学 位 論 文
Doctor's Thesis

The role of Neuropilin-1 in vascular development
(Neuropilin-1の血管新生における役割)

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２００２年３月

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２００２年３月

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Summary

The blood vessel is formed by different two steps, termed vasculogenesis (formation of new blood vessels de novo) and angiogenesis (branching of preexisting blood vessels). During the last several years, a great deal of progress to define vasculogenesis and angiogenesis has been made by isolating factors such as a family of vascular endothelial cell growth factors (VEGFs), angiopoietins and ephrins. Among them, VEGFs and cognate receptor tyrosine kinases, VEGFR-1 and VEGFR-2, which are expressed specifically on the surface of endothelial cells (ECs) have been demonstrated to be required for both vasculogenesis and angiogenesis. Recent reports show that neuropilin-1 (NP-1) is expressed on ECs and tumor cells and that it is the third VEGF receptor.

First, we examined NP-1 functions on ECs. Some reports indicate that NP-1 works as a coreceptor with VEGFR-2 on ECs and enhances the function of $VEGF_{165}$, however, mechanism of signaling of NP-1 was not well understood. So we analyzed knock out mice of NP-1 and revealed that *np-1*$^{-/-}$ mutant mice are lethal at around embryonal (E) 12.5 day and embryonic death may be due to anomalies in the cardiovascular system and vascular defects in the central nervous system (CNS). We also detected that NP-1 deficient embryos are defective in angiogenesis, especially in the CNS and pericardium. To better analyze the function of NP-1 in ECs, we examined the vasculogenesis and angiogenesis of NP-1 deficient embryos by using *in vitro* P-Sp (para-aortic

splanchnopleural mesodermal) culture system. Using this culture system, we found that a monomer of soluble NP-1 inhibits vascular development in wild type P-Sp culture, but a dimer of soluble NP-1 can enhance the vasculogenesis. We also demonstrated that a dimer of soluble NP-1 together with VEGF effectively phosphorylate VEGFR-2 on ECs, although VEGF alone did not. Furthermore, we provided the evidence that a dimer of soluble NP-1 rescued the vascular defects of the *np-1*$^{-/-}$ embryos observed in CNS and pericardium.

Second, we investigated whether a dimer of soluble NP-1 exists or not in vivo. Although it was reported that truncated form of monomer soluble NP-1 existed in vivo, we could not detect a dimer form of it. On the other hand, instead of soluble NP-1, we found that CD45^{+} hematopoietic cells express NP-1 in fetal liver and adult bone marrow. Hematopoietic cells move freely in the vessels and reach to the point wherein the vessel formation is required, so we considered that they might work as a dimer form of soluble NP-1. At first, we confirmed the binding ability of CD45^{+}NP-1^{+} cells to $VEGF_{165}$ and CD45+NP-1+ cells together with VEGF165 induced that phosphorylation of VEGFR-2. Next, we indicated that CD45^{+}NP-1^{+} cells together with $VEGF_{165}$ effectively rescued the vasculogenesis and angiogenesis in vitro NP-1$^{-/-}$ P-Sp culture and induced vessel proliferation and migration in vivo matrigel assay. Finally, we showed that dimerization of NP-1 was critical for enhancing the signaling of VEGFR-2. We constructed L cell possessed various kind of extracellular domain of NP-1. In addition of L cells possessed only "a" domain or "a" and "b" domains to NP-1$^{-/-}$

P-Sp culture, defective vascularity was not rescued at both adherent and non-adherent area of L cells. On the other hand, in addition of L cells possessed "a", "b", and "c" domains to NP-$1^{-/-}$ P-Sp culture, defective vascularity was rescued at adherent area of L cells, however, it was not rescued at non-adherent area of L cells.

Taken together, we concluded that dimer type of soluble NP-1 enhanced vascular development exogenously, and $CD45^{+}$ cells worked as dimer type of soluble NP-1 in vivo and deliver $VEGF_{165}$ to the area where was required the vessel formation.

Publication list

1. Yamada Y[1,2], Ogawa H[2], Oike Y[1], Fujisawa H[6], Suda T[1], Takakura N[1]. Neuropilin-1 on Non-Endothelial Cells As a Source of Vascular Development. ***Blood*** (in revised)
2. Yamada Y[1,2], Takakura N[1], Yasue H[2], Ogawa H[2], Fujisawa H[6], Suda T[1]. Exogenous clustered neuropilin 1 enhances vasculogenesis and angiogenesis. ***Blood***, Vol.97, Number6, 1671-1678, 2001
3. Takakura N[1], Watanabe T[3], Suenobu S[1], Yamada Y[1], Noda T[4], Ito Y[5], Satake M[3], Suda T[1]. A Role for Hematopoietic Stem Cells in Promoting Angiogenesis. ***Cell***, Vol.102, 199-209, 2000

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Acknowledgments

These series of investigations were performed from 1998 to 2002, in the Department of Cell Differentiation, Kumamoto University School of Medicine.

I wish to extend my warmest thanks to Professor Toshio Suda, Chairman of the Department of Cell Differentiation, Kumamoto University School, who generously gave me help and advice throughout my study.

My great thanks are due to Dr. Nobuyuki Takakura, who was Associate Professor in the Department of Cell Differentiation, Kumamoto University School (now he is Professor of the Department of stem cell biology cancer research institute Kanazawa University). He introduced me to this field and gave me many advices and supports.

I also thank to Professor Hisao Ogawa, and pre-Professor Hirofumi Yasue, Chairman of the Department of Cardiovascular Medicine Kumamoto University School of Medicine, to give me an opportunity to learn about the field of vascular biology.

Finally, I'd like to dedicate this work to my father and mother, my wife Tomomi and my daughter Risa, especially my wife, who always listened to and encouraged me, and was a constant source of moral support.

Abbreviations

NP-1, neuropilin-1

EC, endothelial cell

P-Sp, para-aortic splanchnopleural mesodermal region

VEGF, vascular endothelial growth factor

$VEGF_{121}$, 121 amino-acid form of vascular endothelial growth factor

$VEGF_{165}$, 165 amino-acid form of vascular endothelial growth factor

PlGF, placental growth factor

VEGFR-1, tyrosine-kinase VEGF receptor-1

VEGFR-2, tyrosine-kinase VEGF receptor-2

VEGFR-3, tyrosine-kinase VEGF receptor-3

TIE1, tyrosine kinase with Ig and EGF homology domain 1

TIE2, tyrosine kinase with Ig and EGF homology domain 2

CNS, central nervous system

mAb, monoclonal antibody

Introduction

1. Construction of blood vessels

The blood vessels of the cardiovascular system are classified into arteries (elastic and muscular), resistance vessels (small arteries and arterioles), capillaries, venules and veins (Fig. 1).

Large blood vessels share a common three-layered structure that is intimal, medial, and adventitial layer. Intimal layer comprises an endothelial cell monolayer supported by connective tissue. The endothelial cells play a crucial role in controlling vascular permeability, vasoconstriction, angiogenesis (growth of new blood vessels) and regulation of coagulation. Intimal layer is relatively thicker in larger arteries, and contains some smooth muscle cells in large and medium sized arteries and veins. The thick middle layer is separated from the intima, by fenestrated sheath, the intimal elastic lamina, mostly composed of elastin. The medial layer contains smooth muscle cells embedded in an extracellular matrix composed mainly of collagen, elastin and proteoglycans. An external elastic lamina separates the medial layer from the outer layer, the adventitial layer. This contains collagenous tissue supporting fibroblasts and nerves. In large arteries and veins, the adventitial layer contains vasa vasorum, small blood vessels which also penetrate into the outer portion of the media and supply the vascular wall with oxygen and nutrients.

