt-1, VEGFR-3/Flt-4 and co-receptor neuropilin-1 (NP-1). These VEGF receptors are characterized by the presence of seven immunoglobulin (Ig)-like domains in their extracellular parts and a split tyrosine-kinase domain in their intracellular part, and therefore be regarded as a new subfamily of tyrosine-kinase receptors (Shibuya et al. 1990; Devries et al. 1992; Pajusola et al. 1992; Terman et al. 1992; Soker et al. 1998).

VEGF, particularly the $VEGF_{165}$ isoform is a potent mitogen and chemoattractant for EC (Keyt et al. 1996). Disruption of gene for VEGF revealed embryonic lethality even in the heterozygous state (Carmeliet et al. 1996; Ferrrara et al. 1996). The heterozygous mutant embryos die in utero between E11 and E12, and are characterized by defects in most of the development of the cardiovascular system. These observations suggest that the VEGF exerts dose-dependent effects during the cardiovascular system development.

This dose-dependence was also suggested by studies of over-expression of VEGF. Microinjection of $VEGF_{165}$ into quail embryos led to the formation of a hyperfused network of vessels and the development of vessels in areas that are usually avascular (Drake and Little 1995). Inhibition of VEGF activity by neutralizing antibodies or by introduction of dominant negative VEGF receptors into ECs of tumor-associated blood vessels resulted in the inhibition of tumor growth and in tumor regression (Kim et al. 1993; Millauer et al. 1994). Likewise, disruption of the genes encoding VEGF receptors Flk-1 and Flt-1 results in severe abnormalities of blood vessel formation in homozygous animal (Shalaby et al. 1995; Fong et al. 1995; Ferrara et al. 1996; Carmeliet et al. 1996; Shalaby et al. 1997). Flk-1 is expressed primarily by the endothelium, and it is required for the differentiation of ECs and for the movement of primitive precursors of ECs from the posterior primitive streak to the yolk sac, as a precondition for the subsequent formation of blood vessels (Shalaby et al. 1997). Flk-1-deficient mice die in utero between embryonic day (E) 8.5 and E9.5 due to lack of both EC and a developing hematopoietic system (Shalaby et al. 1995). The vascular network formation in the dorsal aorta, yolk sac and other smaller vessels are all absent, indicating the absolute requirement for Flk-1 in the growth and establishment of the endothelial lineage. Flt-1 null mice also display embryonic lethality between E8.5 and E9.5 (Fong et al. 1995). These embryos have normal hematopoietic progenitors and abundant EC. The defect appears to lie more in the organization of EC into tube-like structure than in the differentiation of the endothelium itself. Activation of the Flt-1 promotes cell migration but dose not seem to induce cell proliferation efficiently (Seetharam et al. 1995; Barleon et al. 1996). Flt-4 expression is restricted largely to lymph vessels and binds VEGF-C and VEGF-D, and contributes to angiogenesis and lymphangiogenesis

(Dumont et al. 1998; Jussila et al. 1998). Targeted disruption of the gene encoding Flt-4 resulted in defective blood vessel development in early mouse embryos. Vasculogenesis and angiogenesis occurred, but large vessels become abnormally organized with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure at E9.5 (Dumont et al. 1998). NP-1 has a wide tissue contribution that includes some tumour-derived cells and ECs. Studies about NP-1 suggested that it also contributes to angiogenesis and vasculogenesis. Target disruption of NP-1 leads embryos to die at E10.5-12.5 due to cardiovascular anomalies (Kitsukawa et al. 1997; Dumont et al. 1998). Overexpression of NP-1 causes mice to die at E17.5 with an excessively high density of blood vessel (Kitsukawa et al. 1995). Altogether, above data suggested that the VEGFs and their receptors plays quite important roles in vascular development.

5.2 Angiopoietins and the Tie receptor family

Angiopoietins and their EC-specific receptors, Ties, have been suggested to be important for vascular remodeling and cardiac development (Dumont et al. 1994; Suri et al. 1996; Vikkula et al. 1996). Tie receptors consist of two members, Tie1 and Tie2. Tie1 mRNA is first detected in the mouse at E8.5 in angioblasts of the head mesenchyme, in EC of the dorsal aorta, and in the blood islands of the yolk sac (Korhonen et al. 1994). Independently generated of Tie1 null embryos die over a variable period, ranging from E14.5 to perinatally, due to hemorrhages and abnormal edema (Sato et al. 1995; Puri et al. 1995), implicating the Tie1 signal in control of fluid exchange across capillaries and in hemodynamic stress resistance. Tie2 mRNA is detected in E8.0 embryos in the endocardium dorsal aorta, maternal decidual blood

vessels, and in the yolk sac vasculature (Sato et al. 1993). Tie2 homozygous mutant embryos were dead by E9.5 to E10.5, and displayed a distinct phenotype from that of the VEGF receptor knockouts. ECs are present in normal numbers and are assembled into tubes, but the vessels are immature, lacking branching networks and proper organization into large and small vessels. Notably, the vessels lack an intimate encapsulation by periendothelial cells. The dorsal aorta was ruptured and disorganized. The heart did not possess organized trabeculae and the endocardium and myocardium do not show tight association (Dumont et al. 1994; Sato, et al. 1995; Vikkula et al. 1996). Thus, Tie2 appears to control the capability of EC to recruit stromal cells to stabilize the structure and modulate the function of blood vessels. Ligands for Tie2 have been identified and named as angiopoietins (Davis et al. 1996).

Angiopoietin was identified as a family of growth factors specific for the endothelium, which include angiopoietin-1 (Ang1), angiopoietin-2 (Ang2), angiopoietin-3 (Ang3) and angiopoietin-4 (Ang4) and all bind to the Tie2 receptor with similar affinity, but it is still unclear as to whether they utilize the closely related receptor Tie1 (Davis et al. 1996; Maisonpierre et al. 1997; Valenzuela et al. 1999). Ang1 is an activating ligand for Tie2, and induces autophosphorylation of Tie2 in cultured EC; Ang2, an antagonist for Ang1, competitively inhibits Ang1-induced kinase activation of the Tie2 (Maisonpierre et al. 1997). Ang1 is first expressed between E9 and E11, and promotes EC sprouting *in vitro*, but could not induce tubule formation in cultured ECs (Davis et al. 1996; Koblizek et al. 1998). The Ang1 null embryos display angiogenic deficits reminiscent of those seen in Tie2 mutations and die at E12.5. The principal defect is a failure to recruit smooth muscle and pericyte precursors. The primary vascular plexus dose not fully remodel into large and small caliber vessels (Suri

et al. 1996). Consistent with *in vitro* results, Ang1 seems to have a later role in vascular development. Another major defect was in the heart where endocardium and trabecular network are very immature. It is characterized by incomplete association of the endothelial layer with the underlying myocardial wall. Transgenic overexpression of Ang1 in the skin leads to striking hypervascularization, presumably by promoting vascular remodeling events and perhaps by decreasing normal vascular pruning (Suri et al. 1998). Transgenic mice overexpressing of the negative ligand Ang2 during embryogenesis, also die with similar vascular defects to $Ang1^{-/-}$ or $Tie2^{-/-}$ mice. (Maisonpierre et al. 1997). Altogether, the defects in mice lacking Ang1 or Tie2, and in the overexpression of Ang2 suggest that this system is critical for normal remodeling, maturation, and stabilization of the developing vasculature.

5.3 Eph receptors and their ephrin ligands

One of the most important events in vascularization is the development of arteries and veins. Generally, the differences between arteries and veins have been defined by functions, anatomy, pressure and blood flow direction. Recent studies have shown that the arterial and venous EC are molecularly distinct from the earliest types of blood vessels. This distinction is revealed by a tyrosine kinase receptor, EphB4, that is dominantly expressed on venous EC, and its obligate membrane-bound ligand, ephrin-B2, is expressed on arterial EC (Wang et al. 1998; Gerety et al. 1999). The Eph receptor and ephrin ligand families mediate numerous developmental processes in both invertebrates and vertebrates (Flanagan and Vanderhaeghen 1998; Holder and Klein 1999). Eph receptor family consists of at least 14 members, and nine ephrin ligands for these receptors have described. They can be grouped into two subclasses based on

structure, sequence homology and binding specificity. Ephrin-A ligands (EphrinA1-A5), which are tethered to the cell surface via a glycosyl phosphatidylinositol (GPI)-anchor and preferentially bind to the EphA receptor subfamily, and ephrinB ligands (Ephrin-B1-B3), which are inserted into the plasma membrane via a transmembrane region along with conserved cytoplasmic tyrosine residues, and bind to the corresponding EphB receptor subfamily (Introductions Fig 3) (Gale et al. 1996a; Bruckner et al. 1997; Brambilla et al. 1999). There are two exceptions that EphA4 binds not only the ephrin-A ligands but several of the ephrin-Bs as well (Gale et al. 1996a; Gale et al. 1996b), and EphB4, selectively binds ephrin-B2 and not other ephrin-B ligands (Brambilla et al. 1995; Sakano et al. 1996). These ephrins are different from ligands for other receptor tyrosine kinases in that they need to be membrane-bound to activate their receptors, but do not function in a soluble form (Davis et al. 1996; Gale and Yancopoulos 1999). Recombinant soluble ligands appear to act as antagonists and can be converted to an agonistic reagent by artificial clustering (Davis et al. 1994; Stein et al. 1998). Notably, signaling between ligands and receptors in the B subfamily appears to be reciprocal, because ephrin-B ligands not only activate their bound receptor, but also in return are activated by their receptors in a neighboring cell (Holland et al. 1996; Bruckner et al. 1997).

A functional analysis of Eph-ephrin signals has been conducted in the circulatory and nervous systems. Targeted disruption of the ephrin-B2 gene leads to embryonic lethality at around E11, due to a defect in both arterial and venous vessel remodeling. This defect was accompanied by a failure of intercalation between the arteries and veins (Wang et al. 1998). Additional defects in the primordial vein suggested that ephrin-B2 is not only required for angiogenesis but also for vasculogenesis (Adams et al. 1999). EphB4

homozygous mutants have a symmetric phenotype with ephrin-B2 homozygous embryos in the cardiovascular system (Gerety et al. 1999). In ephrin-B2 mutant mice, the vessels were no longer following the somitic borders and to form numerous sprouts penetrating into the somites (Adams et al. 1999). These results were confirmed in *Xenopus* embryos. Disruption of interactions between EphB4 on intersomitic vessels and ephrin-B ligands on somites leads to patterning defects which is similar to ephrin-B2 mutant mouse (Helbling et al. 2000). Those reciprocal expression patterns and the functions of EphB4 and ephrin-B2 in vasculo-angiogenesis not only provide the earliest known markers defining arterial and venous endothelium, but suggest that some of bi-directional signaling is occurring between these sets of cells, demonstrating reciprocal functions for the two molecules during angiogenesis and vascular development. Yancopoulos et al supposed that at the early stage of vascular development, the Eph receptor and ephrin ligand are engaged in "cis" configuration, i.e., between neighboring arterial and venous cells, and may act locally to form a boundary that prevent cell mixing. They may also interact in "trans", i.e., arterial cells in one vessel interact with adjacent venous cells, and may prevent large vessels from inappropriately fusion in the absence of small branches, perhaps by providing repulsive cues (Introductions Fig 4) (Yancopoulose et al. 1998). Thus signaling between arteries and veins mediated by EphB4 and ephrin-B2 may be required for proper morphogenesis of the intervening capillary beds and network as well as for interdigitation and differential growth of arterial and venous vessels (Wang et al. 1998; Yancopoulose et al. 1998).

Other Eph receptors and ephrin ligands are also involved in angiogenesis. Ephrin-A1 was identified as the product of an early response gene induced by tumor necrosis factor-α in ECs (Holzman et al. 1990). It is chemoattractive for ECs *in vitro* and

promotes tubule formation of human umbilical vein endothelial cells (HUVECs), and induces sprouting of blood vessels in a cornea pocket assay (Pandey et al. 1995; Daniel et al. 1996). Ephrin-B1 is expressed in both arterial and venous vessel wall cells during embryonic development (Adams et al. 1999; Bruckner et al. 1999). It promotes the formation of EC capillary-like structures, cell attachment, and sprouting angiogenesis *in vitro* (Stien et al. 1998). An *in vitro* sprouting assay also shows that purified ephrinB1-Fc induces a significant increase in the number of sprouts in adrenal-cortex-derived microvascular endothelial (ACE) cells. This activity is completely blocked by EphB1-Fc and EphB2-Fc (Adams et al. 1999). EphB3 and ephrin-B3 are coexpressed with EphB4 in venous EC. EphB3 is also expressed in some arteries, and EphB2 and ephrin-B2 are coexpressed in mesenchymal cells adjacent to EC. But this overlapping of expression is unable to compensate for the lack of ephrin-B2 or EphB4. No vascular defects are found in either EphB2 or EphB3 homozygous mutant mice. Whereas, EphB2 and EphB3 double mutant mice have shown vascular defects only with 30% penetrance, which are similar, but not identical to those in ephrin-B2 mutants (Adams et al. 1999). It has been suggested that the complex cell-to-cell interaction via Eph receptors and ephrin ligands on EC is mainly restricted by EphB4/ephrin-B2, but also involves other Eph receptors and ephrin ligands.

In the nervous system, Eph receptors and ephrin ligands function has been studied in most detail and is best understood. The functional studies have shown that in variety developmental contexts, cell repulsion is a major consequence of signaling between Eph receptors and ephrins in nervous system. Localized expression of class A ephrins in the posterior optic tectum and class B ephrins in the posterior half of each somite is required for repulsion axons from EphA receptor expressing retinal ganglion cells and

EphB receptor expressing trunk motoneurones, respectively (Monschau et al. 1997; Bruckner et al. 1998). Eph and their ligands take a role in the molecule responses for rhombomere boundary formation. In Zebrafish, the EphB4 and ephrin-B2 are expressed in complementary stripes throughout the presumptive hindbrain and the interactions between EphB4a and ephrin-B2a mediate cell sorting and boundary formation in the segmenting caudal hindbrain (Cooke, et al. 2001). Many members of Eph family are expressed in rhombomere-restricted patterns (Flanagan et al. 1998). Disruption of Eph signaling results in embryos showing a loss of the normal segmental restriction of gene expression within the developing hindbrain (Xu et al. 1995). Also mosaic activation of Eph molecules leads to sorting of cells at the rhombomere boundaries (Xu et al. 1999). Eph receptors and ephrin ligands involved in pathfinding and bundling of outgrowing nerve fibers, patterning of hindbrain rhombomeres, and the guidance of migrating neural crest cells (O'Leary et al. 1999). They regulate topographic map formation in the visual system (Cheng et al. 1995; Drescher et al. 1995; Nakamoto et al. 1996; Frisen et al. 1998). The bi-directional activations between Eph receptor and ephrin-B ligands is important for the patterning of the embryonic structure of the brain and somites (Xu et al 1995; Gale et al. 1996a; Holland et al. 1996; Xu et al. 1996; Durbin et al. 1998). It is implicated in the repulsion that guides the migration of cells and growth cones to specific destinations, and maintains the boundaries between cell groups in the neuronal system (Wang and Anderdon 1997; Brennan et al. 1997). Recent studies have revealed that a clear border is formed between the Eph and ephrin-B expressing cell populations. Bi-directional signaling between Eph/ephrin-B restricts the intermingling of adjacent cell populations and maintenance of the boundaries (Xu et al. 1999; Mellitzer et al. 1999).

5.4 Other growth factors and vascular myogenesis

Although ECs are important for vasculo-angiogenesis, they alone can initiate but can not complete the angiogenesis process. Once the primitive EC tubes are formed, a process termed vascular myogenesis is critical for vascular further maturation and development, in which the pericytes and vascular smooth muscle cells (SMCs) are recruited to form vascular wall. They can inhibit EC proliferation and migration and can produce extracellular matrix for stabilize nascent vessels. They also provide hemostatic control and protect new endothelium-lined vessels against rupture or regression (Benjamin et al, 1998).

Vascular SMCs have a complex origin; they can differentiate from mesoderm, transdifferentiate from EC, bone marrow precursors or macrophage, transform from epicardial cells to form coronary arterial SMC, and differentiate from neural crest into vascular SMCs (Rosenquist et al. 1990; DeRuiter et al. 1997; Bergwerff et al. 1998; Hungerford and Little 1999; Vrancken Peeters et al. 1999). The process of vascular SMCs to contribute to vessel wall formation may be divided as differentiation, recruitment, growing and remodeling. Platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β have been reported to be associated with pericyte proliferation and movement (Heimark et al. 1986; Holmgren et al. 1991; Leveen et al. 1994; Dickson et al. 1995).

PDGF consists of two homologous but distinct peptides termed PDGF-A (17 kDa) and PDGF-B (14 kDs) chains and may exist as PDGF-AA, PDGF-BB and PDGF-AB. The receptors, in manner similar to their ligands, consist of dimers of the α and β subtype of the PDGF tyrosine kinase receptor. The α receptor can bind both PDGF

chains, whereas the β receptor is selective for the PDGF-B chain. Therefore, αα receptor dimers can bind all three isoforms PDGF, αβ heterodimers can bind AB or BB, and the ββ receptor binds only PDGF-BB. In vitro studies have suggested that cultured vascular SMCs autocrine products PDGF could promote vascular SMC proliferation and PDGF expression (Nilsson et al. 1985; Sjolund et al. 1988). Both the PDGF-B and the PDGF β receptor deficient mice reveal similar phenotype (Leveen et al. 1994; Soriano et al. 1994). They die perinatally from hemorrhage and some vascular dilation. Especially there have no pericyte (mesangilal cells) in the kidney vasculature.

The TGF-βs are a large family of homodimeric peptides; three vertebrate TGF-β isoforms have been identified and termed TGF-β1, TGF-β2 and TGF-β3. TGF-β has been shown to inhibition of EC and SMC growth and migration (Heimark et al. 1986; Sato et al. 1989; Battegay et al. 1990). TGF-β1 and endoglin (an endothelial TGF-β binding protein) were also shown to stimulate vascular SMC differentiation, extracellular matrix deposition and strengthen EC-SMC interactions (Dickson et al. 1995; Li et al. 1998). In vitro studies have suggested that TGF-β can promote or inhibit growth of vascular SMC. At low concentration of TGF-β could increase expression of PDGF-A and PDGFβ-receptor to promote vascular SMC growth. At higher concentrations, TGF-β inhibits growth by the down regulation of PDGF-A and PDGFβ-receptor expression (Gronwald et al. 1989; Battegay et al. 1990; Janat et al. 1992). TGF-β mutant mouse analysis revealed that nearly 50% of TGF-β -/- and 25% of TGF-β +/- embryos die in utero and have defective vasculogenesis in the yolk sac and improper interactions between epithelial and mesenchymal cells (Dickson et al. 1995). However, other growth factors such as epidermal growth factor (EGF) act as a

chemoattractants for vascular SMCs. Endothelin (ET)-1 appears to have an important role in migration and differentiation of vascular SMC from neural crest cells (Yanagisawa et al. 1998). Tissue factor and heparin binding EGF-like factor (HBEGF) also play important role in vascular SMC differentiation and growth.

Although the field of vascular development has blossomed in the past decade, the molecules and mechanisms that regulate this developmental pathway are not well defined. Especially the work on Eph/ephrin functions in the vasculature is only just beginning. The molecular mechanism and how such ligands and receptors work for vasculogenesis and angiogenesis remain to be solved. In this study, I focused on the molecular interactions between the EphB4 and ephrin-B2 in angiogenesis, I have generated ephrin-B2 and EphB4 over-expressing stromal cell lines OP9, and co-cultured P-Sp (para-aortic splanchnopleural mesoderm) explants with transfected OP9 cells. This system can support both vasculogenesis and angiogenesis in vitro, and provides us with opportunities to observe the interaction of EC and surrounding cells during vasculogenesis and angiogenesis (Takakura et al. 1998; Hirashima et al. 1999; Takakura et al. 2000).

6. Materials and Methods

6.1 Cell lines and animals

OP9 stromal cell line (a gift from Dr. H. Kodama, Bayer Yakuhin Ltd., Nara, Japan) was established from newborn calvaria of the (C57BL/6xC3H) F2-*op/op* mouse which lacks a functional macrophage colony-stimulating factor (Yoshida et al. 1990; Kodama et al. 1994). The OP9 cells were maintained in α-modified minimum essential media (α-MEM, GIBCO-BRL, Gaitherburg, MD) containing 20% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS (Nakano et al. 1994). Pregnant C57BL/6 mice (purchased from SLC, Shizuoka, Japan), green mice (green fluorescent protein (GFP) was over-expressed under transcriptional control of a CAG promoter, a gift of Dr. Okabe, Osaka Univ. Japan) (Okabe et al. 1997) and ephrin-B2$^{LacZ/+}$ heterozygous mutant mice (ephrin-B2$^{LacZ/+}$ mice in which β-galactosidase (LacZ) expression is under the transcriptional control of an ephrin-B2 promoter) (Gale et al. 2001) were housed in environmentally controlled rooms at a facility of Kumamoto University under the guidelines of Kumamoto University for animal and recombinant DNA experiments.

6.2 Transfections

In the day before transfection, OP9 cells which 80% confluence in a dish were divided into two culture dishes. In the day of transfection, plasmid DNA transfection mixture was prepared according to the Effectene Transfection method manufacturer's instructions. OP9 cells were whashed once with PBS, then transfected with plasmid pCAGneo-ephrinB2 containing full-length ephrinB2 cDNA (a gift from Dr. Tohn G. Flanagan, Harvard Medical School, Boston, MA) (OP9/ephrinB2) or expression vector

pCAGneo (OP9/vector). And expression vector pRK5-EphB4 (a gift from Dr. Axel Ullrich, Max-Planck-Institute für Biochemie, Martinstried, Germany) containing full-length EphB4 cDNA, was co-transfected with expression vector pCAGneo (OP9/EphB4) into OP9 cells by the Effectene Transfection method (Qiagen GmbH, Hilden, Germany). The selection medium α-MEM/20% FCS containing 500μg/ml of geneticin disulfate (G418, Life Technologies, Inc., Grand Island, NY) was added to the transfected OP9 stromal cells after 48 hours of transfection for select the EphB4 or ephrin-B2 overexpression clones. The protein expression of selected clones was checked by western blot analysis (Results Figs 1A and 2A). We selected 3 clones each showing high levels of protein expression for further use.

6.3 Western blot analysis

OP9, OP9/ephrin-B2, OP9/EphB4 and OP9/vector cells were lysed with 1% Triton lysis buffer (50 mM/L Hepes [pH7.4], 1% Triton X-100, 10% glycerol, 10 mM/L sodium pyrophosphate, 100 mM/L sodium fluoride, 4 mM/L EDTA, 2 mM/L sodium orthovanadate, 50 μg/ml aprotinin, 1 mM/L phenylmethylsulfonyl fluoride, 100 μM/L leupeptin, and 25 μM/L pepstatin A) and incubated at 4°C for 30 min. Cell lysates were then clarified by centrifugation for 15 min at 4°C, then collected the supernatant as protein lysates for western blot analysis. After measuring the proteins contain, an equal amount of each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred to a polyvinylidene diflouride membrane (PVDF; Millipore, Yonezawa, Japan). After blocking of the residual binding sites by incubation of the membrane with 5% bovine serum albumin (BSA, Sigma., St. Louis, MO) in PBS-T (0.1% triton X-100 in phosphate buffered saline) for 1 hour at

room temperature, the primary antibody, rabbit anti-Lerk-2 PolyAb (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-EphB4 PolyAb (1:500, made in our laboratory) in 3% BSA/PBS-T were incubate with the membrane for 1 hour at room temperature. After wash the membrane with PBS-T for four times, the HRP-conjugated anti-rabbit secondary antibody in 3% BSA/PBS-T were incubated with the membrane for 1 hour at room temperature, after more four times washing, the membrane were treated for exposure by using a chemiluminescence detection system (Amersham, Arlington Heights, IL), according to the manufacturer's instructions.

6.4 *In vitro* co-culture of para-aortic splanchnopleura (P-Sp) explants or single cell suspensions on stromal cells

The method of P-Sp dissection and P-Sp/stromal cell co-culture system were described previously (Methods Figs 1 and 2).(Takakura et al. 2000). Embryos were dissected from pregnant females C57BL/6 mice at 9.0-9.5 days post coitum (dpc) (E9.5). Ephrin-B2$^{LacZ/+}$ embryos were generated from crosses of ephrin-B2$^{LacZ/+}$ and wild type mouse. By convention, the morning the vaginal plug was detected was defined as embryonic day 0.5 (E0.5). Mouse genotyping for ephrin-B2 was confirmed by LacZ staining in yolk sacs and RT-PCR using a LacZ-specific primer. Then the wild type or ephrin-B2$^{LacZ/+}$ P-Sp explants were dissected and cultured on confluents OP9/ephrin-B2, OP9/EphB4 or OP9/vector stromal cells that prepared in the day before co-culture in RPMI1640 (GIBCO-BRL, Gaithersburg, MD) containing 10% FCS and 10^{-5} M 2-mercaptoethanol (2ME, Sigma, St. Louis, MO), and supplemented with IL-6 (20 ng/ml), IL-7 (20 U/ml) (a gift from Dr. T. Sudo, Toray Industries Inc., Kamakura, Japan), stem cell factor (SCF, 50 ng/ml, a gift from Chemo-Sero-Therapeutic Co., Ltd., Kumamoto,

Japan), and erythropoietin (Epo, 2U/ml, a gift from Snow-Brand Milk Product Co, Tochigi, Japan) in 12-well plates, then the plate was incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 to 14 days.

For single cell suspension culture, after isolating the E9.0-9.5 P-Sp regions, I prepared single cell suspensions by incubating the tissue with 2.4 U/ml DISPASE (GIBCO, Grand Island, NY) and passing the tissues through a 23-gauge needle for 10 times. After wash the prepared cell suspensions with RPMI1640/10% FCS twice, the single cells were co-cultured with OP9/ephrin-B2 or OP9/vector stromal cells in RPMI1640 medium containing 10% FCS, 10^{-5} M 2ME, 5 ng/ml recombinant vascular endothelial cell growth factor (VEGF, Pepro Tech EC Ltd., London, England) and 50 ng/ml SCF for 7 to 14 days in 12-well plates at 37°C in a humidified 5% CO_2 atmosphere incubator.

6.5 Immunohistochemistry of culture plates

Vascular formation from P-Sp explants was analyzed on OP9/ephrin-B2, OP9/EphB4 and OP9/vector feeder layers and anti-platelet endothelial cell adhesion molecule-1 monoclonal antibody (PECAM-1 MoAb) immunohistochemical staining. PECAM-1, also known as CD31, is a transmembrane molecule expressed on EC, and therefore a good marker for identification of capillary vessels. (Albelda et al. 1991). After co-culturing the P-Sp with OP9/ephrin-B2, OP9/EphB4 or OP9/vector stromal cells for 4, 7, 10, and 14 days, immunohistochemistry was performed as described (Takakura et al. 1997 and 1998). In brief, the cultures were fixed in situ with 4% paraformaldehyde in PBS for 10 min at 4°C, washed twice with PBS, and incubated with 0.3% H_2O_2 in PBS at room temperature for 30 min to block endogenous peroxidase activity. To block

nonspecific reactions, cultures were incubated with 1% normal goat serum and 0.2% bovine serum albumin (BSA, Sigma) in PBS-MT (PBS-T containing 2% skin milk) at room temperature for 30 min. Then the fixed dishes were incubated with PECAM-1 monoclonal antibody (1 μg/ml) (MoAb, MEC13.3, rat anti-mouse monoclonal, Pharmingen, San Diego, CA) in PBS-MT canting 1% normal goat serum and 0.2% BSA for overnight at 4°C, washed with PBS-MT 3 times, and incubated with peroxidase-conjugated anti-rat IgG (1:100) (BioSource, Camarillo, CA) in PBS-MT for 1 hour at room temperature. After three more washes with PBS-MT, the samples were visualized by using the AEC substrate system (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Alternatively, they were soaked in PBS-T containing 300 μg/ml diaminobenzidine (Dojin Chem., Kumamoto, Japan) in the presence of 0.05% $NiCl_2$ for 10-30 min at room temperature, then the hydrogen peroxide was added to about 0.01%, and the color reactions were visualized. To detect the SMC, anti-α-smooth muscle actin (α-SMA) antibody (DAKO, Glostrup, Denmark) was used for immunohistochemical staining in this culture system.

6.6 Single cell suspensions preparing from fetal liver

To obtain single mononuclear cells from E13.5 fetal liver, I dissected pregnant females C57BL/6 mice at 13.5 dpc and the fetal livers were moved from the embryos. After isolating the fetal liver, I prepared single cell suspensions by incubate the fetal liver with 2.4 U/ml DISPASE and passed the tissues through each 18, 21 and 23-gauge needle for 10 times. After wash twice with 10% FCS/RPMI medium, the cells were re-suspended in 3ml FCS and add slowly into a 15 ml Falcon tube containing 4 ml Lymphep™ (NYCOMED PHARME AS, Oslo, Norway) and centrifuge at 1800 rpm at

room temperature for 20 min, then remove the mononuclear cells in the upper layer according to the manufacture's instructions. After wash the obtained mononuclear cells twice with 5% FCS/PBS, the cells were immunofluorescence stained for FACS (fluorescence-active cell sorting) sorting.

6.7 Immunofluorescence staining, endothelial cell (EC) sorting and single EC culture

Single cell suspensions for cell sorting were prepared from the E9.0-9.5 P-Sp and E13.5 fetal liver regions as described above. The cells were first incubated with CD16/CD32 (Fc γ III/II receptor) (Phamingen) for 20 min on ice to block the no specific binding. Then the sample were incubated for 30 min on ice with biotin-anti-Flk-1 MoAb (AVAS12, rat anti-mouse monoclonal antibody, a gift from Dr. S.-I. Nishikawa, Kyoto University, Kyoto, Japan), and washed twice with washing buffer (5% FCS/PBS). Subsequently the cells were incubated with PE-conjugated PECAM-1 antibody (Pharmingen) and PE-conjugated streptavidine (Caltag Laboratories, South San Francisco, CA) for 30 min on ice. Then the cells were washed more than twice and suspended in washing buffer for cell sorting. The stained cells were sorted by FACSvantage (Becton Dickinson Immunocytometry System, San Jose, CA) to obtain Flk-1$^+$PECAM1$^-$ (R2) and Flk-1$^+$PECAM1$^+$(R3) EC populations. Then the sorted R2 and R3 cells were co-cultured with OP9/ephrin-B2, OP9/EphB4 or OP9/vector stromal cells. The cell cultures were performed at 300 cells /well in 24-well plates for E9.5 P-Sp derived cells and 500 cells/well for E13.5 fetal liver derived cells, respectively, in RPMI1640 medium containing 10% FCS, 10^{-5}M 2ME, 5 ng/ml VEGF, and incubate the plates at 37°C in a humidified 5% CO_2 atmosphere incubator for 10 days.

For assessing the single EC precursor development on the OP9 stromal cells, after co-culture the EC with OP9 cells for 10 days, the cultured cells were in situ fixed by 4% paraformaldehyde in PBS for 10 min at 4°C. Then the immunohistochemistry with anti-PECAM-1 MoAb to detect the EC growth was performed as done in the P-Sp explants cultures. Then the two manners of PECAM-1 positive EC structure, sheet-like or cord-like structures were enumerated.

6.8 Reverse transcription-polymerase chain reaction (RT-PCR) analysis

For RT-PCR analysis, total RNA were isolated from whole yolk sac (as a control), R2 and R3 cells sorted from E9.0-9.5 P-Sp regions using the GIAtmp RNaeasy mini kit (Qiagen GmbH, Germany). First-strand cDNAs were generated by subjected the RNA to reverse transcription with an Advantage RT for PCR kit (Clontech). Control reactions without reverse transcriptase were performed for each RNA sample. After reverse transcript the cDNA was amplified in a final volume of 20 μl with an Advantage polymerase mix PCR kit (Clontech Laboratories, Inc., Palo Alto, CA) in a GeneAmp PCR system model 9700 (Perkin-Elmer Inc., Norwalk, CT). The amplification parameters were 94°C for 30 sec, 70°C for 4 min for 25 to 30 cycles, followed by 3 minutes at 70°C for final extension (check the temperature). All PCR reactions included reverse transcriptase negative controls and a blank with no template. To detect the expression of ephrin-B2 and EphB4, the following primers were used: ephrin-B2, 5'-TCTGTGTGGAAGTACTGTTGGGGACTTT-3' (sense), 5'-TGTACCAGCTTCTAGCTCTGGACGTCTT-3' (antisense); EphB4, 5'-CGTCCT-GATGTCACCTATACCTTTGAGG-3' (sense), 5'-GAGTACTCAACTTCCCTCCCA-TTGCTCT-3' (antisense), for amplification.

6.9 Preparation of recombinant fusion protein

To generate cDNA encoding the full-length EphB4 extracellular domain, an expression plasmid vector containing full-length mouse EphB4 cDNA (pRK5-EphB4) was used as a template for polymerase chain reaction (PCR) using the Advantage 2 PCR kit (Clontech). Sense and antisense primers that had Sal I and EcoR I restriction enzyme sites at their 5' ends for reconstruction were designed: 5'-ACGCGTCGACATGGA-GCTCCGAGCGCTGCTG-3' (sense), 5'-CGGAATTCTGCT CCCGCCAGCTCTCGCTCTC -3' (antisense). Amplified cDNA fragments were sequenced (ABI Prism™ 310) to confirm the precise amplification, and the cDNA were subcloned into the expression vector pCAGneo-human-Fc (Fc part of human IgG1, EphB4-Fc). EphB4-Fc or CD4-Fc protein was prepared from COS7 cell supernatant in GIT medium (Wako Pure Chemical Ind., Ltd., Osaka, Japan), as previously described (Yano et al. 1997). In brief, COS7 cell were transfected with expression plasmid DNA pCAGneo-EphB-Fc or pCAGneo-CD4-Fc, then incubated at 37°C in a humidified 5% CO_2 atmosphere. Protein in the conditioned medium of COS7 cell was collected in day 4 and 8 of culture and the Fc fusion protein were purified using protein A Gel (Bio-Rad Laboratories., CA) according to the manufacturer's instructions.