Although veins share the basic three-layered structure, the layers are less distinct in the venous system. Compared to arteries, veins have a thinner

tunica media containing a smaller amount of smooth muscle cells, which also tend to have a more random orientation.

Capillaries and postcapillary venules are tubes formed of a single layer of overlapping endothelial cells. This is supported and surrounded on the external side by the basal lamina, a 50-100nm-thick layer of fibrous proteins including collagen, and glycoproteins. Pericyte, isolated cells which can give rise to smooth muscle cells during angiogenesis, adhere to the outside of the basal lamina, especially in postcapillary venules.

2. Cells constructing blood vessels

1) Origin of endothelial cell and hematopoietic cells

During the primitive streak stage, groups of mesodermal cells aggregate in the developing yolk sac to form the extraembryonic blood islands. Cells at the periphery of these aggregates differentiate into angioblast, the precursors of the blood vessels, while cells in the interior become hematopoietic stem cells (HSCs), the precursors of all the blood cells.[1] The close spatial and temporal association between the developments of these two cell lineages has led to the hypothesis that they have a common precursor, the "hemangioblast" (Fig.2, 3, ref.2, 3, 4). Although the yolk sac contains hematopoietic activity as early as 7-8.5 days postcoitum (dpc), these progenitors have limited potential.[5,6,7,8] Recent evidence suggests that pluripotent HSCs with long-term repopulating potential (LTR-HSC) populate the yolk sac secondarily from an intraembryonic source.

LTR-HSC can develop from a para-aortic splanchnopleural mesoderm (P-Sp) or dorsal aorta, genital ridge/gonads and pro/mesonephros (aorta-gonad-mesonephros, AGM) by 10.5 dpc.[9] HSCs and angioblasts from this region are then presumed to colocalized both the yolk sac and fetal liver, where they give rise to definitive hematopoietic precursors after 12.5 dpc.[10] Finally, HSCs and angioblasts are produced in bone marrow.

2) Origin of the mural cells

Mural cells are divided into two cell types. One is smooth muscle cell, which is largely distributed surrounding at arteries and resistance vessels. The other is pericytes, which are largely distributed surrounding at capillaries and venules. They arise from two different primary lineages in development. One lineage descends from the cardiac neural crest and is therefore ectodermal in origin.[11] The other lineage originates from lateral mesoderm-derived mesenchyme that surrounds the developing outflow elastic arteries (Fig.3, ref.11,12, 13).

3. Formation of blood vessels

1) Vasculogenesis

The early vascular plexus forms from mesoderm by differentiation of angioblasts, which subsequently generate primitive blood vessels. The molecular mechanisms responsible for this process are termed vasculogenesis (Fig.3, 4, ref.14).

During vasculogenesis, blood vessels are created de novo from the lateral plate mesoderm (Fig.3). In the first phase of vasculogenesis, groups of splanchnic mesoderm cells are specified to become hemangioblasts, the precursors of both the blood cells and the blood vessels (Fig.4, ref.15). In the second phase, the angioblasts multiply and differentiate into endothelial cells, which form the lining of the blood vessels. In the third phase, the endothelial cells form tubes and connect to form the primary capillary plexus, a network of capillaries (Fig.4, ref.16).

Three growth factors may be responsible for initiating vasculogenesis. One of these, basic fibroblast growth factor (bFGF) is required for the generation of hemangioblasts from the splanchnic mesoderm (Fig.3). When cells from quail blastodiscs are dissociated in culture, they do not form blood islands or endothelial cells.[17] However, when these cells are cultured in bFGF, blood islands emerge and form endothelial cells.[17] The second protein is vascular endothelial growth factor (VEGF). VEGF appears to enable the differentiation of the angioblasts and their multiplication to form endothelial tubes (Fig.3, 4). VEGF is secreted by the mesenchymal cells near the blood islands, and the hemangioblasts and angioblasts have receptors for VEGF.[18] If mouse embryos lack the genes encoding either VEGF or VEGFR-2 (Flk-1), yolk sac blood islands fail to appear, and vasculogenesis fails to take place.[19,20,21] Mice lacking genes for VEGFR-1 have differentiated endothelial cells and blood islands, but these cells are not organized into blood vessels (Fig.5, ref.22). A third protein,

angiopoietin-1 (Ang-1), mediates the interaction between the endothelial cells and the pericytes – smooth muscle like cells they recruit to cover them.[23,24,25]

2) Angiogenesis

After the primary vascular plexus is formed, more endothelial cells are generated, which can form new capillaries by sprouting or by splitting from their vessel of origin in a process termed angiogenesis (Fig.3, 4). There are at least two different types: true sprouting of capillaries from pre-existing vessels, and non-sprouting angiogenesis or intussusception (Fig.4).

For endothelial cells to emigrate from their resident site, they need to loosen interendothelial cell contacts and to relieve periendothelial cell support; that is, mature vessels need to become destabilized. Angiopoietin-2 (Ang2), natural antagonist of Angiopoietin-1 (Ang-1), inhibits binding of Ang-1 to Tie-2 and induces detachment of smooth muscle cells from endothelial cells and looseness the matrix.[26,27] Proteinases of the plasminogen activator, especially urokinase-type plasminogen activator (u-PA), matrix metalloproteinases (MMP), especially MMP-1,2,3,7,9,14, which are produced from endothelial cells, influence angiogenesis by degrading matrix molecules and by activating or liberating growth factors (bFGF, VEGF and IGF-1).[28] The exposed endothelial cells proliferate and sprout from their resident site, eventually forming a new vessel. New vessels can also be formed in the primary capillary bed by splitting an existing vessel in two. The loosening of the cell-cell contacts may also allow

the fusion of capillaries to form wider vessels – the arteries and veins. Eventually, the mature capillary network forms and is stabilized by TGF-β, which strengthens the extracellular matrix, Ang-1 and platelet-derived growth factor (PDGF), which are necessary for the recruitment of the pericytes that contribute to the mechanical flexibility of the capillary wall.[29]

A key to our understanding of the mechanism by which veins and arteries form was the discovery that the primary capillary plexus in mice actually contains two types of endothelial cells. The precursors of the arteries contain EphrinB2 in their cell membranes. The precursors of the veins contain one of the receptors for this molecule, EphB4 tyrosine kinase, in their cell membranes.[30] It is thought that EphB4 interacts with its ligand, EphrinB2, during angiogenesis in two ways. First, at the borders of the venous and arterial capillaries, it ensures that arterial capillaries connect only to venous ones. Second, in non-border areas, it might ensure that the fusion of capillaries to make larger vessels occurs only between the same types of vessel.