6.10 LacZ staining

For LacZ staining, the ephrin-B2$^{LacZ/+}$ P-Sp cultures were fixed in a solution containing 2% formaldehyde and 0.2% glutaraldehyde in PBS for 10 min at room temperature, and washed with PBS three times, then they were stained at room temperature for 30-60 min in a solution containing 1 mg/ml X-Gal (Nacalai Tesque,

Inc., Kyoto, Japan), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS (X-Gal staining solution). For detect the EC growth from P-Sp explants, the stained plates were re-fixed in 4% formaldehyde for 5 minutes and wash twice with PBS, then the re-immunohistochemical staining with anti-PECAM-1 MoAb was done as described above.

For whole mount LacZ staining of the yolk sac, specimens were removed from E9.5 embryos and fixed in cold 4% paraformaldehyde/PBS for 5-10 min at 4°C, washed twice with PBS and stained with X-Gal staining solution at room temperature for 30-60 min. For *in vivo* LacZ /ephrin-B2 expression analysis, the venae cava and aortas, along with surrounding tissues, were removed from adult ephrin-$B2^{LacZ/+}$ or wild type mice, and the ephrin-$B2^{LacZ/+}$ or wild type embryos were removed at E9.0-9.5. Then the removed samples were fixed in cold 4% paraformaldehyde/PBS for 30-60 min for adult tissues and 5-10 min for embryos, respectively, at 4°C, and washed twice with PBS. The tissues were then stained from 4 hours to overnight at room temperature in a LacZ staining solution. After LacZ staining, the tissues were post-fixed in 4% formaldehyde/PBS for 4 hours at 4°C, then washed with PBS, dehydrated with methanol, embedded in polyester wax, and transverse sectioned of 8μm-thick sections for further staining. The immunohistochemical staining for PECAM-1 and α-SMA in sections were carried out as previously described (Takakura et al. 1997).

6.11 Immunohistochemical staining

Wash the tissue twice with PBS for 30-60 minutes after LacZ staining and re-fixed with 4% paraformaldehyde. Gradient dehydrated with 30%, 50% and 70% methanol in PBS for 10-30 min, and 100% methanol for10-30 min according to the size of tissues.

Then the samples were transferred to 50% wax in ethanol for 30-60 minutes then move the sample to 100% wax for 2-3 hours in 40 °C incubator and embedded in 100% wax. After the wax was extensive solidified in 4°C. the samples were transverse sectioned at 8 μm-thick and the sections were air dried in room temperature for 1 hour, then move the sample to 4°C for overnight for further drying.

For section staining, the air dried sections were put in 100% ethanol for 3 minutes to de-wax, then move the sections to 100% methanol containing 0.15% azide and 0.5% H_2O_2 for 20min at room temperature to inactive the endogenous peroxidase activity. Then the sections were move to 70% ethanol for 3 min and PBS 10 minutes to re-hydrate them. The sections were then incubated with 5% normal goat serum and 1% bovine serum albumin in PBS (blocking solution) at room temperature for 30 min to block nonspecific activity. Then the sections were incubated with the primary antibody anti-PECAM-1 monoclonal antibody for overnight at 4°C in blocking solution. After 3 time washes with PBS-T, the sections were incubated with secondary antibody peroxidase-conjugated goat anti-rat IgG for 1 hour at room temperature. After three times more washes, the sections were visualized by using the AEC substrate system as described above. To detect the SMC, peroxidase-conjugated anti-α-SMA antibody was used in the section staining and it was visualization by using AEC substrate system.

6.12 Quantitative analysis of vascular network areas

After PECAM-1 staining in the culture plate, the images were integrated using a color camera (Hamamatsu Photonics, Shizuoka, Japan). Image-processing (NIH image 1.62/power Macintosh G3, National Institutes of Health, Bethesda, MD) was used to determine alterations in the size of vascular networks (Hamada et al. 2000). In P-Sp

cultures, PECAM-1$^+$ EC formed a sheet-like structure (vascular beds) beside the P-Sp explant. Subsequently, PECAM-1$^+$ EC sprouted from the sheet and formed a cord-like structure (vascular networks). Since the outside border of the vascular network is not in regular pattern, I delimited the outside border with curve lines. Then I measured the areas between the outside border of vascular network and the boundary line of vascular bed and network, as a vascular network area. Three vascular networks from each P-Sp explant were measured under 12.5x magnification. The average value and standard deviation for each period was calculated, and the statistical significance was tested using an unpaired *t* test in Startview.

7. Results

7.1 Function of ephrin-B2 in vasculogenesis and angiogenesis

The OP9 stromal cell line was established from newborn calvarias of the (C57BL/6xC3H) F2-*op/op* mouse, which lacks a functional macrophage colony-stimulating factor (Yoshida et al. 1990; Kodama et al. 1994). According to previous studies (Takakura et al. 1998; Hirashima et al. 1999; Takakura et al. 2000), OP9 stromal cells are suitable for *in vitro* analysis of angiogenesis compared with other stromal cells such as NIH3T3, Balb/c3T3 and PA6. To examine the function of ephrin-B2 in angiogenesis, I overexpressed the full-length ephrin-B2 cDNA into OP9 stromal cells, which did not express ephrin-B2. The predicted molecular weight for ephrin-B2 is 37kD, but three bands of 38kD, 46kD and 48kD were detected in OP9/ephrin-B2 stromal cells by western blot analysis (Results Fig 1A). Consistent with a previous paper, this slow electrophoretic mobility may be due to the modification of glycosylation (Holland et al. 1996).

P-Sp region has been shown to harbor EC progenitors, which develop into vascular structure *in vitro* (Takakura et al. 1998 and 2000). To test the function of ephrin-B2 during angiogenesis, the P-Sp explants from E 9.0-9.5 embryos were co-cultured with the OP9/vector or OP9/ephrin-B2 stromal cells in the presence of IL-6, IL-7, SCF and Epo. Immunohistochemical staining with PECAM-1 MoAb to detect EC was performed after culturing for 4, 7, 10 and 14 days. In P-Sp culture, PECAM-1^{+}Flk-1^{+}Tie2$^{low/-}$ EC first to form a sheet-like structure (vascular beds) beside the P-Sp explant. Subsequently, PECAM-1^{+}Flk-1^{+}Tie2^{+} EC sprouted from the sheet and formed a cord-like structure (vascular networks) (Takakura et al. 2000). EC forming the sheet-like structure are an

immature phenotype, which assembles into vascular channels. EC forming a cord-like structure are the mature phenotype, which expands the vascular network. In this culture system, the early (days 4 and 7) and late (days 10 and 14) vascular network formation from P-Sp explants were enhanced in all three OP9 stromal cell clones that over-expressed ephrin-B2 compared to OP9/vector stromal cell clones (Results Fig 1B). The vascular formation on the OP9/vector stromal cells was the same intensity as that on the parent OP9 cells. Moreover, I measured the area of vascular networks from multiple P-Sp cultures at each time point using NIH Image software. The vascular network areas were 25, 1.6 and 2.4 times higher in OP9/ephrin-B2 stromal cells than that in OP9/vector stromal cells on days 7, 10 and 14, respectively (Results Fig 1C). It is evident that OP9 stromal cells expressing ephrin-B2 promoted EC sprouting from an early stage to late stage in this co-culture system.

7.2 Effect of EphB4 during angiogenesis in P-Sp culture system

Although it has been reported that mesenchymal cells do not express EphB4 *in vivo* (Adams et al. 1999), to investigate the interactions between environmental EphB4-positive cells and EC, EphB4 was transfected into OP9 cells and OP9 cells that expressed high levels of EphB4 were cloned (OP9/EphB4), while parent OP9 cells expressing little endogenous EphB4 (Results Fig 2A). To confirm the function of EphB4 in angiogenesis in the OP9 culture system, E9.0-9.5 P-Sp explants were co-cultured with OP9/EphB4 or OP9/vector stromal cells, as described above. After 14 days of co-culture, immunohistochemistry with the anti-PECAM-1 antibody was performed to detect the EC development from P-Sp explants. The level of vascular network formation in all three of the EphB4 over-expressing clones was severely

suppressed compared with that seen on OP9/vector stromal cell clones, however, the vascular bed formation was not changed (Results Fig 2B).

When the chimeric protein EphB4-Fc was added to the P-Sp and OP9/ephrin-B2 co-cultures, vascular network formation was also inhibited (Results Fig 2B); however, CD4-Fc as a control did not affect vascular formation (data not shown). On the other hand, when 20 μg/ml of ephrin-B2-Fc was added to this co-culture system, vascular EC sprouting/remodeling from the P-Sp explant increased in both OP9/EphB4 and OP9/vector stromal cells, and was strongly increased in OP9/ephrin-B2 stromal cells (Results Fig 2C). In contrast, monomer ephrin-B2 chimeric protein (ephrin-B2-FLAG) did not affect vascular formation (data not shown). These results indicate that, contrary to OP9/ephrin-B2, OP9/EphB4 inhibited vascular network formation from P-Sp explants, but did not affect development of vascular beds. The signals from a soluble form of ephrin-B2-Fc also promoted EC sprouting/remodeling, but soluble EphB4-Fc inhibited it.

7.3 Effect of OP9/ephrin-B2 stromal cells on P-Sp single-cell suspension cultures

To analyze the effect of ephrin-B2 on endothelial precursor cells, dissociated E9.0-9.5 P-Sp cells were seeded on OP9/ephrin-B2 or OP9/vector stromal cells in the medium containing VEGF and SCF. EC proliferated by two different means in this condition. One consisted of spreading EC networks, and the other of aggregated EC. The former was named a 'cord-like' and the latter a 'sheet-like' structure. In the OP9/ephrin-B2 stromal cells, most of the PECAM-1$^+$ EC formed a cord-like structure, while in OP9/vector stromal cells EC formed a sheet-like structure (Results Fig 3A). The number of sheet-like structures in OP9/vector stromal cells was about 5 times

greater than that in OP9/ephrin-B2 stromal cells. On the other hand, the number of cord-like structures was about 2.5 times higher in OP9/ephrin-B2 stromal cells than in the OP9/vector stromal cells (Results Fig 3B). These observations further indicated that OP9/ephrin-B2 stromal cells supported EC sprouting.

7.4 Effect of OP9/ephrin-B2 stromal cells on single-EC suspension cultures

P-Sp explants contain various kinds of cells, such as endothelial precursors, hematopoietic cells and mesenchymal cells. To examine whether OP9/ephrin-B2 stromal cells affect EC precursors directly, ECs were sorted from the E9.0-9.5 P-Sp regions by FACSvantage (Results Fig 4A). Flk-1$^+$PECAM-1$^-$ (R2) and Flk-1$^+$PECAM-1$^+$ (R3) cells were co-cultured with OP9/ephrin-B2 or OP9/vector stromal cells and showed EC growth on these stromal cells. After culturing for 10 days, I counted the number of PECAM-1-positive cord-like or sheet-like EC structures. The cord-like structures from R2 and R3 cells were 2.8 times and 14 times higher than sheet-like structures on OP9/ephrin-B2 stromal cells, respectively; but no significant difference was seen between the two kinds of vascular structure on OP9/vector stromsl cells (Results Fig 4B). These data indicated that the stromal cells expressing ephrin-B2 could promote EC spreading from a single EC precursor. RT-PCR showed that R2 cells expressed neither ephrin-B2 nor EphB4. R3 express both ephrin-B2 and EphB4, according to previous studies that R3 population may contain ephrin-B2 positive arterial EC and EphB4 positive venous EC (Results Fig 4C) (Wang et al. 1998; Gerety et al. 1999; Gale et al. 2001). These results when considered together, further indicated that the OP9 expressing ephrin-B2 directly promoted EC sprouting.

Further, the Flk-1$^+$PECAM-1$^+$ ECs from E13.5 fetal liver cells were sorted by

FACSvantage, and co-cultured with OP9/vector, OP9/ephrin-B2 and OP9/EphB4 stromal cells to investigate the effect of stromal cells expressing EphB4 and ephrin-B2 on EC growth. The ECs formed more cord-like endothelial cells on OP9/ephrin-B2 stromal cells, sheet-like on OP9/EphB4 stromal cells, and both cord-like and sheet-like on OP9/vector stromal cells, respectively (Results Fig 5). It is further indicated that OP9 expressing ephrin-B2 promoted EC sprouting, and EphB4 inhibited it.

7.5 Ephrin-B2 was expressed on both arterial ECs and smooth muscle cells (SMCs)

Vascular ECs consist of arterial EC and venous EC. Recent studies have shown that the two kinds of ECs are molecularly distinct. A ligand ephrin-B2 is dominantly expressed on arterial EC, and EphB4, a receptor specific for ephrin-B2 is expressed on venous EC (Wang et al. 1998; Gerety et al. 1999; Gale et al. 2001). To analyze the *in vivo* ephrin-B2 expression on the artery or vein, the LacZ and anti-PECAM-1/CD31 or anti-α-SMA double staining was done in the wild type or ephrin-B2 heterozygous embryos, the abdominal aorta/thoracic aorta and superior/inferior vena cava containing the surrounding tissues that derived from adult ephrin-B2 heterozygous or wild type mice. Serial staining of tissue sections revealed that in the E9.5 embryos the ephrin-B2 was absolutely expressed in the dorsal aorta ECs and SMA-positive surrounding cells, but not in the anterior cardinal vein cells, as a control no LacZ positive cells was observed in the wild type embryos (Results Fig. 6). In the adult, the ephrin-B2 was not only expressed on the artery ECs, but also dominantly expressed on the artery smooth muscle cells (SMCs). By contrast, no ephrin-B2 was detected in the venous endothelium, and a very few ephrin-B2 positive cells were observed on venous SMCs, as a control no LacZ positive cells was observed in the wild type mice tissues (Results

Fig 7). This result provided the evidence that the differences between the artery and vein keep not only on the ECs, but also on the SMCs that marked by the ephrin-B2 expression. Moreover, this result suggested that the ephrin-B2 expressed in the EC and surrounding of EC plays a crucial role not only in the ECs, but also surrounding cells during vascular development.

7.6 Effect of ephrin-B2 and EphB4 on ephrin-B2$^+$ cells

Next, to determine which types of EC (ephrinB2$^+$ or EphB4$^+$ EC) were supported or inhibited by ephrin-B2 or EphB4 on OP9 cells, and how they interact with each other in this system. The ephrin-B2$^{LacZ/+}$ embryos were obtained from a cross of wild type and ephrin-B2$^{LacZ/+}$ mice. After genotyping of the embryo by LacZ staining of the yolk sac (Results Fig 8A), the E9.0-9.5 ephrin-B2$^{+/+}$ or ephrin-B2$^{LacZ/+}$ P-Sp was co-cultured with OP9/ephrin-B2, OP9/EphB4 or OP9/vector stromal cells. After culturing for 10-14 days, I examined the formation of vascular networks by immunohistochemical staining with PECAM-1 MoAb and LacZ staining to detect the ephrin-B2 positive cells. Ephrin-B2$^+$ EC were detected as PECAM-1 and LacZ-double positive cells. As shown in Results Fig 8B, the OP9/vector stromal cells form a fine vascular network, which consisted of both PECAM-1-single positive cells and LacZ/ephrin-B2-PECAM-1-double positive cells. But the vascular network formation on OP9/ephrin-B2 stromal cells was promoted than in OP9/vector stromal cells and mainly formed by LacZ/ephrin-B2 and PECAM-1-double positive cells. In contrast, OP9/EphB4 stromal cells did not support vascular network formation and almost no LacZ/ephrin-B2$^+$ cells were observed. This indicates that OP9/ephrin-B2 promotes vascular network formation and supports the proliferation and sprouting of ephrin-B2$^+$ cells, but that OP9/EphB4 inhibits ephrin-B2$^+$ cell

proliferation.

7.7 Function of ephrin-B2 and EphB4 in SMC recruitment

During vascular development, an important event is the formation of vessel walls by recruitment of mural cell precursors, especially in the artery. To investigate SMC recruitment in this culture system, I performed immunohistochemical staining with anti-α-smooth muscle actin (α-SMA) antibody. On day 14 of culturing, the number of α-SMA$^+$ SMC increased in the surrounding EC formed on OP9/ephrin-B2 stromal cells. Nevertheless, no α-SMA$^+$ cells were detected in OP9/EphB4 stromal cells, while a small number of α-SMA$^+$ cells were detected in OP9/vector stromal cells (Results Figs 9A and B). SMCs have a complex origin depending on their location. It has been reported that SMC develop from mesodermal cells, neural crest cells or trans-differentiate from other cells (Hungerford and Little 1999). It was further confirmed that the SMA$^+$ cells in the vascular network was derived from P-Sp explants, but not from OP9 stromal cells by using P-Sp obtained from "Green mice" harboring GFP ubiquitously under the transcriptional control of a CAG promoter in this co-culture system (Results Fig 9C). This result suggested that the ephrin-B2$^+$ EC and stromal cells have the ability to promote SMC recruitment and proliferation. However, EphB4$^+$ EC might be unable to promote SMC recruitment. This further suggests that ephrin-B2/EphB4 signaling is important for the interaction between the EC and surrounding cells.

8. Discussion

In this paper, I examined the function of EphB4 receptors and ephrin-B2 ligands in the interaction of EC with mesenchymal cells using an *in vitro* P-Sp and stromal cell co-culture system. To address this issue, I analyzed angiogenesis using OP9 stromal cells that were over-expressed with ephrin-B2 or EphB4. Using these sublines of OP9 cells, I observed several pieces of evidence. 1) Environmental expression of ephrin-B2 supports the proliferation of ephrin-$B2^+$ ECs and suppresses the proliferation of ephrin-$B2^-$ ECs. 2) Environmental expression of ephrin-B2 supports the proliferation of SMCs. 3) Environmental expression of EphB4 suppresses the proliferation of ephrin-$B2^+$ ECs, and does not promote the proliferation of SMCs. 4) Environmental expressing ephrin-B2 also promote single EC sprouting and environmental expressing EphB4 suppresses single EC sprouting.

The EphB4 receptor and its cognate ligand, ephrin-B2, are crucial for successful cardiovascular development during embryogenesis (Wang et al. 1998; Yancopoulos et al. 1998; Gerety et al. 1999). EphB4 is exclusively expressed in venous ECs, whose cognate ligand ephrin-B2 is complementary expressed in arterial ECs. Ephrin-B2 null mutant mice display defects in angiogenesis by both arteries and veins in the capillary networks of the head, yolk sac and myocardial trabeculation, and was lethality 100% around E11 (Wang et al. 1998). The EphB4 homozygous mutants have a symmetric phenotype with ephrin-B2 homozygous embryos in the cardiovascular system, and embryos were lethally around E10 (Gerety et al. 1999). To elucidate the interactions between the EC and surrounding cells, which expressing ephrin-B2 or EphB4, we established an *in vitro* OP9 stromal cell and P-Sp co-culture assay for analyses of angiogenesis. Ephrin-B2-transfected stromal cells enhanced the vascular network

formation from P-Sp, while EphB4-transfected stromal cells inhibited it. RT-PCR analyses have shown that ephrin-B2, ephrin-B1, EphB4, EphB3 and EphB2 are expressed in the PECAM-1^{+}CD45^{-} EC population, which are grown from P-Sp explants in OP9 and P-Sp co-culture systems (data not shown). However, other Eph receptors and ephrin ligands may also be involved in angiogenesis. Ephrin-B1, ephrin-B3, EphB2 and EphB3 are expressed in the vascular ECs or the surrounding cells *in vivo*. But from the gene mutant result that this overlapping of expression is unable to compensate for the lack of ephrin-B2 or EphB4. No vascular defects were seen in either EphB2 or EphB3 homozygous mutant mice. Whereas, EphB2 and EphB3 double mutant mice have shown the vascular defect only with 30% penetrance (Adams et al. 1999). In combination, these results suggest that angiogenesis is dependent upon the appropriate expression and function of ephrin-B2 and EphB4.

In this culture system, I found that both ephrin-B2-positive and -negative EC proliferated. For the detection of ephrin-B2 ligand-positive cells, I used the mice that lacZ gene is inserted in the ephrin-B2 locus. In the LacZ staining of P-Sp cultures using ephrin-B2$^{LacZ/+}$ embryos, I found that OP9/ephrin-B2 stromal cells exclusively promoted the proliferation and sprouting of LacZ/ephrin-B2^{+} EC while OP9/EphB4 stromal cells inhibited it. This might suggest that over-expression of ephrin-B2 on stromal cells primarily suppress the proliferation of EphB4^{+} ECs and subsequently supported the proliferation of arterial ECs. In contrast, the over-expression of EphB4 on stromal cells suppressed ephrin-B2-positive EC proliferation and vascular network formation. Indeed, mesenchymal cells surrounding the artery, especially the dorsal aorta, widely expressed ephrin-B2 and might stimulate proliferation or sprouting of ephrin-B2^{+} EC, which also express EphB2 or EphB3 (Results Figs 6 and 7) (Gale et al. 2001). The results

suggested that vascular development on ephrin-B2 or EphB4-over-expressing stromal cells is mediated by signaling between EphB4 and ephrin-B2 expressed on the endothelial or stromal cells. Also, it may indicate that over-expression of ephrin-B2 might permit the proliferation of ephrin-B2$^+$ EC through EphB receptors by preventing contact inhibition with EphB4$^+$ EC. Recently Helbling. et al., reported that EphB4 and B-class ephrins act as regulators of angiogenesis, possibly by mediating repulsive guidance cues to migrating EC (Helbling et al. 2000). The interaction between EphB4-expressing EC and adjacent ephrin-B-expressing somatic tissue is therefore likely to be of a repulsive nature. Also, it suggests that signaling between EphB4 and ephrin-B2 is involved in controlling EC sprouting and vascular boundary formation during vascular development, as is the case in the nervous system. In Zebrafish, the expression domain of EphB4 and ephrin-B2 in the developing somite and hindbrain are complementary and can bind together in *in vivo* and is required for somite and hindbrain boundary formation (Durbin et al. 1998; Wang and Anderson 1997; Brennan et al. 1997; Cooke et al. 2001). Moreover, the expression of EphB4 and ephrin-B2 is mediated by Val bzip transcription factor, in Val mutant embryos. A low level of EphB4a expression is some times seen in the caudal hindbrain at early somite stages. In contrast to the downregulation of EphB4a, ephrin-B2 is upregulated in the caudal hindbrain. In Val mutant embryos there is loss of EphB4a and ephrin-B2 interfaces and a corresponding loss of boundaries; otherwise ectopic EphB/ephrin-B interface correlates with ectopic boundary formation (Cooke et al; 2001). These suggested that the complementary pattern of EphB4 and ephrin-B2 are not only in the expression, but also in the regulation. And the repulsive interactions between the receptor-ligand pair at the interface of their expression domains are important for proper boundary formation

during embryo development. Mutual repulsive or growth-inhibitory interactions between EphB4 and ephrin-B2-positive EC or surrounding cells simultaneously need other Eph receptors and ligand signals to establish a balance between these two kinds of EC to maintain appropriate sprouting and remodeling during vascularization (Adams et al. 1999).

To analyze the directly function of stromal cell expressing ephrin-B2 or EphB4 during single EC differentiation/proliferation, I sorted the EC cell with monoclonal antibodies against to Flk-1 and PECAM-1 from E9.0-9.5 P-Sp region and E13.5 fetal liver. Flk-1, one of the receptor for VEGF, is a marker for the lateral plate mesoderm and the earliest differentiation marker for EC and hematopoietic cells (Yamaguchi et al. 1993; Shalaby et al. 1995; Eichmann et al. 1997). PECAM-1, also known as CD31, is a transmembrane molecule expressed on ECs, and therefore a good marker for identification of capillary vessels. (Albelda et al. 1991). I co-cultured the sorted single EC suspension with OP9/vector, OP9/EphB4 or OP9/ephrin-B2 stromal cells, and observed that the single EC's sprouting also supported by stromal cells expressing ephrin-B2, and suppressed by the stromal cell expressing EphB4. According to the RT-PCR results that the Flk-1$^+$PECAM-1$^+$ EC population expressed both EphB4 and ephrin-B2, suggesting that the stromal cells expressing EphB4 or ephrin-B2 interact directly with the EC expressing Eph or ephrins in P-Sp region or fetal liver.

During angiogenesis, although the ECs have attracted most attention, they alone can initiate, but not complete angiogenesis. One important event is the formation of the vascular wall by the recruitment of pericytes and SMCs from mesenchymal progenitor cells and neural crest cells to complete the angiogenesis (vascular myogenesis) (Kirby and Waldo 1995; Hungerford et al. 1996). The bi-directional signaling between ECs and

the surrounding mesenchymal cells is important (Folkman and D'Amore 1996). During vascular myogenesis, mural cells stabilize nascent vessel by inhibiting endothelial proliferation and migration, and by stimulating production of extracellular matrix. They thereby provide hemostatic control and protect new endothelium-lined vessels against rupture or regression. And the interaction between EC and mural cells is essential for vascular development and maintenance (Suri et al. 1996; Folkman and D'Amore 1996; Darland and D'Amore 1999; Carmeliet 2000). ECs derive from Flk-1-positive mesoderm cells (Yamaguchi and Dumont 1993), and the mural cells derive from mesoderm, neural crest or epicardial cells (Topouzis and Majesky 1996; Mikawa and Gourdie 1996; Jiang et al. 2000). Peri-endothelial supporting cells are recruited to encase the endothelial tubes, providing maintenance and modulatory functions to the vessel. They are pericytes for small capillaries, SMCs for larger vessels, and myocardial cells in the heart. SMCs have a complex origin depending on their locations. SMCs can transdifferentiate from ECs, differentiation from meshenchymal cells in situ in response to as-yet-unidentified endothelial-derived stimuli, or from bone marrow precursors or macrophages. During the course of development and maturation, the vascular SMCs serve as a biosynthetic, proliferative, and contractile components of the developing vessel wall. Although the developmental program for vascular SMCs is poorly understood, recent findings in the vascular biology field have focused attention on potential mechanisms of vascular SMC recruitment/differentiation. Progress in elucidating the mechanism of SMC recruitment, proliferation and differentiation was achieved by identification of a number of smooth muscle-specific proteins and their expression in SMC lineage cells (Skalli et al. 1986; Lee et al. 1997). EC can modulate phenotypic change, regulate proliferation, and induce migration of SMC (Powell et al.

1996a; Powell et al. 1996b). These processes are regulated by growth factors, such as PDGF and TGF-β. PDGF-BB is a chemoattractant for SMCs (Lindahl et al. 1998), PDGF derived from EC acts as a mitogen for mesenchymal cells (Westermark et al. 1990), VEGF also promotes mural cell accumulation, presumably through the release of PDGF-BB or binding to VEGF receptors (Bejamin et al. 1998; Carmeliet et al. 1999), whereas TGF-β induces differentiation of neural crest cells into SMC (Shah et al. 1996). However, SMC/pericytes also take an active part during vascular development. SMCs are capable of synthesizing angiopoietin-1 (Ang1), an essential factor for angiogenesis (Takakura et al. 2000). Ang1 and Tie2 affect growth and maintenance of blood vessels by stabilizing of the interaction of mural cells with ECs, and by inducing branching and remodeling (Suri et al. 1996; Maisonpierre et al. 1997; Gale and Yancopoulos 1999).

Vascular SMC produce and organize extracellular matrix molecules within the developing vessel wall. In the *in vitro* assays, OP9/ephrin-B2 promoted not only proliferation and sprouting of ephrin-B2$^+$ EC, but also promoted the recruitment and proliferation of α-SMA$^+$ SMC. α-SMA is the earliest marker expressed by SMCs, cardiomyocytes, and skeletal muscles (Skalli et al. 1986; Hungerford and Little 1999). However, its expression is lost in striated muscle and becomes specifically associated with vascular and visceral SMCs. By contrast, OP9/EphB4 inhibited the α-SMA$^+$ SMC recruitment and/or proliferation. Moreover, studies by ourselves and others have shown that differences between the artery and vein apply not only to the EC, but also to the SMC marked by the ephrin-B2 expression (Results Figs 6 and 7) (Gale et al. 2001). In ephrin-B2 knockout mice, it was observed that the mesenchymal cells and pericytes appeared poorly associated with ECs in the yolk sacs, and that these cells exhibited a rounded morphology compared with those in wild-type yolk sacs (Wang et al. 1998). It

suggested that the ephrin-B2 positive arterial EC may play a crucial role in SMC recruitment and proliferation. It is likely that ephrin-B2 expressing mesenchymal cells surrounding the dorsal aorta was recruited by ephrin-B2-expressing ECs, which also coexpress EphB2 and EphB3 during the early stage of angiogenesis. Then, the ephrin-$B2^+$ EC and SMC stimulate each other during arteriogenesis. Molecular cues as to how such ephrin-$B2^+$ EC and SMC interact with each other have not been clarified. However, co-expressions and interactions of ephrin-B1, EphB3. and EphB4 on vein primordia, and co-expression of ephrin-B1, ephrin-B2, EphB2 and EphB3 on arteries may also be required for venous and arterial formation.

In summary, blood vessels are formed by the recruitment and migration of mesenchymal cells outside of the endothelial layer. Therefore, from our results and others, it is reasonable to conclude that the initial commitment of ephrin-B2 or EphB4-positive EC from angioblasts is the trigger for determining vessels that become arteries or veins (Wang et al. 1998; Gerey et al. 1999; Gale et al. 2001). During this process, along with other factors (Holmgren et al. 1991; Leveen et al. 1994; Heimark et al. 1986; Dickson et al. 1995; Sata et al. 1990; Hirschi et al. 1998), Ephs /ephrins, and in particular EphB4 /ephrin-B2, are the key regulators in the recruitment and migration of SMC precursors *in vivo*, and maintain the balance of proliferation activity. Based on previous studies and our own findings, I propose a model (Conclusion Fig) to account for the role of EphB4/ephrin-B2 signaling in the interaction between endothelial and mesenchymal cells during vasculogenesis, angiogenesis and vascular morphological change.

9. Conclusions

1. Surrounding cells expressing ephrin-B2 supports the proliferation of ephrin-B2$^+$ ECs and suppresses the proliferation of ephrin-B2$^-$ ECs.
2. Surrounding cells expressing ephrin-B2 support the proliferation of SMC.
3. Surrounding cells expressing EphB4 suppress the proliferation of ephrin-B2$^+$ ECs, and do not promote the proliferation of SMC.
4. Surrounding cells expressing ephrin-B2 also promote single EC sprouting and surrounding cells expressing EphB4 suppress single EC sprouting.

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11. Figures

Introduction Fig 1

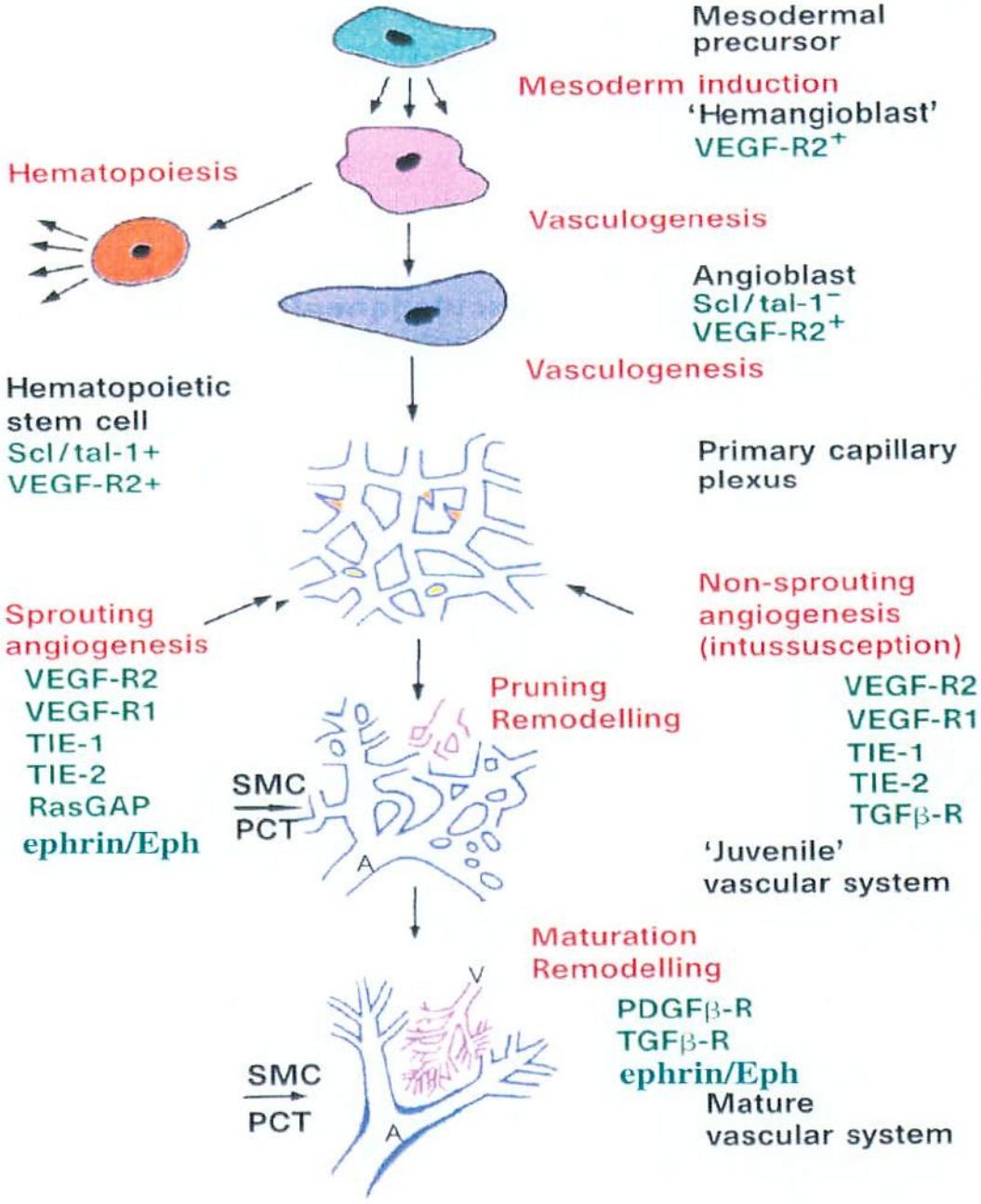


Fig 1. The processes (red labels), molecules (green labels) and appearances (black labels) involved in vascular development. Red tips in the primary capillary plexus represent sprouts, yellow circles represent splitting pillars. A: arteriole; V: venule, SMC: smooth muscle cells; PCT: pericytes. (Risau., Nature, 1997)