4. Key regulators for vasculogenesis and angiogenesis

1) The VEGF family

There are five characterized VEGF relatives in mammals, which are VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF, and they display differential interactions with three related receptor tyrosine kinases, which are VEGFR-1/Flt-1, VEGFR-2/Flk-1, VEGFR-3/Flt-4, and ancillary receptor components,

such as the neuropilins (Fig.7). VEGFR-1 and VEGFR-2 are restricted largely to vascular endothelium in their expression. Interestingly, although VEGFR-3 is restricted largely to lymphatic endothelium (Fig.7, ref.31), mice that contain a knockout of the gene for VEGFR-3 display early embryonic lethality due to defects in the organization of large vessels prior to the emergence of lymphatics.[32]

The roles of VEGF-A and its receptors, VEGFR-1 and VEGFR-2, have been well characterized. In vitro, VEGF-A seems to have the ability to induce endothelial cell proliferation as well as migratory and sprouting activity, and to help promote endothelial cells to form tubule-like structures; these effects seem to be mediated largely by the VEGFR-2. Consistent with these in vitro actions, VEGF-A and VEGFR-2 are absolutely critical for the earliest stages of vasculogenesis in vivo, as blood islands, endothelial cells, and major vessel tubes fail to develop in appreciable numbers in embryos lacking either VEGF-A or VEGFR-2 (Fig.4,7,9, ref.19, 20, 21). Interestingly, even loss of single VEGF-A allele results in embryonic lethality, demonstrating a remarkably strict dose-dependence for VEGF during development (Fig.4, ref.20, 21). Mice lacking VEGFR-1 revealed a rather late role for this receptor as compared to VEGFR-2, as vessels do form in these mutant embryos, but are organized abnormally, with seemingly excess levels of endothelial cells being generated and entering into the lumens of the abnormal vascular channels.[22] Thus, VEGFR-1 may actually be involved in down-regulating VEGF activity to ensure

that the right numbers of endothelial cells are generated. Remarkably, mutant mice containing a VEGFR-1 lacking its tyrosine kinase domain allow for normal vascular development[33], indicating that only the binding portion of this receptor may be necessary to support its major actions during vascular development.

2) The Angiopoietin family

The angiopoietins were discovered as ligands for the Ties, family of receptor tyrosine kinases that are as selectively expressed within the vascular endothelium (despite expression in some other cells, such as in the hematopoietic lineage) as are the VEGF receptors (Fig5, ref34,35,36,37). There are now four definitive members of the angiopoietin family, although Ang-3 and Ang-4 may represent widely diverged counterparts of the same gene locus in mouse and human.[38] All of the known angiopoietins bind primarily to Tie-2, and it is unclear whether there are independent ligands for the second Tie receptor, Tie-1 (Fig.5).

In vitro analyses on cultured endothelial cells revealed that Ang-1 could not elicit standard mitogenic responses as can VEGF-A nor induce tubule formation, although it could apparently promote endothelial cell sprouting in vitro.[23,39]

Consistent with these in vitro results, in vivo studies reveal that Ang-1 seems to act in complementary and coordinated fashion with VEGF, having a later role in vascular development (Fig.3, 4). Thus, in mouse embryos lacking either Ang-1 or Tie-2, the early stages of VEGF-dependent vascular development

appear to occur rather normally, resulting in the formation of a primitive vasculature.[24,40,41] However, remodeling and stabilization of this primitive vasculature is severely perturbed, leading to embryonic lethality.[24,41,42] These defects are thought to result from disruptions in Ang-1-mediated interactions between endothelial cells and surrounding support cells such as smooth muscle cells; support cells produce the Ang-1 that paracrinely acts on endothelial cells expressing Tie-2 receptors (Fig.3,4).

Shortly after the discovery of Ang-1, the cloning of Ang-2 was described.[27] Remarkably, although Ang-2 bound to the Tie-2 receptor as did Ang-1, it could not activate it (Fig.5). Instead, Ang-2 provided the first example of a naturally occurring antagonist for a vertebrate tyrosine kinase. Consistent with the notion that Ang-2 acts as a natural antagonist for the Ang-1/Tie-2 interaction, transgenic overexpression of Ang-2 during embryogenesis leads to a lethal phenotype reminiscent of that seen in embryos lacking either Ang-1 or Tie-2, with severe disruptions in vascular development.[27,43]

3) The ephrins

The Eph receptor tyrosine kinases comprise the largest known family of growth factor receptors, and use the similarly numerous ephrins as their ligands.[26]

The ephrins are unlike ligands for other receptor tyrosine kinases in that they must be tethered to the membrane to activate their Eph receptors.[26,44] Although

initially characterized in the nervous system as well as neuropilins, recent knockout studies have suggested key roles for ephrin-B2 and EphB4 receptor during vascular development (Fig.5, ref.30,45,46). Mouse embryos lacking ephrin-B2 and EphB4 suffer fatal defects in early angiogenic remodeling that are somewhat reminiscent of those seen in mice lacking Ang-1 or Tie-2. Moreover, ephrin-B2 and EphB4 display remarkably reciprocal distribution patterns during vascular development, with ephrin-B2 marking the endothelium of primordial arterial vessels while EphB4 marks the endothelium of primordial venous vessels.[30,45,46]

These distributions suggested that ephrin-B2 and EphB4 are involved in establishing arterial versus venous identity, perhaps in fusing arterial and venous vessels at their junctions, and that defects in these process might account for the early lethality observed in mouse embryos lacking these proteins.[30,45,46]

4) Neuropilins

Among many blood vessel related proteins, NP-1 has a unique character. NP-1 had been first described as a cell surface glycoprotein expressed on axons in the developing nervous system[47] and shown to be a receptor for the semaphorin/collapsin[48,49], a family of transmembrane and secreted glycoproteins that act as mediators of neuronal guidance.[48,50] Secreted collapsin-1 (Sema3A) binds to NP-1 on axons, repels neurons, and induces the collapse of DRG neuronal growth cones (Fig.6). Recently, plexin has been identified as a

second semaphorin-binding protein.[51,52] Several analyses indicated that NP-1 and Plex1 form a complex with enhanced affinity for Sema3A (Fig.6, ref.53). Furthermore, this NP-1/Plex1 complex, but neither protein alone, can mediate a morphologic response to Sema3A in nonneuronal cells.[53] Other than neuronal system, NP-1 was identified another VEGFR which binds $VEGF_{165}$ but not $VEGF_{121}$ (Fig.7, ref.54). When the NP-1 was co-expressed on VEGFR-2 positive ECs, binding to $VEGF_{165}$ and chemotactic activity by $VEGF_{165}$ for these cells were enhanced, compared with ECs expressing VEGFR-2 alone suggesting that in ECs, NP-1 acts as coreceptor for VEGRF-2. Moreover, Sema3A inhibited the motility of porcine aortic ECs in NP-1 dependent manner by disrupting the formation of lamellipodia and inducing depolymerization of F-actin.[55] $VEGF_{165}$ and Sema3A are competitive inhibitors of each other in binding, EC motility, and DRG collapse assay[55], suggesting that the two ligands have overlapping NP-1 binding sites, possibly the b/coagulation factor homology domain.[56] Recently, a 2.2-kb truncated NP-1 cDNA was cloned that encodes a 644-aa soluble NP-1 (sNP-1) isoform containing just the a/CUB and b/coagulation factor homology extracellular domains of NP-1 (Fig.8, ref.57). sNP-1 is secreted by cells as a 90-kDa protein that binds $VEGF_{165}$, but not $VEGF_{121}$. It inhibits $VEGF_{165}$ binding to endothelial and tumor cells and $VEGF_{165}$-induced tyrosine phosphorylation of VEGFR-2 in ECs.

Many evidences were shown that modification of the *np-1* gene altered vascular development in vivo. Overexpression of *np-1* gene in the mouse led

excess capillary and blood vessel, malformed heart.[58] Targeted disruption of *NP-1* led embryonic lethality at E10.5 to E12.5 and vascular defects such as impairment of neural vascularization, transposition of large vessels, and insufficient development of vascular networks in the yolk sac (Fig.9, ref.59). These results suggest that NP-1 is one of the key regulators in vascular development.