Introduction Fig 2

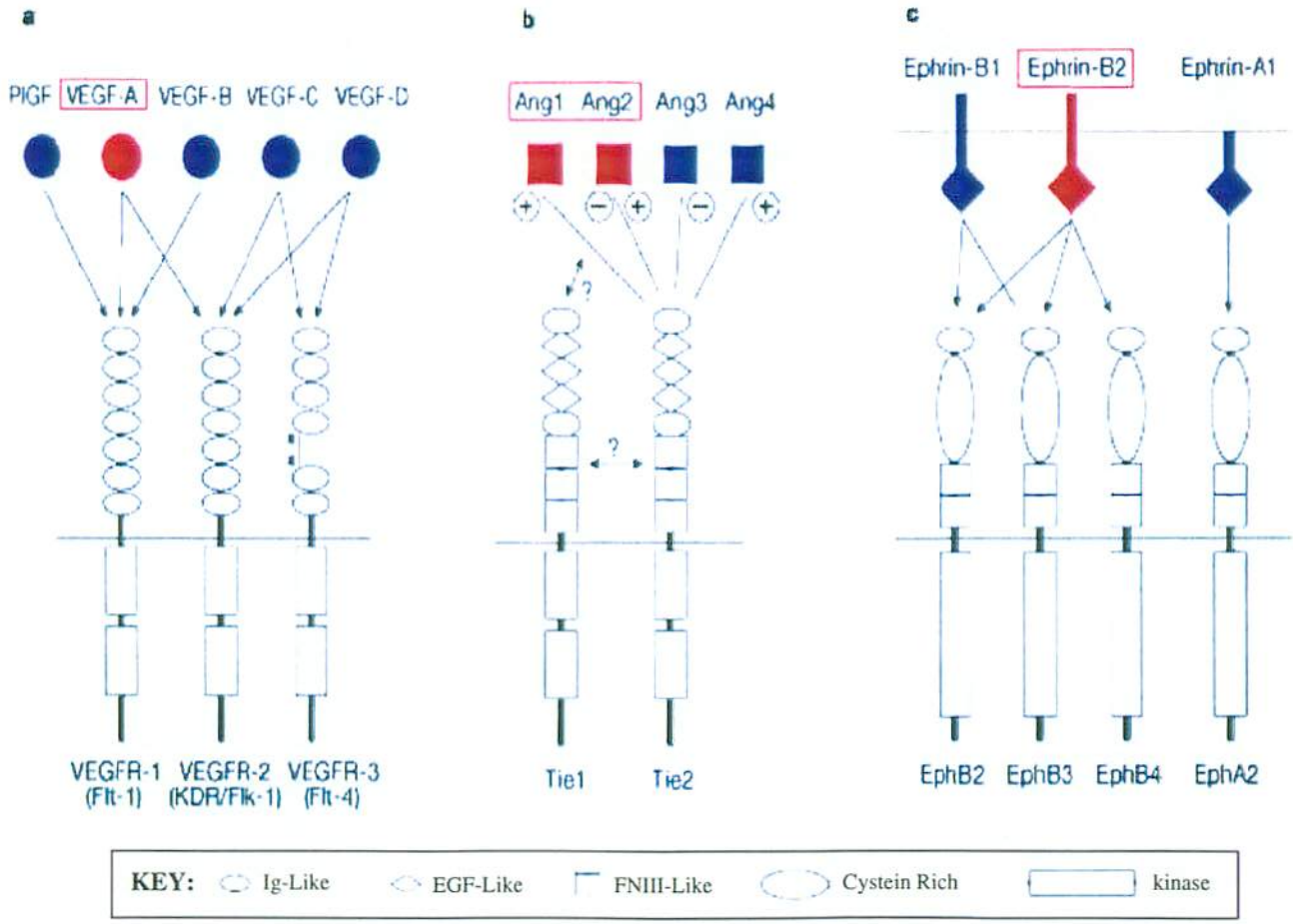


Fig 2. Ligands and RTK families involved in vascular development. (A) VEGFs and the VEGF receptors. (B) The Angiopoietins (Ang) and Tie receptor family. (C) The ephrins and Eph receptors. Arrows indicate documented interactions among ligands with their receptors. The four factors the highlighted in red was discussed in more in vascular development. '+' or '-' indicates whether the particular angiopoietin activates or block the Tie2 receptor. '?' Indicates that a potential interaction has not yet been confirmed experimentally. (Yancopoulos et al., Nature, 2000)

Introduction Fig 3

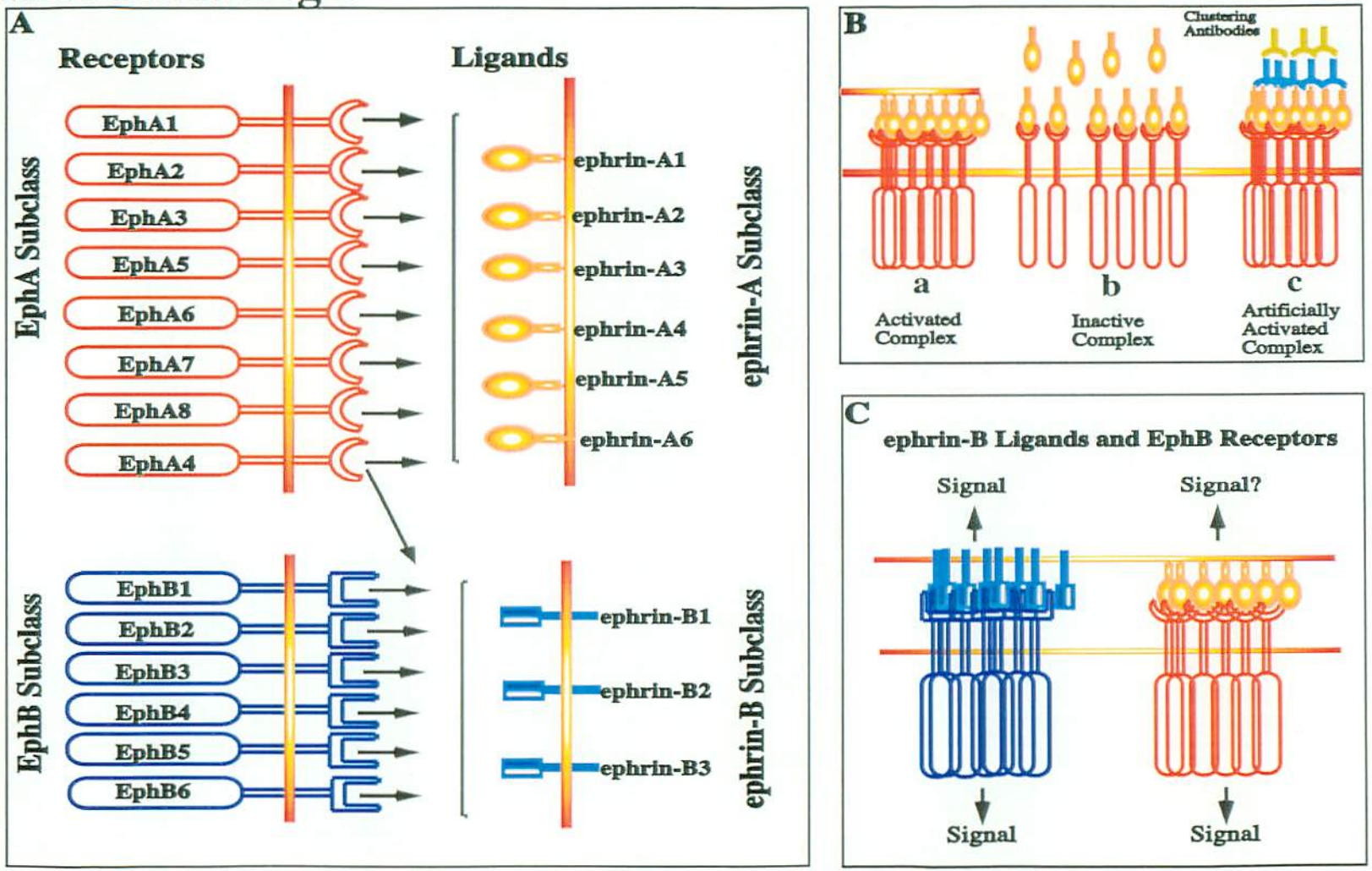


Fig 3. (A) Schematic summary of Eph receptor and ephrin ligand interactions. Arrows drawn from a receptor to a ligand group denote binding of that receptor to the ephrins indicated by the brackets. Lack of arrows drawn from receptor to ligand indicates that the proposed interactions have not yet been tested. (B) Ephrin clustering and receptor activation. a) Ephrins and their receptors normally come into interact with each other at the surface between two juxtaposed cells. b) Soluble versions of ephrins are capable of interacting with their receptors but are unable to direct the formation of an active receptor complex. c) The artificial clustering of soluble versions of ephrins with antibodies is sufficient to give them receptor activation potential. (Modified from Nicholas WG and Yancopoulos GD., Cell Tissue Res, 1997)

Introduction Fig 4

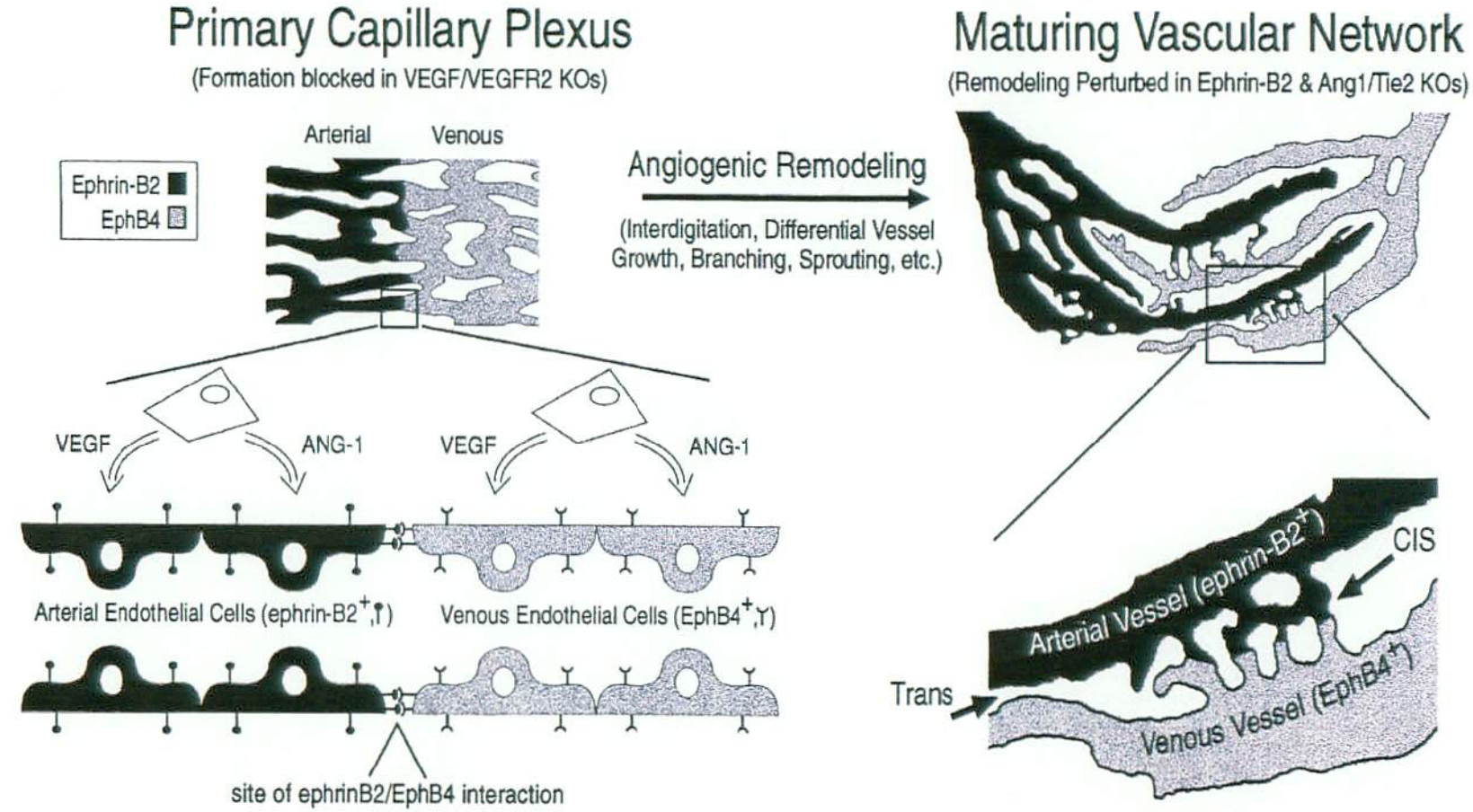


Fig 4. Presumed distributions and sites of action of epnrin-B2 and EphB4 in the primary capillary and plexus and during later maturation of the vascular network. Both ephrin-B2 and EphB4 act only as membrane-bound protein, they presumably interact with each other only at the junction of the arterial and venous ECs as a manner termed 'cis' (left panel). But they may also interact as a 'Trans' manner (right panel) at the surface between interdigitating lager vessels as they pass each other. (Yancopoulos et al., Cell, 1998)

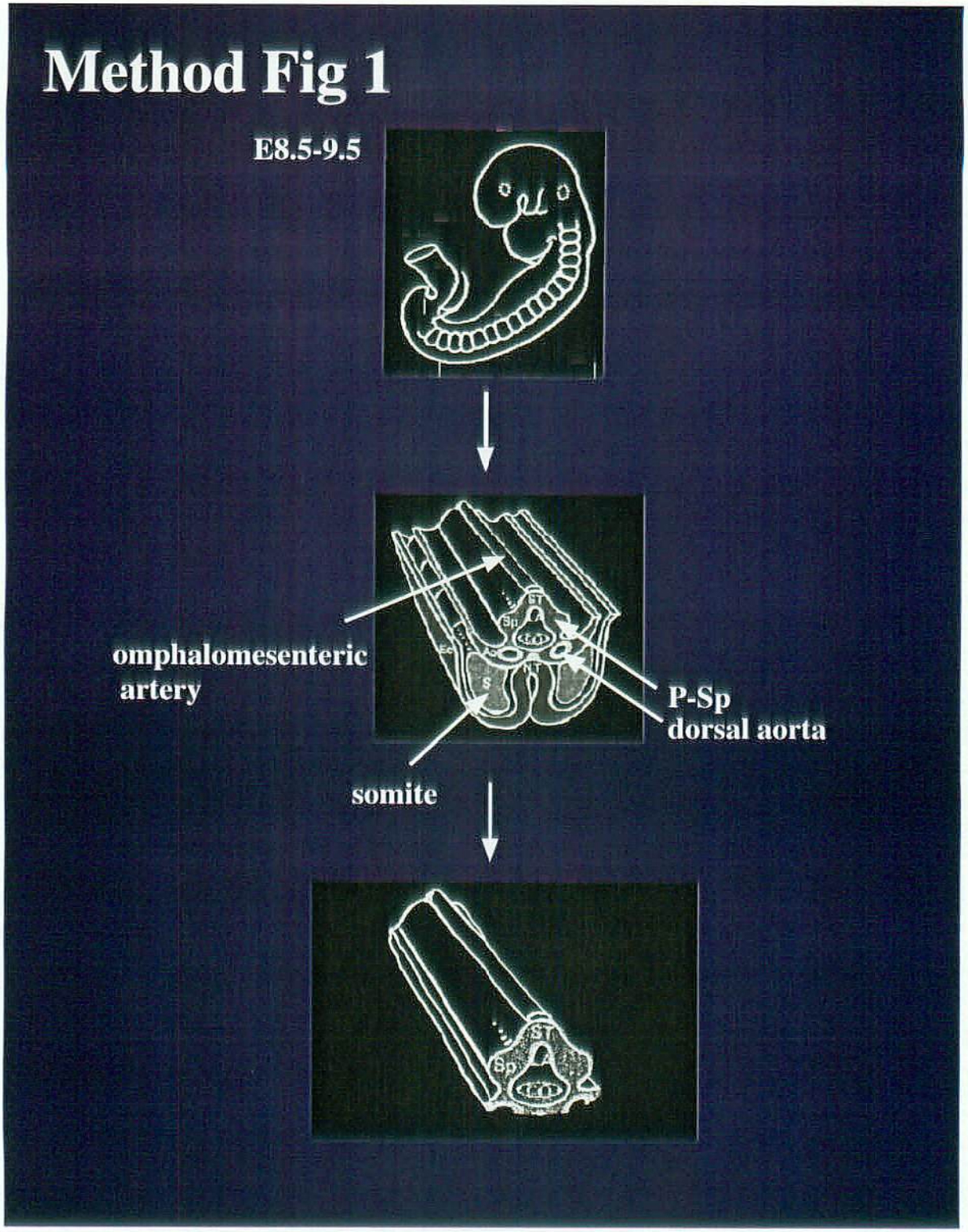


Fig 1.Dissection of P-Sp Region from Murine Embryo

Method Fig 2

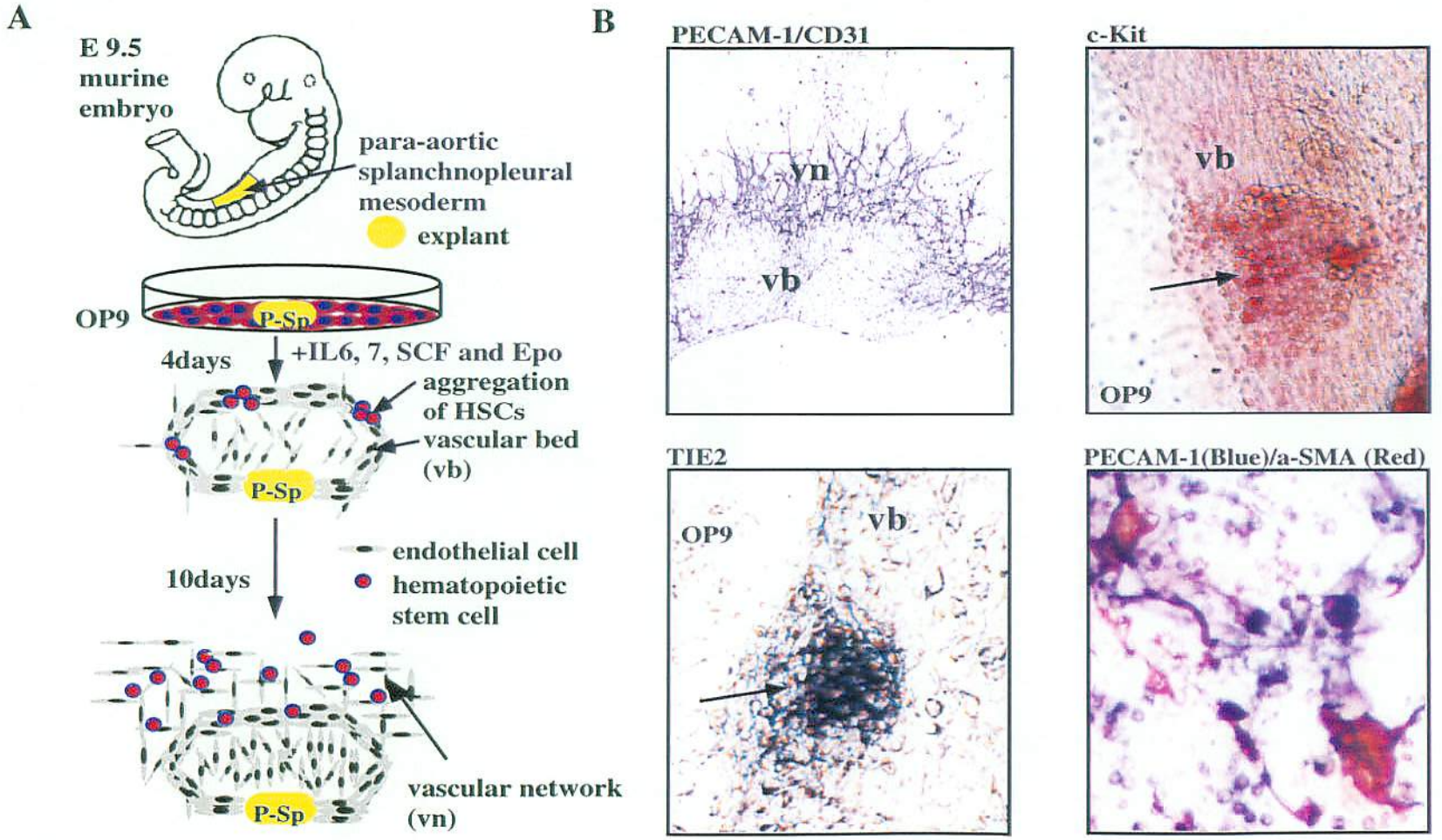


Fig 2. P-Sp Culture System Supports Vasculo-angiogenesis and Hematopoiesis. (A) P-Sp culture system. (B) Immunohistochemistry to detect the ECs (PECAM-1), hematopoietic cells (c-kit) and SMCs that development from P-Sp explants.

Result Fig 1

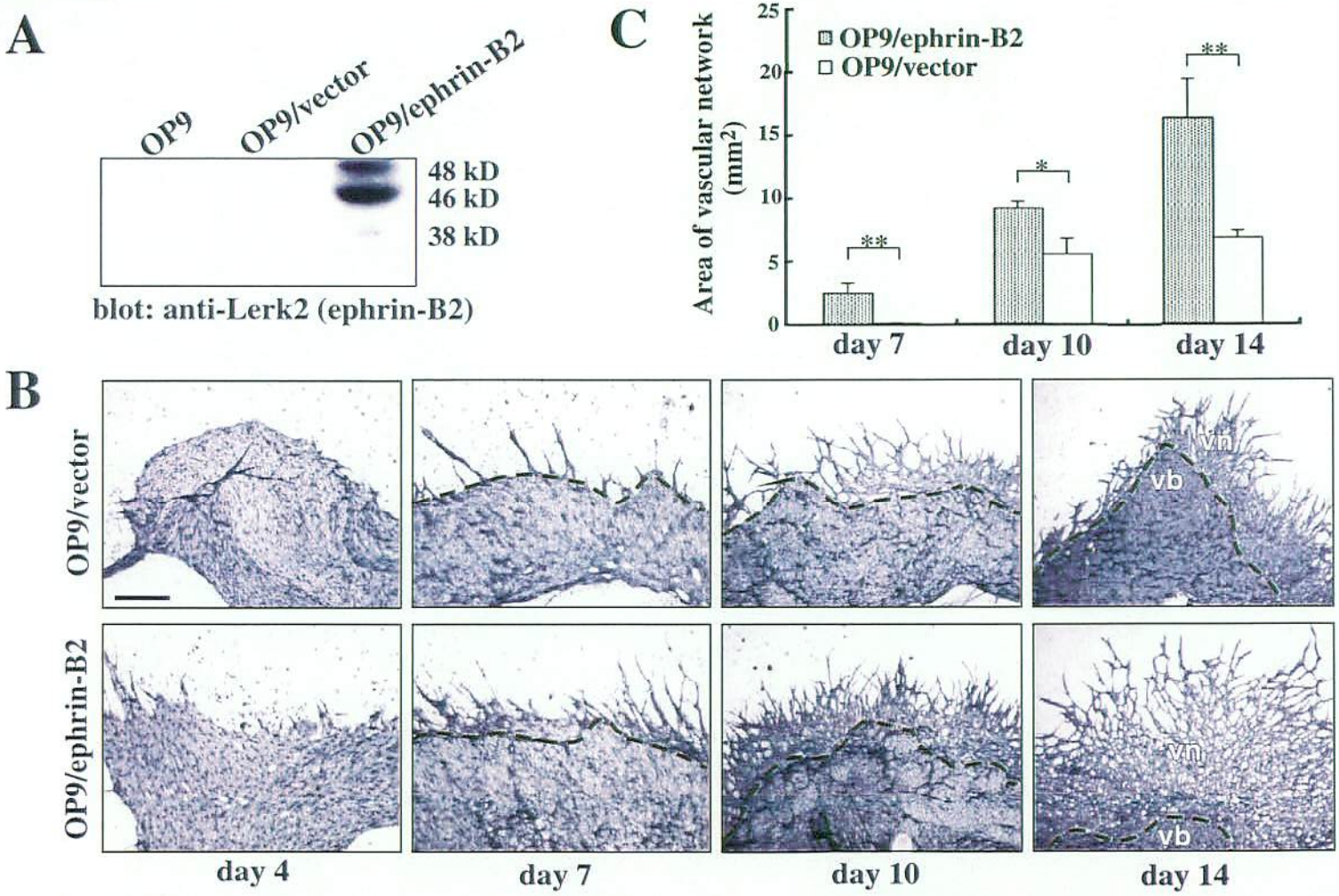


Fig 1. OP9/ephrin-B2-enhanced vascular network formation from E9.5 P-Sp explants. (A) OP9 cells were stably transfected with expression plasmid DNA pCAGneo-ephrin-B2 (OP9/ephrin-B2) or expression vector pCAGneo alone (OP9/vector) and individual G418-resistant clones were selected. (B) P-Sp explants were co-cultured with OP9/ephrin-B2 or OP9/vector stromal cells. After the indicated period, the cultures were immunostained by anti-PECAM-1/CD31 MoAb. The formation of vascular networks was enhanced more on the OP9/ephrin-B2 stromal cells than on OP9/vector stromal cells. Abbreviations: vb, vascular bed; vn, vascular network. The bar indicates 500 μm (day 4 and day 7) and 200 μm (day 10 and day 14). The dotted line indicate the border of vascular bed and vascular network. (C) Quantitative analysis of vascular network area by NIH Image software. The results represent the mean ± SD of triplicate cultures. $^{*}P < .05$; $^{**}P < .01$.

Result Fig 2

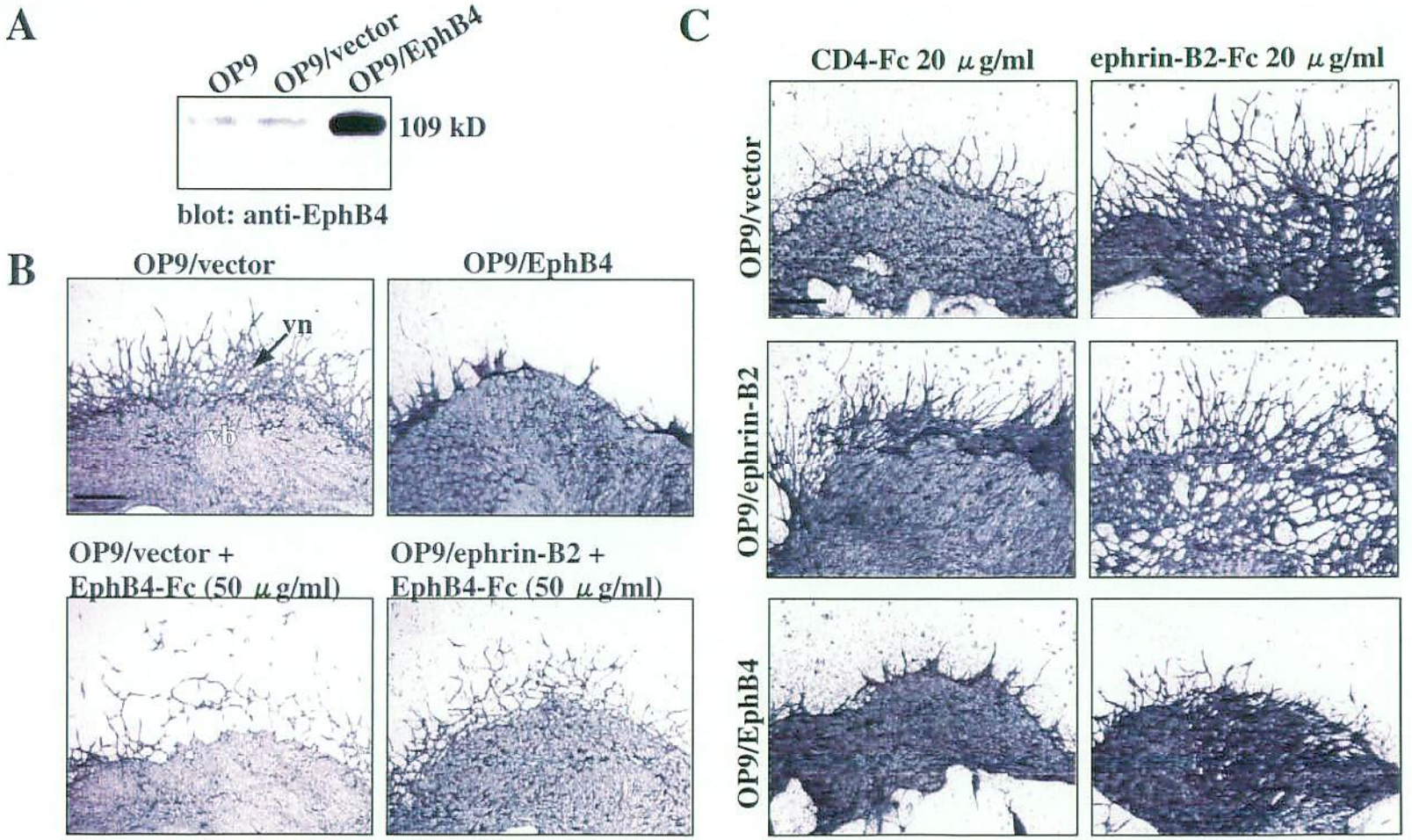


Fig 2. OP9/EphB4 stromal cells inhibited vascular network formation from E9.5 P-Sp explants. (A) OP9 cells were co-transfected with expression plasmid DNA pRK5-EphB4 (OP9/EphB4) and pCAGneo or expression vector pCAGneo alone (OP9/vector). (B) P-Sp explants were co-cultured with OP9/EphB4 or OP9/vector stromal cells. After 14 days, the cultures were immunostained by anti-PECAM-1 MoAb. The formation of vascular networks (vn) was inhibited on the OP9/EphB4 stromal cells compared to the OP9/vector stromal cells. Vascular network formation was also inhibited by the addition of EphB4-Fc in both the OP9/vector and OP9/ephrin-B2 stromal cells. The bar indicates 500mm. (C) P-Sp explants were co-cultured with OP9/ephrin-B2, OP9/EphB4 or OP9/vector stromal cells in the presence of chimeric protein ephrin-B2-Fc for 10 days. The vascular EC sprouting and remodeling from P-Sp explants was promoted by the addition of ephrin-B2-Fc, especially in OP9/ephrin-B2 stromal cells. The bar indicates 500mm.

Result Fig 3

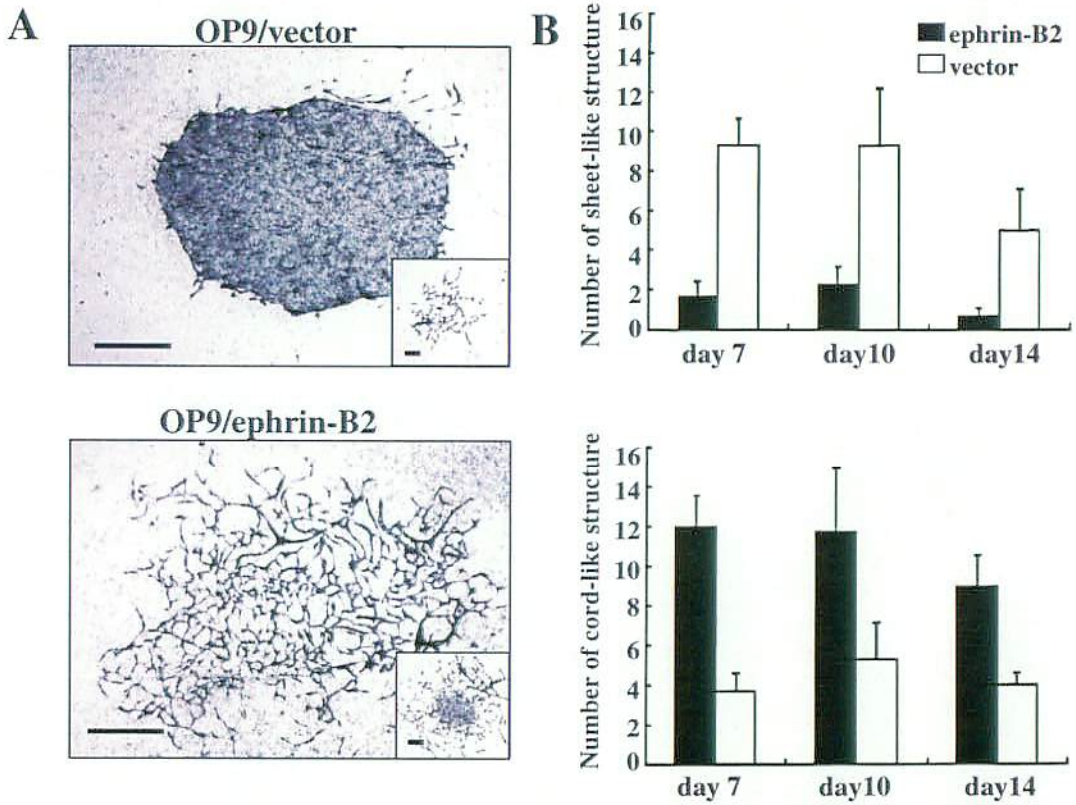


Fig 3. OP9/ephrin-B2 promoted EC sprouting in cultures using dissociated cells from P-Sp explants. The E9.5 P-Sp region was dissociated enzymatically and co-cultured with OP9/ephrin-B2 or OP9/vector stromal cells in the presence of SCF and VEGF. Immunostaining by anti-PECAM-1 MoAb was performed at the indicated times. (A) The PECAM-1+ EC formed mostly sheet-like structures on OP9/vector stromal cells (inset shows the cord-like structures in OP9/vector stromal cells), whereas the PECAM-1+ EC formed cord-like structures on OP9/ephrin-B2 stromal cells (inset shows the sheet-like structure in OP9/ephrin-B2 stromal cells). The bar indicates 500mm. (B) The number of sheet-like or cord-like structures on OP9/ephrin-B2 or OP9/vector stromal cells. The results represent the mean±SD of triplicate cultures.