5. Interaction of non-endothelial cells with endothelial cells for vessel formation

1) Neural cells

Several anatomical parallels between the vascular and nervous systems can be readily identified. For example, like blood vessels with their mural and endothelial cells, nerves comprise two basic and intimately associated units: neurons and glia. As with blood vessels, nerves are pervasive, ramifying throughout nearly all domains of the body. Analogous to arterial and venous blood flow, the directionality of information in the nervous system is controlled by subdivision of nerves into interconnected motor and sensory pathways. And, in addition to sharing several morphological features, nerves and blood vessels have a clear physical relationship, as seen with the vasa nervorum, and the perivascular autonomic nerve fibers which control vascular tone (Fig.10, ref.60,61).

There are an increasing number of examples where the two organ systems

appear to use related developmental mechanisms. In terms of behavior, it has been long known that both nerves and blood vessels often follow similar routes and modes of migration during embryogenesis (Fig.10,ref62,63). In terms of molecules, observations in mouse and zebrafish have provided increasing evidence that blood vessel architecture is, partly, determined by families of proteins and signaling relays which have first been described in the developing nervous system, such as ephrin/Eph and NP-1 signaling. Therefore, angiogenesis and neurogenesis may have some interactions through the utilization of these same molecular tools (Fig11).

2) Hematopoietic cells

Hematopoietic cells have been suggested to be one of the regulators of the development of blood vessels. Recently, we reported that angiopoietin-1, a ligand for TIE2, is produced by hematopoietic stem cells and that it regulates the formation of the vascular network in the restricted region *in vivo*.[64] Moreover, VEGF and PlGF (placental growth factor) expressed in erythroblasts have been suggested to be involved in blood vessel formation[65], and macrophages and mast cells have been reported to be involved in tumor angiogenesis.[28]

In this report, we found that $CD45^+$ hematopoietic cells in the murine fetal liver and adult bone marrow express NP-1. These hematopoietic cells bind $VEGF_{165}$ and phosphorylate VEGFR-2 positive endothelial cells exogenously. Moreover, we found that $CD45^+$ hematopoietic cells rescued the in vitro and in

vivo defective vascular formation of NP-1 mutants by P-Sp culture and matrigel assay, respectively. These results suggest that hematopoietic cells, including HSCs, are one of the important inducers of angiogenesis wherein vessel formations are required.

3) Stromal cells

Stromal cells form the backbone of the bone marrow and provide a complex array of extracellular matrix proteins that facilitate cell-cell interactions. In addition, stromal cells are instrumental in providing various soluble or resident cytokines for controlled differentiation and proliferation of early hematopoietic progenitors. Molecules mediating interactions between hematopoietic cells and stromal cells are either transmembrane or secreted proteins.

Recent reports indicated that NP-1 is expressed in MS-5 stromal cells, in other hematopoietic cell lines, and in primary stromal cells[66], however, precise mechanism how NP-1 on stromal cells regulates hematopoiesis was not understood. In our reports, we used OP9 stromal cells overexpressing NP-1 and showed that NP-1 on stromal cells enhanced vasculogenesis and angiogenesis. This indicates that NP-1 on stromal cells may regulate vascular development as well as hematopoiesis.

4) Tumor cells

Tumorigenesis is invariably a multistep process,[67,68] that involves not only

transformed cells but also an assemblage of normal support cells, including stromal fibroblasts, and endothelial cells.[69,70] Tumor growth demonstrably depends on angiogenesis, whereby concomitant increases in the tumor vasculature supply nutrients and oxygen to the expanding neoplastic mass.[71] Some tumor cells, such as 231 breast carcinoma cells and PC3 prostate carcinoma cells express NP-1 abundantly. On the other hand, these tumor cells do not express VEGFR-2 or Flt-1, so NP-1 is the only VEGF receptor associated with these cells. The role of NP-1 in tumors in the absence of high affinity receptors is unknown.

It is reported that NP-1 supported VEGF autocrine function, which protected tumors from hypoxia-induced apoptosis, in cells lacking VEGFR-2 expression by stimulating the PI3-kinase pathway.[72] This finding raises the possibility that NP-1 functions either alone or in concert with other tyrosine kinase-linked receptors to transducer VEGF signaling in tumors.

6. Design of this study

Recently, NP-1 was identified as the third VEGF receptor and enhanced endothelial cells growth as a co-receptor of VEGFR-2.[54] We have not known the precise mechanism of signal transduction through NP-1, because the intracellular domain of NP-1 is short and contains no motifs known to participate in signal transduction.[73] Moreover, smooth muscle cells[58], stromal cells[66] and tumor cells[54] have been reported that they also express NP-1, however, the

significance why they express NP-1 is not well understood.

In this study, we started to analyze the function of NP-1 in vascular development using NP-1 mutant embryos. To better analysis, we established in vitro P-Sp culture system which recapitulated vasculogenesis and angiogenesis during embryonic stage (Fig.12,13,14). Using this system, we elucidated that NP-1 was important for vasculogenesis and angiogenesis in embryonic stage. We also clarified that dimerization of NP-1 was important for inducing the signal transduction through NP-1 and tried to clarify the function of NP-1 expressing smooth muscle cells, stromal cells, and tumor cells.

Based on these analyses, we advance our research as a goal that we clarify the function of NP-1expressing various kinds of cells in vascular development

Materials and Methods

Animals

C57BL/6 mice were purchased from Japan SCL (Shizuoka, Japan). *Np-1* heterozygous mutant mice[74] were housed in environmentally controlled rooms of our facility at Kumamoto University School of Medicine under the university guidelines for animal and recombinant DNA experiments. Genotype analysis of the neuropilin-1 mutants was performed by PCR as described.[74]

Immunohistochemistry

1. Embryo

Immunohistochemistry on embryos was performed as previously described.[75] In brief, embryos were fixed in 4% paraformaldehyde at 4°C overnight. The fixed embryos were then rinsed in phosphate-buffered saline (PBS), dehydrated in a methanol series, and stored in 100% methanol at -80°C. The dehydrated embryos were bleached in methanol plus 5% vol/vol hydrogen peroxide for 4 to 5 hours at 4°C. The bleached embryos were rehydrated and blocked in PBSMT (2% instant skim milk, 0.1% Triton X-100, PBS) for 2 x 1 hour. The embryos were then incubated with a 1:500 dilution anti-PECAM-1 antibody (MEC13.3, rat-anti-mouse monoclonal; Pharmingen, SanDiego, CA) in PBSMT overnight at 4°C. The next day, the embryos were washed with PBSMT at 4°C 5 x 1 hour and incubated at 4°C with 1μg/ml horseradish peroxidase-conjugated goat anti-rat IgG (Biosource; Camarillo, CA) in PBSMT overnight at 4°C.

Embryos were then washed with PBSMT 5 x 1 hour at 4°C and in PBST (0.1% Triton X-100, PBS) 3 x 20 minutes at room temperature. Peroxidase staining was performed by incubating the embryos in 0.3mg/ml DAB (Dojin Chem.; Kumamoto, Japan) and 0.8mg/ml $NiCl_2$ (Wako Pure Chemical Ind., Ltd., Osaka, Japan) in PBST for 20 minutes, and then adding hydrogen peroxide at a final concentration of 0.05%. Rinsing in PBST stopped the staining reaction.