Result Fig 4

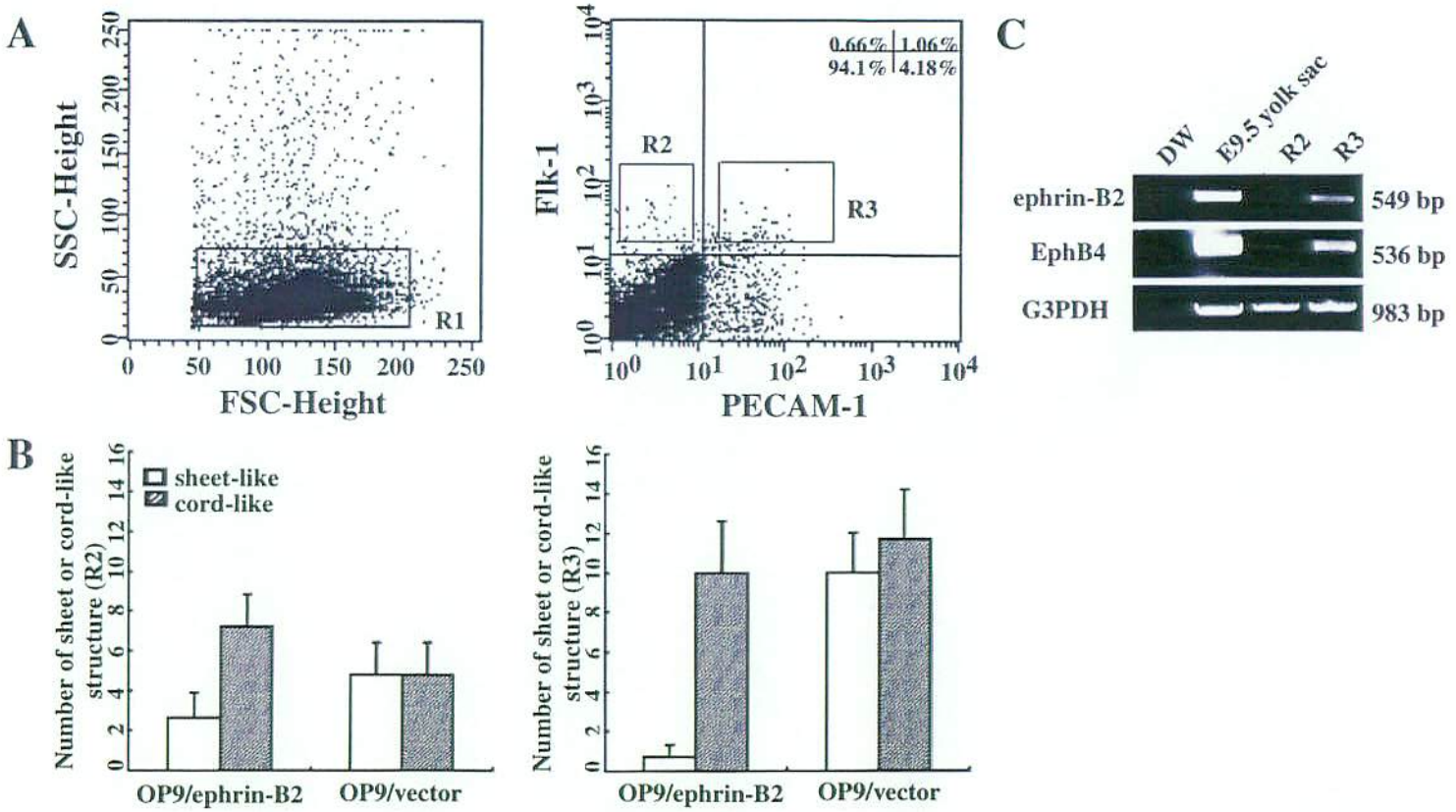


Fig 4. OP9/ephrin-B2-promoted endothelial cell sprouting from a single EC. (A) Flk-1+PECAM-1- (R2) and Flk-1+PECAM-1+ (R3) cells were sorted from E9.0-9.5 P-Sp regions stained whith Flk-1 and PECAM1 antibodies. (B) The sorted R2 and R3 cells were co-cultured with an OP9/ephrin-B2 or OP9/vector in the presence of VEGF for 10 days, and PECAM-1 immunostianing was performed. The cord-like structures from R2 and R3 cells were 2.8 times and 14 times higher than the sheet-like structure on OP9/ephrin-B2 stromal cells, respectively. But almost no difference was seen between the two types of structure on OP9/vector stromsl cells. (C) Expression of ephrin-B2 and EphB4 was detected by RT-PCR analysis. Total RNA was extracted from R2 and R3 cells and the RNA extracted from E9.5 yolk sac was used as a positive control. R3 cells expressed both ephrin-B2 and EphB4, while cells R2 did not express either.

Result Fig 5

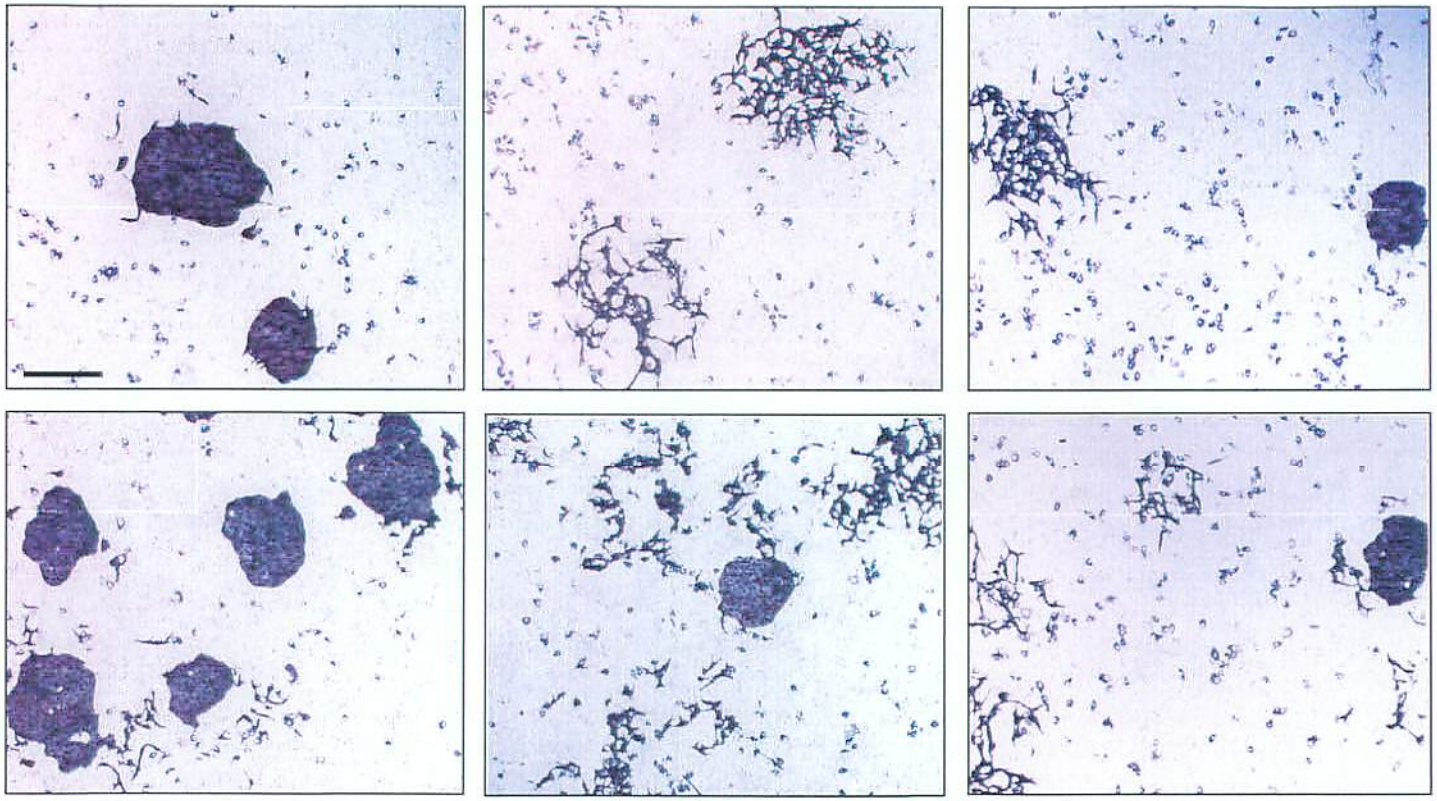


Fig 5. OP9/ephrin-B2 promoted EC sprouting from single fetal liver EC, whereas OP9/EphB4 inhibited it. Flk-1^{+}PECAM-1^{-} (uper panel) or Flk-1^{+}PECAM-1^{+} (lower panel) EC precursors were sort from E13.5 fetal liver cells were co-cultured with OP9/EphB4, OP9/ephrin-B2, or OP9/vector stromal cells for 10 days. The EC formed cord-like structure on OP9/ephrin-B2 stromal cells, sheet-like structure on OP9/EphB4 stromal cells and both structure on OP9/vector stromal cells. The bar indicates 500 μm.

Result Fig 6

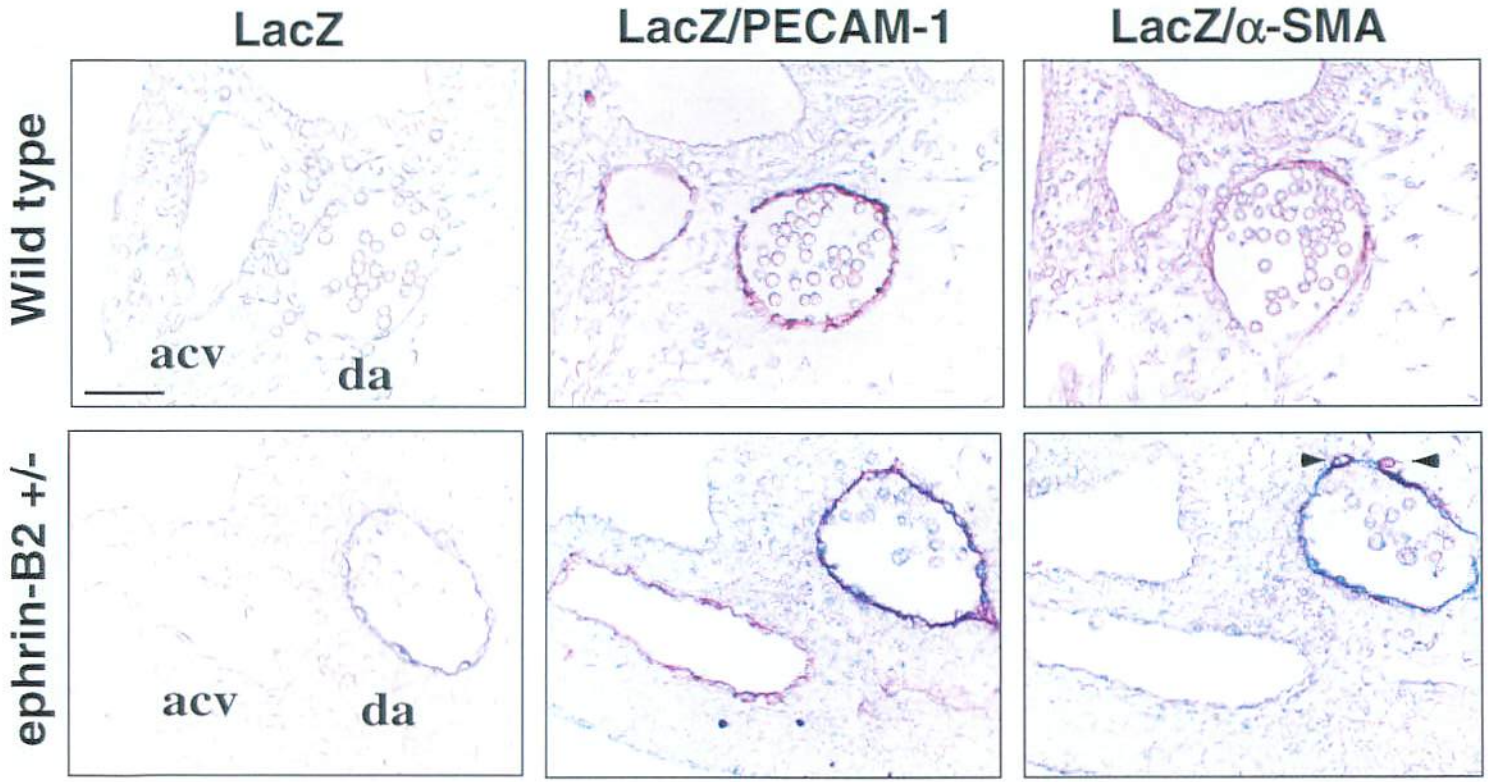


Fig 6. LacZ/ephrin-B2 expression in the embryos. Ephrin-B2 heterozygous and wild type embryos were removed at E9.0-9.5, then fixed and stained by LacZ staining solution. After LacZ staining the embryos were post-fixed, embedded and sectioned. Then the vascular endothelial cells and surrounding cells/SMCs was examined by immunochemical staining with PECAM-1/CD31 and α-SMA antibodies. In wild type embryos dorsal aorta or other tissues no LacZ staining (blue) was observed, when the PECAM-1/CD31 (red) and α-SMA (red) positive cells was clearly observed. In ephrin-B2 heterozygous embryos the LacZ/ephrin-B2 is obviously expressed in the dorsal aorta (da) endothelial cells and surrounding cells with PECAM-1/CD31 and α-SMA, but not in the anterior cardinal vein (acv). The bar indicates 25μm.

Result Fig 7

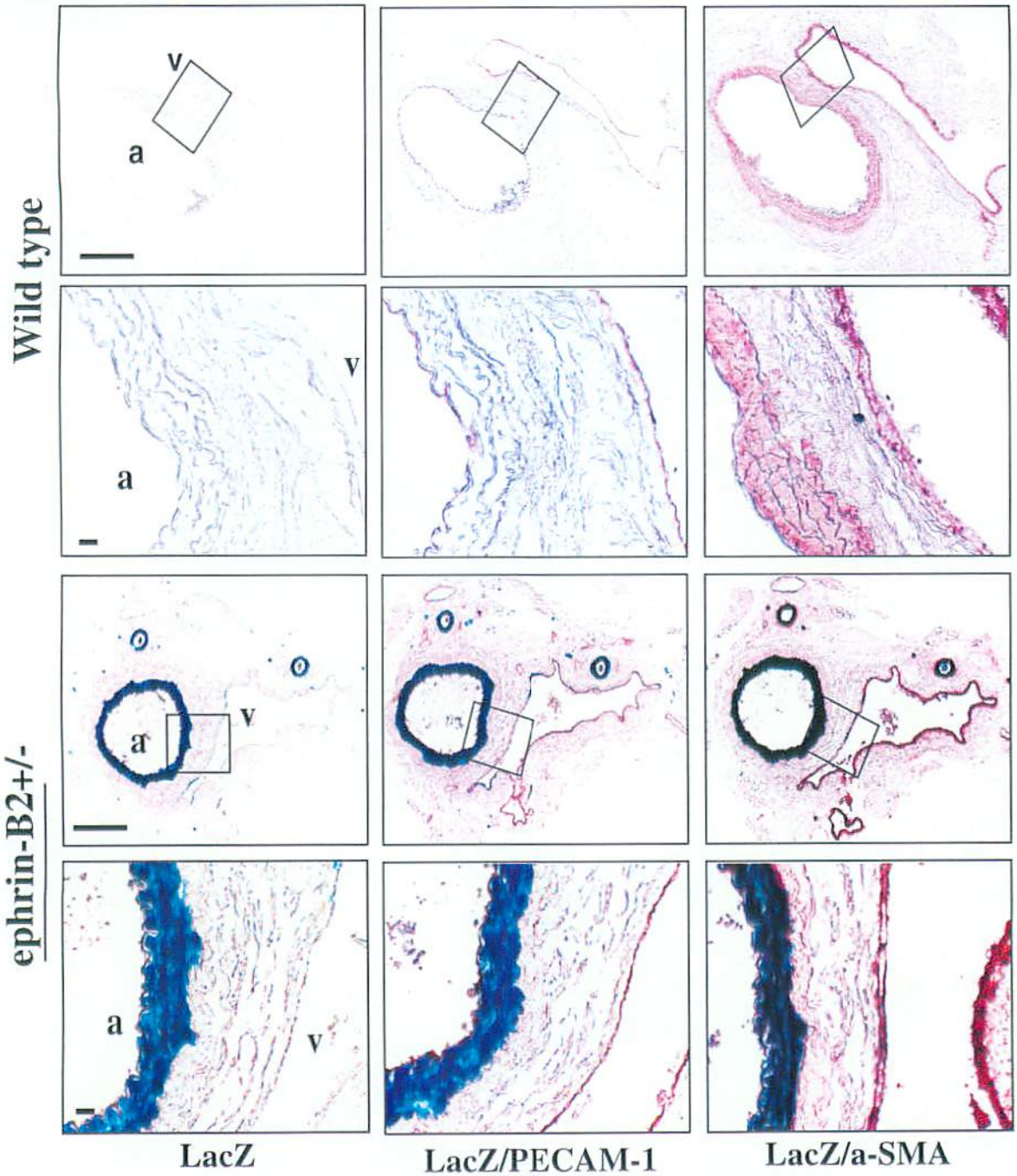


Fig 7. Expression of ephrin-B2 in the both of adult mouse arterial ECs and SMCs. The abdominal / thoracic aorta, superior / inferior vena cava and surrounding tissues from adult wild type or ephrin-B2 heterozygous mice were stained with LacZ/ephrin-B2. After LacZ staining, the tissues were re-fixed and sectioned, the double-stained by anti-PECAM-1 or α-SMA antibodies. The LacZ/ephrin-B2 (blue) was co-expressing with PECAM-1/CD31 (red) and α-SMA (red) in the arterial ECs and SMCs, but not venous ECs and SMCs, the PECAM-1/CD31 and α-SMA stained cells were identical with the expression in arterial ECs and SMCs and ECs and SMCs in wild type mice. A: aorta; V: vena cava. Each low panel are the high magnification of up panel. The bar indicates 25μm.

Result Fig 8

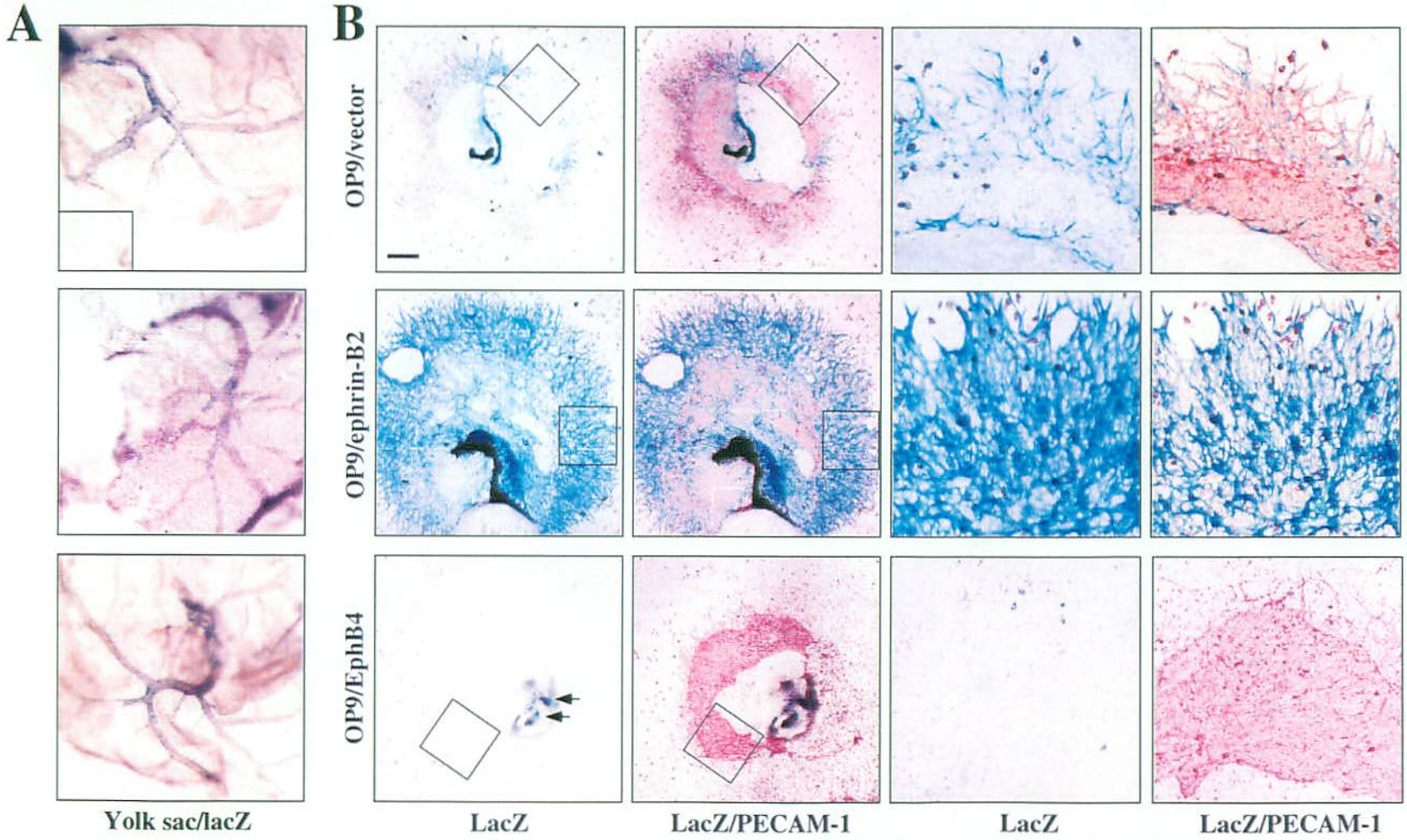


Fig 8. OP9/ephrin-B2 promoted ephrin-B2+ cell proliferation and sprouting, whereas OP9/EphB4 caused inhibition. (A) Whole-mount LacZ staining of the E9.0-9.5 yolk sac. The blue color in the vascular region indicates LacZ-positive staining. The phenotype in each panel corresponds to the panel in B. The inset shows LacZ staining in the wild-type yolk sac. (B) E9.5 ephrin-B2$^{+/LacZ}$ P-Sp explants were co-cultured with OP9/ephrin-B2, OP9/EphB4 or OP9/vector stromal cells for 14 days. LacZ and PECAM-1 staining have shown that the vascular network was formed by LacZ/ephrin-B2 (blue)/PECAM-1 (red) double-positive EC on OP9/ephrin-B2 stromal cells, and almost no LacZ/ephrin-B2^{+} EC were detected on OP9/EphB4 stromal cells. The vascular network was formed by LacZ/ephrin-B2 (blue)/PECAM-1 (red) double-positive EC and PECAM-1 single positive cells on OP9/vector stromal cells. The right two panels are a higher magnification of the area indicated by the box in the left two panels. The bar indicates 1mm (left two panels) and 80 μm (right two panels). The arrow on the OP9/EphB4 stromal cell plate indicates where some LacZ positive cells are available.

Result Fig 9

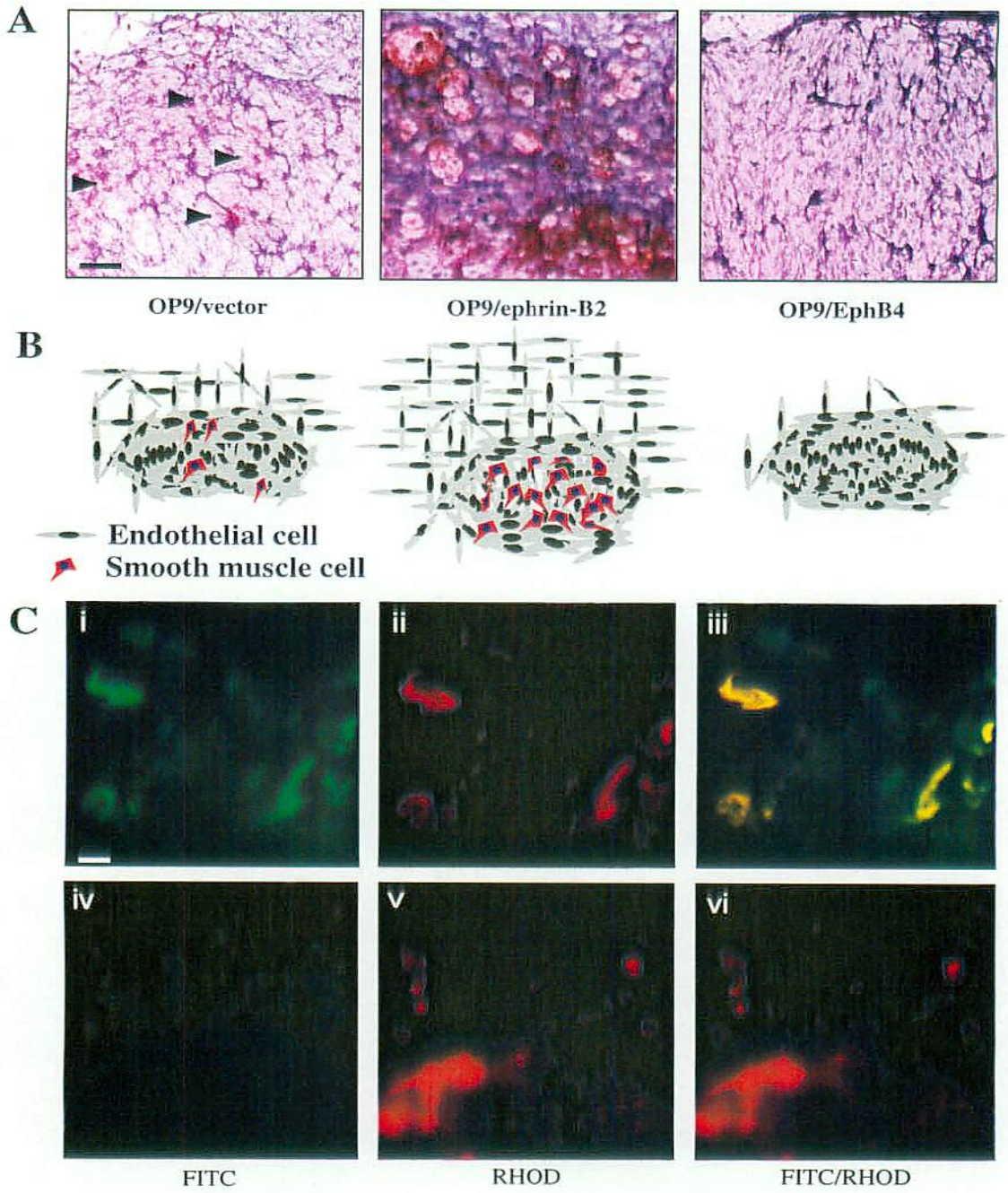
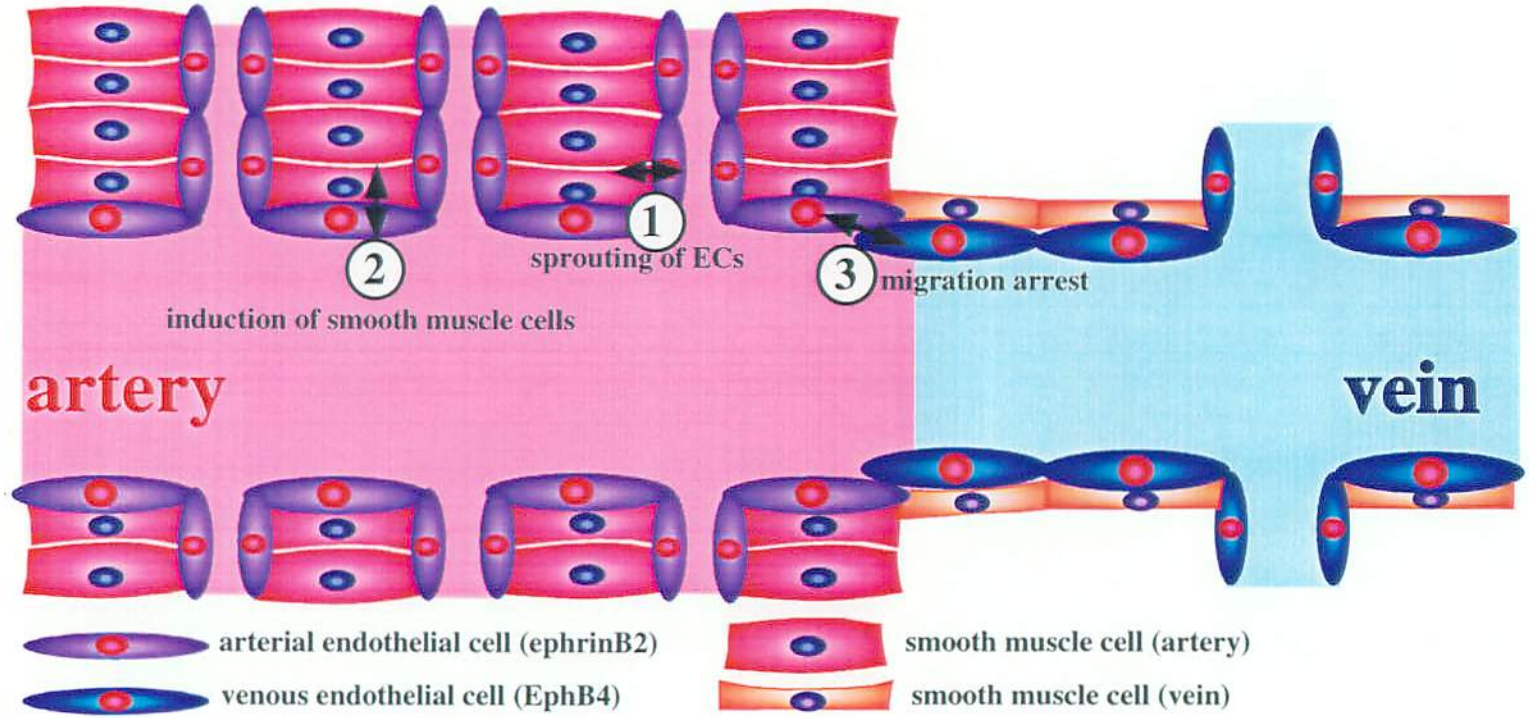


Fig 9. OP9/ephrin-B2 supported SMC recruitment, whereas OP9/EphB4 and EphB4-Fc inhibited it. (A) The vascular networks in the P-Sp explant cultures were visualized by PECAM-1 staining (blue), and the anti-a-SMA antibody was used to detect the SMC on the P-Sp culture system after co-culture for 14 days. The a-SMA+ (red) cells were located more abundantly in the vascular bed on OP9/ephrin-B2 stromal cells than in OP9/vector stromal cells (arrowheads indicate the a-SMA+ cells), and almost no a-SMA+ (red) cells were detected in the OP9/EphB4 stromal cells. The bar indicates 100 μm. (B) Schematic presentation of SMC recruitment under various conditions. Figures correspond to the upper panels in figure A. (C) P-Sp explants from embryos of 'green mice' wich expressing GFP ubiquitously and wild type embryos were co-cultured with OP9 stromal cells, and the culture plates were immunostained with Cy3-conjugated anti-a- SMA antibody. The green color indicates cells from GFP P-Sp explants, red indicates a-SMA+ cells, and yellow color indicates GFP and a-SMA double positive cells. i and iv are FITC-specific, ii and v are Rhodamine-specific wavelengths, and the merged configuration in iii and vi. The bar indicates 25 μm.

Conclusion



Model of ephrinB2-EphB4 signals in vasculo-angiogenesis.
1) EphrinB2 on SMC support proliferation and sprouting of arterial EC. 2) EphrinB2+ EC promot the recruitment of SMC near EC. Molecular cues for such induction are unknown; however, Tie2/Ang1, PDGF-BB and TGF-β may be involved in this process. 3) When the ephrin-B2+ EC (arterial EC) and EphB4+ (venous EC) face each other at the boundary of a capillary, cell proliferation of EC may be suppressed, and the migratory ability may be arrested there.