2. Sections and culture dishes

Immunohistochemistry on tissue sections and culture dishes were performed as previously described.[75] Tissue fixation procedures were basically the same as described.[76] Fixed specimens were embedded in polyester wax and sectioned at 8μm. An anti-PECAM-1 antibody (Pharmingen), anti-NP-1 polyclonal antibody[58], and anti-myc monoclonal antibody (Invitorogen, Carlsbad, CA) were used in this assay. In brief, anti-PECAM-1 antibody was developed with horseradish peroxidase-conjugated anti-rat IgG antibody (Biosource; Camarillo, CA). In the final step of staining, samples were soaked with PBS containing 250μg/ml diaminobenzidine (Dojin Chem) in the presence of 0.05% $NiCl_2$ for 10min, and hydrogen peroxidase was added to 0.01% for the enzymatic reaction. An anti-NP-1 polyclonal antibody was developed by incubating Envision alkaline phosphatase (DAKO). The enzymatic reaction with BCIP/NBT stock solution (Boehringer Mannheim, Mannheim, Germany) was allowed to proceed until the desired color intensity was reached. Finally, the

sections were observed and photographed under microscope (IX-70, Olympus, Tokyo, Japan). An anti-myc antibody was developed by incubating Envision peroxidase (DAKO). In the final step of staining, samples were soaked with PBS containing 250μg/ml diaminobenzidine, and hydrogen peroxidase was added to 0.01% for the enzymatic reaction.

FACS analysis

1. Cell sorting

Embryos were staged by somite counting. The whole mount embryos of wild type and *np-1* mutants were dissociated by DispaseII (Boehringer Mannheim, Mannheim,Germany) and drawn through a 23G needle. The cell-staining procedure for the flow cytometry was as described previously.[77] The monoclonal antibodies (mAbs) used in immunofluorescence staining were anti-VEGFR-2 antibody (Pharmingen), and anti-PECAM-1 antibody (Pharmingen). All mAbs were purified and conjugated with PE (phypoerythrin) or biotin. Biotinylated antibodies were visualized with PE-conjugated streptavidin (GIBCO-BRL) or APC-conjugated streptavidin (CALTAG, Burlingame,CA). Cells were incubated for 15 min on ice with CD16/32 (FcγIII/II Receptor) (1:100) (Fcblock™, Pharmingen) prior to staining with primary antibody. Cells were incubated in 5% FCS/PBS (washing buffer) with primary antibody for 30 min on ice, and washed twice with washing buffer. Secondary antibody was added and the cells were incubated for 30 min on ice. After incubation, cells were

washed twice with, and suspended in washing buffer for FACS analysis. The stained cells were analyzed and sorted by FACSvantage (Becton Dickinson, San Jose, CA). Sorted VEGFR-2$^+$ PECAM-1$^+$ cells were cultured on OP9 cells in 10% FCS and 10^{-5}M 2ME containing RPMI1640 supplemented with VEGF (1ng/ml) and NP-1-Fc (50μg/ml) or CD4-Fc (50μg/ml) at 37°C in a 5%CO_2 incubator.

2. Analysis the expression of $VEGF_{165}$ receptor

Fluorokine biotinylated VEGF (R&D systems,Inc) was used for this binding assay. This assay was performed according to the protocol provided by R&D system. In brief, cells from E12.5 fetal liver were stained with a PE conjugated anti-CD45 mAb (Pharmingen) and an APC conjugated anti-B220 mAb (Pharmingen) and then, CD45$^+$B220$^+$ cells were sorted using FACSvantage (Becton Dickinson, San Jose, CA). 10μl of biotinylated VEGF reagent was added to 25μl of the CD45$^+$B220$^+$ cells ($4x10^6$ cells/ml). As a negative control, an identical sample of cells was stained with 10μl of biotinylated negative control reagent. Cells were incubated for 60 minutes on ice. Then, 10μl of avidin-FITC reagent was added to each sample, and incubated the reaction mixture for a further 30 minutes on ice. After incubation, cells were washed twice with 2ml of 1xRDF1buffer (wash buffer) to remove unreacted avidin-FITC and resuspend the cells in 0.2ml of 1xRDF1 buffer for flow cytometric analysis. The stained cells were analyzed and sorted using FACSvantage. Total RNA from sorted

$CD45^+B220^+VEGFR^+$ cells was isolated by RNeasy mini kit (QIAGEN). Then, RT-PCR and PCR using VEGFRs specific primers were performed as below.

Immunoprecipitation and immunoblotting

Dissociated cells from E12.5 wild-type or *np-1* mutant embryo as described above were cultured on OP9 cells in 10% FCS and 10^{-5}M 2ME containing RPMI1640 supplemented with VEGF (10ng/ml) and b-FGF (10ng/ml) (Pepro Tech EC LTD,London,UK) at 37°C in a 5%CO_2 incubator. After 7 days, cells were harvested, labeled with PECAM-1-PE and VEGFR-2-biotin antibodies and sorted with FACSvantage. PECAM-1 and VEGFR-2 double positive cells were transferred to fibronectin (FN)- coated dishes (IWAKI GLASS, Chiba, Japan) and cultured in serum-free conditioned medium for 24 hr. ECs of wild type or *np-1* mutants were stimulated with 1ng/ml VEGF in the presence or absence of 50μg/ml NP-1-Fc for 10min at 37°C. Immunoprecipitation and Immunobloting were performed as previously described.[78]

The bEND3 cell lines were cultured in serum free medium 12hr before stimulation. $CD45^+$ cells from E12.5 fetal liver of NP-1 wild and mutants were incubated with various concentration of VEGF, and, or NP-1 flag for 30minutes on ice, and were washed twice with PBS (-) to remove unreacted VEGF. Then, bEND3 cells were stimulated with incubated $CD45^+NP\text{-}1^+$ cells or $CD45^+NP\text{-}1^-$ cells for 10minutes at 37℃. The cells were solubilized with lysis buffer (20mM HEPES, pH 7.4, 137mM NaCl, 5mM EDTA, 1mM Na3VO4, 1% Triton X-100,

and protease inhibitors). Immunoprecipitation and immunobloting were performed as previously described.[78]

Cell culture

1. Production of recombinant fusion protein

Recombinant fusion proteins of the full length extracellular domain of murine surface molecules and the Fc part of human IgG and FLAG epitope were designed (Fig.16, ref.79). NP-1-Fc, CD4-Fc, NP-1-FLAG, and control-FLAG were produced by COS7 cells in serum-free conditioned medium as previously described.[79,80] Fc fusion protein was purified over a protein-A column (Bio-Rad Richmond, CA) and FLAG fusion protein was purified over an anti-FLAG M2 column (Scientific Imaging System Eastern Kodak Company, Rochester, NY). Their purity and disulfide-linked dimerization were assessed by Coomassie Brilliant Blue staining of 6% SDS gels.

2. Preparation of retroviral vectors expressing Neuropilin-1

Neuropilin-1 expression retroviral vectors were generated by ligating full-length cDNA into the pMY-IRES-GFP vector provided by Dr. T. Kitamura (University of Tokyo, Institute of Medical Science). Retroviral vectors were transfected into Phoenix-ecotropic cells by Lipofectamine plus (Gibco-BRL) in Dulbecco's modified essential medium (DMEM,GIBCO-BRL) containing 10 % FBS. The medium was collected after 24 and 48 hours incubation as a virus

solution and stored at –80°C. For infection of OP9 stromal cells, retronectin coated plates (Takara, Otsu, Japan) were used. $2x10^5$ OP9 stromal cells were cultured on these plates with 10 MOI of retroviral constructs. After 24 hours of infection, the culture medium was changed to fresh medium. After 5 days of cultivation, GFP positive cells were detected and sorted by FACS Calibur (Becton Dickinson, San Jose, CA) and re-cultured at 37°C in a humidified 5%-CO2 air.

3.Expression of mutant NP-1 proteins in L cells

Production of various mouse NP-1 mutants cDNA transfected into the L cells were described previously.[80] In brief, the myc-tag sequence GGEQKLISEEDL in the NP-abcm, NP-abm and NP-am was introduced as follows. An Xbal site was added to the 3'-end of the coding region of NP-1 by PCR, and then the Xbal-myc- tag-stop codon adapter was ligated. In all mutant NP-1 cDNAs, the signal sequence was retained intact. To isolate cells that stably express truncated NP-1, L cells, a mouse fibroblastic cell line, were co-transfected with the truncated NP-1 cDNAs and pST-neoB[81] according to the calcium phosphate method and selected with GENETICIN (GIBCO-BRL).

4. In vitro culture of P-Sp

The stromal cell line, OP9[82], was maintained in α–modified minimum essential media (α-MEM, GIBCO-BRL, Gaitherburg, MD) supplemented with 20% fetal calf serum (FCS) (JRH Bioscience, Lenexa, KS). E9.5 P-Sp explants containing a part of the omphalomesenteric artery (OA) were cultured on OP9 stromal cells in 10% FCS and 10^{-5}M 2-mercaptoetanol (2-ME) (GIBCO-BRL) containing RPMI1640 (GIBCO-BRL) with or without full-length VEGF (Pepro Tech EC Ltd, London, UK) and NP-1-Fc or CD4-Fc, and NP-1-FLAG or control-FLAG. After 14 days in culture, an anti-PECAM-1 antibody (MEC13.3; rat-anti-mouse monoclonal; Pharmingen, SanDiego, CA) was used to visualize ECs.

In vivo angiogenesis assay

1.Injection of NP-1-Fc into the mice

We mated *np-1*$^{+/-}$ male and female mice, and checked for plugs the next morning (designated as 0.5 embryonal days.). From E9.5 to E11.5, pregnant mice were given daily intraperitoneal injections of 150μl of a 2mg/ml solution of NP-1-Fc or CD4-Fc (control protein). Embryos were harvested at E12.5 and immunohistochemistry on whole mount embryos or sections was performed by PECAM-1 monoclonal antibody as previously described.[75]

2.In vivo neovascularization using matrigel

Preparation, injection, and processing of matrigel (Becton Dickinson) were as

described[83] with some modifications. Briefly, 8-weel-old C57BL mice were injected subcutaneously with 0.5ml matrigel and 40 units heparin/ml (Sigma), 20ng/ml VEGF (Pepro Tec), and $5x10^4$ CD45+B220+ cells from the fetal liver of E12.5 NP-1 wild or mutant embryos. Matrigels were dissected on day 4 from mice, and gel sections were stained with FITC-conjugated anti-PECAM-1 mAb (Pharmingen).

RT-PCR analysis

RNeasy mini kit (Qiagen GmbH, Hilden, Germany) was used for isolation of total RNA from whole embryos, $CD45^+$ cells and $CD45^-$, $PECAM\text{-}1^+$ cells. Total RNA was reverse transcribed using a RT for PCR kit (Clontech, Palo Alto, CA). The cDNA were amplified using Advantage polymerase mix (Clontech) in a GeneAmp PCR system model 9700 (Perkin-Elmer Inc., Norwalk, CT) by 30 cycles. Sequences of gene specific primer for RT-PCR were as follows: 5'-mNP-1 (ACTGACAGCGCAATAGCAAAAGAAG), 3'-mNP-1 (TCGGACAAAT CGAGTATCAGTGGT), 5'-mVEGFR-1 (CTTCCTACAGCACAGCAGATGTGAA), 3'-mVEGFR-1 (CACGTTTACAATGAGAGTGGCAGTG), 5'-mVEGFR-2 (TACAC AATTCAGAGCGATGTGTGGT), 3'-mVEGFR-2 (CTGGTTCCTCCAATGGGATA TCTTC), 5'-mVEGF165 (CTTTACTGCTGTACCTTCACCATGC), 3'-mVEGF165 (AACAAGGCTCACAGTGATTTTCTGG), 5'-G3PDH (TGAAGGT CGGTGTGAACGGATTTGGC), 3'-G3PDH (CATGTAGGCCATGAGGTCCACC AC).

Each cycle consisted of 30sec denaturation at 94°C, 4min annealing/extension at 70°C.

Quantitative analysis of vascular areas

The method of immunohistochemistry in the P-Sp culture using PECAM-1 antibodies was performed as described above. After PECAM-1 immunohistochemical staining, images were integrated using a color chilled 3CCD camera (Hamamatsu Photonics, Shizuoka, Japan). Image processing software (NIH image 1.62 / Power Macintosh G3: National Institutes of Health, Bethesda, MD) was used to determine alterations in the size of vascular areas. Three vascular areas from each P-Sp explants were measured under each culture condition. The values of all parameters are shown as the mean and standard deviation. P values were calculated by two-tailed Student t test analysis.

Results

P-Sp explant culture system reflects the vascular defect in *np-1* mutant embryos.

To examine the vascularity of the homozygous *np-1* mutant (*np-1*$^{-/-}$) embryos, E12.0 *np-1*$^{-/-}$ and the wild-type (*np-1*$^{+/+}$) embryos from the same litter were stained with PECAM-1 mAb to visualize all ECs. No growth retardation was observed in mutants (Fig.15a, b). However, the vascularity of mutant embryos compared to wild type embryos was impaired in the CNS and pericardial regions (Fig.15d, f). At E12.0, vascular sprouting into the CNS and pericardium was observed in wild-type embryos, however few capillaries and branches were observed in mutants. In order to examine the vasculogenesis and angiogenesis of the *np-1*$^{-/-}$ embryo more precisely, P-Sp explants from E9.5 wild-type and homozygous mutant embryos were cultured with OP9 stromal cells supplemented with interleukin (IL)-6, IL-7, SCF, and Epo. We previously reported that this culture system supported the growth of both hematopoietic and endothelial cells.[64, 75] We also showed that this system recapitulated in vivo vasculogenesis and angiogenesis. Inhibition of VEGF signaling in this system using soluble VEGFR-2 led to lack of EC development, and inhibition of Ang1-tie-2 signaling using soluble tie-2 led to lack of network formation (Fig.14). After 10-14 days of culturing, in the wild-type embryos, ECs migrated from the P-Sp explant and formed vascular beds (sheet-like structure) and networks (cord-like structure) on OP9 cells (Fig.17a). In contrast, though a small number of ECs

developed, neither vascular beds nor networks formed in P-Sp explants from mutant embryos (Fig.17b).

The structure of NP-1 recombinant proteins

To analyze NP-1 function *in vitro* P-Sp culture, we produced recombinant fusion proteins containing the Fc part of human IgG1 or FLAG and the full-length of NP-1 ectodomain (NP-1-Fc and NP-1-FLAG; Fig.16A). We verified the molecular size of each protein by SDS-PAGE. The molecular size of each protein was expected size in reducing and non-reducing condition (Fig.16B). We then confirmed by Western blot (reducing and non-reducing conditions) that NP-1-Fc formed a dimmer NP-1 and NP-1-FLAG formed a monomer NP-1 (Fig. 16C).

Effects of soluble NP-1 on vasculogenesis and angiogenesis in P-Sp culture.

We previously reported that, when soluble receptors, such as VEGFR-2 or TIE-2, were added to the P-Sp culture system and free ligands were saturated in the culture, EC development was suppressed (Fig.14, ref.75). To test whether soluble NP-1 in P-Sp culture prevents vascular development by inhibiting the binding of $VEGF_{165}$ to VEGFR-2, excess amounts of NP-1-FLAG or NP-1-Fc were added to the P-Sp culture of wild-type embryo. We measured VEGF concentration presenting in P-Sp culture by ELISA and confirmed that 1.2±

0.4ng/ml (n=5) VEGF was produced in wild type cultures and 1.5±0.3ng/ml (n=5) VEGF was produced in mutant cultures.

On addition of 50μg/ml NP-1-FLAG to these cultures, vascular bed formation was suppressed (Fig.17c) but unexpectedly, on addition of 50μg/ml NP-1-Fc or NP-1-FLAG+anti-FLAG IgG (dimer form of NP-1-FLAG) to this culture, the vascular bed formation was expanded in wild type (Fig.17e, g). In *np-1* mutants, suppressed EC development was completely rescued by addition of NP-1-Fc or NP-1-FLAG+anti-FLAG IgG (Fig.17f, h), while the same amount of CD4-Fc (data not shown) or FLAG (Fig.17b), or anti-FLAG alone (data not shown), as a control protein, had no effect.

Effect of a dimer of soluble NP-1 on the growth of sorted endothelial cells.

P-Sp explants contain many cell types, including hematopoietic cells, endothelial progenitor cells, and other mesenchymal cells. To clarify whether NP-1-Fc affects ECs directly to induce vasculogenesis and angiogenesis, we sorted the ECs of E12.5 wild type and mutant embryos by FACS and cultured them on OP9 cells in the presence or absence of NP-1-Fc. Dual staining with anti-VEGFR-2 and PECAM-1 mAbs revealed that 0.3% of cells in wild type embryos and 0.5% of cells in mutant embryos were double positive (Fig.18A). VEGFR-2$^+$PECAM-1$^+$ cells were cultured on OP9 stromal cells in the presence of 1ng/ml VEGF (Fig.18B). VEGFR-2$^+$PECAM-1$^+$cells from wild type embryos formed vascular beds and networks (Fig.18Ba). In contrast, EC from mutant

embryos did not proliferate in the presence of 1ng/ml VEGF (Fig.18Bb). However, 50μg/ml NP-1-Fc in addition to 1ng/ml VEGF promoted vascular development (Fig.18Bc) as observed in the culture of ECs of wild type (Fig.18Ba). The same amount of CD4-Fc had no effect (data not shown). These findings indicate that NP-1-Fc enhances the proliferation of individual ECs through VEGFR-2.

To examine the role of NP-1-Fc in VEGFR-2 activation, we evaluated the phosphorylation of VEGFR-2 on the ECs. Whole embryos of the E12.5 wild type and mutant were dissociated and then cultured on OP9 stromal cells in the presence of 10ng/ml VEGF and bFGF for 7 days. The VEGFR-2$^+$ PECAM-1$^+$ ECs were then sorted by FACS, seeded onto FN-coated dishes, cultured for 24h under serum-free conditions, and subsequently challenged with VEGF. Although ECs from mutant embryos expressed slightly more VEGFR-2 than those from wild-type embryos (Fig.18A), this difference was lost after culture *in vitro* (data not shown). Cell lysates were immunoprecipitated with an anti-VEGFR-2 antibody and then subjected to Western blotting using an anti-phosphotyrosine mAb. Phosphorylation of the VEGFR-2 was induced by addition of a low dose of VEGF and NP-1-Fc in ECs from *np-1* mutants (Fig.18C;Lane4). VEGF alone did not induce the phosphorylation of VEGFR-2 in ECs from *np-1* mutants (Fig.18C;Lane3), while VEGF alone did induce phosphorylation in wild type (Fig.18C;Lane1).

Synergistic effect of VEGF and a dimmer of soluble NP-1 in EC growth.

We next examined the synergistic effect of NP-1-Fc and VEGF in P-Sp culture. P-Sp explants from wild type (Fig.19A a, c, e, g) and mutant (Fig.19A b, d, f, h) embryos at E9.5 were cultured on OP9 stromal cells as described above. The suppressed vasculature in the culture of *np-1*$^{-/-}$ embryos (Fig.19Ab) was rescued by addition of 50ng/ml VEGF (Fig.19Ad) or 50μg/ml NP-1-Fc (Fig.19Af). The pattern of the rescue differed between VEGF and NP-1-Fc. When VEGF alone was added to P-Sp culture of *np-1*$^{-/-}$ embryo (Fig.19Ad), the vascular bed was poorly formed compared with that of wild type (Fig.19Aa). In contrast, on addition of NP-1-Fc, the vascular bed and network formation were promoted to a level comparable that of wild type (Fig.19Aa, f). Of particular note, vascular formation was expanded by simultaneous addition of VEGF and NP-1-Fc in both wild type (Fig.19Ag) and *np-1* mutant explants (Fig.19Ah). Vascular areas from each P-Sp explant were measured under each culture condition. Quantitative analyses show that they were increased in the presence of VEGF and NP-1-Fc (Fig.19B). This suggests that VEGF and NP-1-Fc work synergistically in vascular formation.

***In vivo* effect of a dimer of soluble NP-1 in *np-1* mutant embryos**

To examine the *in vivo* effect of NP-1-Fc, we used matrigel assay system. We observed that NP-1-Fc with 20ng/ml VEGF promoted VEGFR-2-positive EC growth in matrigel (Fig.20b, d), although CD4-Fc with 20ng/ml VEGF did not

promote EC growth (Fig.20a, c). Next, we examined that pregnant mice were given daily intraperitoneal injections of 150μl of a 2mg/ml solution of either NP-1-Fc or CD4-Fc from E9.5 to E11.5. Embryos were harvested at E12.5 and immunohistochemistry on whole mount embryos or sections were performed using PECAM-1 monoclonal antibody to observe the vascularity of the embryos. We obtained 11 *np-1*$^{-/-}$ embryos and 56 *np-1*$^{+/+}$ or *np-1*$^{+/-}$ embryos from 6 dams that were injected with NP-1-Fc. Injection of CD4-Fc as a control into the *np-1* mutants had no effect (Fig.21a, c). On injection of NP-1-Fc vascularity in *np-1*$^{+/+}$ and *np-1*$^{+/-}$ was slightly enhanced; however, *np-1*$^{-/-}$ embryos exhibited significant recovery of vascularity, and interestingly, the caliber of the rescued capillaries was larger than that of wild type (Fig.15c, e and Fig.21b,d). We confirmed that NP-1-Fc bound to ECs in CNS and pericardium by staining with anti-human-IgG (data not shown). These findings indicate that NP-1-Fc affects endothelial cell proliferation and migration directly in *np-1*$^{-/-}$ embryos.

NP-1 expression on hematopoietic cells

Based on these experiments, we searched for naturally occurring dimer form of NP1 in vivo. Although a monomer of it has been already reported to exist in vivo, we could not detect a dimer form of NP1. Instead of a dimer form of NP1[57], we found NP-1 expression in the surface of hematopoietic cells among the components flowing in the blood vessels.

Firstly, to define which hematopoietic cells express NP1, several fractions of

CD45-positive cells from murine fetal liver at E12.5 or adult bone marrow were sorted and examined the expression of NP-1 mRNA by RT-PCR. In fetal liver, high expression of Np-1 was detected in CD45-Ter119+ erythrocyte, CD45+Mac-1+ monocyte, and CD45+B220+ B lymphocyte lineage. In contrast, in CD45+CD4+CD8+ T lymphocyte fraction and CD45+CD34+c-kit+ stem cell fraction, Np-1 expression was less abundant. In adult bone marrow, expression of NP1 was different from that in fetal liver. High expression of Np-1 is detected in CD45+CD4+CD8+ cells, Lin-c-kit+Sca-1+ stem cell fraction, and CD45+B220+ cells. We could not detect NP-1 expression in CD45-ter119+ erythrocyte fraction (Fig.22A). In case of the expression of $VEGF_{165}$, high expression of $VEGF_{165}$ was detected in $CD45^{+}Mac\text{-}1^{+}$ monocyte and $CD45^{-}Ter119^{+}$ erythrocyte lineage as previously reported.[66] Next, we investigated whether non-endothelial cells expressing NP-1 located at the site of vascular sprouting, focusing on CNS and pericardium, where vascular defects were observed in $NP\text{-}1^{-/-}$ embryos. In the pericardium, some of ECs did not express NP-1 but some of hematopoietic cells were stained by anti-NP-1 antibody, although in the endocardium and epicardium, ECs expressed NP-1 exclusively (Fig.22Ba, b). In the CNS, ECs and round cells in the lumen expressed NP-1 (Fig.22Bc, d), and these round cells were in the hematopoietic lineage confirmed by their expression of CD45 in the serial section (data not shown).

NP-1 on hematopoietic cells binds VEGF165 and induces phosphorylation

of VEGFR2 on ECs

In order to know whether NP-1 on $CD45^+$ hematopoietic cells binds $VEGF_{165}$, we checked the binding by flow cytometric analysis. As shown in Figure22, some population of CD45 hematopoietic cells expressed NP-1. As previously reported, monocytes, such as Mac-1 positive cells express VEGFR-1.[33] As far as we examined, $CD45^+$ $B220^+$ hematopoietic cells did not express VEGFR-1 and VEGFR-2. Therefore, in binding assay for $VEGF_{165}$, we used $CD45^+B220^+$ cells among these CD45+ hematopoietic cells to exclude the expression of other VEGFRs. At first, we obtained $CD45^+B220^+$ cells (2.6%) from fetal liver, and incubated with biotinylated $VEGF_{165}$. Subsequently binding of $VEGF_{165}$ to cells was visualized by staining with streptavidin-FITC (Fig.23A). Interestingly, most of $CD45^+B220^+$ cells bind $VEGF_{165}$. We sorted these $VEGF_{165}$ receptor positive cells (approximately 50% of VEGF binding cells as shown in Fig23A), and mRNA from such cells were analyzed the expression of VEGF receptors by RT-PCR. As expected, $CD45^+B220^+$ cells expressed NP-1 (Fig23B), but did not express other VEGF165 receptor. So we concluded $CD45^+B220^+$, B lymphocytes express NP-1 and bind $VEGF_{165}$.

To examine the role of NP1 on $CD45^+$ hematopoietic cells in VEGFR-2 activation, we evaluated phosphorylation of VEGFR-2 on ECs. For this purpose, we used bEND3 cell line, which have been already established as endothelial cell lines.[84] At first, we checked the expression of VEGFR-2 in bEND3 cell line by FACS and confirmed the expression of VEGFR-2 (Fig.24A).

Next, bEND3 was seeded on fibronectin-coated dishes, cultured for 12 hours under serum-free conditions, and subsequently challenged with $CD45^+$ hematopoietic cells mixed with various amount of $VEGF_{165}$ in the presence or absence of NP1-flag for neutralizing VEGF. Cell lysates were immunoprecipitated with an anti-VEGFR-2 antibody and the subjected to Western blotting using an anti-phosphotyrosine mAb (4G10). Phosphorylation of VEGFR-2 was induced by addition of $CD45^+NP1^+$ cells mixed with VEGF, and specifically blocked by NP1-flag as a dose dependent manner, although addition of $CD45^+NP1^-$ cells mixed with the same amount of VEGF as above did not induce phosphorylation of VEGFR-2 on ECs (Fig.24B). We confirmed that the mRNA expression of angiogenic factors, such as VEGFs, angiopoietin-1 and 2, did not change in $CD45^+NP1^+$ cells and $CD45^+NP1^-$ cells (data not shown). Therefore, we concluded that NP-1 on hematopoietic cells together with $VEGF_{165}$ activates VEGFR2 on EC exogenously.

Hematopoietic cells expressing NP-1 induce vascular development in vitro and in vivo.

As described above, soluble clustered NP-1 enhanced vascular development (Fig.17, ref.85). Since CD45 positive cells expressed NP-1, we hypothesized that CD45 positive cells work as soluble NP-1 receptors, because hematopoietic cells could move freely through the vessels. To address this hypothesis, we used the P-Sp culture system.[75] As described above, this culture system

supports vasculogenesis and angiogenesis, moreover, hematopoiesis. In case of hematopoietic cells, firstly developed hematopoietic stem cells on sheet formation of ECs migrate into network area of ECs and proliferate and differentiate on such network area. Recently we reported the critical role of Angiopoietin-1 produced from hematopoietic stem cells in promoting this network formation.[64] Therefore, if NP-1 on hematopoietic cells worked as like a exogenously added soluble NP-1, such hematopoietic cells should rescue the defective vascularity observed in P-Sp culture of NP-1-/- embryo. Therefore, we tested whether CD45-positive cells expressing NP-1 was able to rescue the defective vascular formation in the culture of NP-$1^{-/-}$ P-Sp explant. In mutant embryos, vascular bed and network formation were defective (Fig.25Aa). Addition of $CD45^{+}NP\text{-}1^{+}$ cells from fetal liver of E12.5 green mice expressing GFP ubiquitously ($5x10^{3}$cells/well) rescued the defective vascular bed and network formation (Fig.25Ab). Exogenously added hematopoietic cells did not differentiate to endothelial cells confirmed by their morphology (Fig.25Ad). On the other hand, addition of $CD45^{+}$ cells from fetal liver of E12.5 NP-$1^{-/-}$ embryo did not rescue the defective vascular formation effectively (Fig.25Ac). Indeed, quantitative analysis shows that the area of vascular bed was expanded by the addition of $CD45^{+}NP\text{-}1^{+}$ cells (Fig.25B).

Next, we examined whether $CD45^{+}$ hematopoietic cells induce angiogenesis in vivo. For this purpose, we used matrigel assay system that was already established as a method for evaluation of angiogenesis in vivo.[83] We observed

that $CD45^+B220^+$ cells from E12.5 fetal liver of wild-type together with $VEGF_{165}$ promoted capillary formation in matrigel (Fig.26b, d), although $CD45^+B\text{-}220^+$ cells from E12.5 fetal liver of NP-1 mutants together with $VEGF_{165}$ did not induce angiogenesis (Fig.26a, c).

Stromal cells expressing NP-1 enhances vascular development

Recent report showed that NP-1 was expressed in MS-5 stromal cell and in primary bone marrow stromal cells.[66] Therefore, we transfected pMY-IRES-GFP vector inserted full length NP-1 into OP9 stromal cells and generated several lines of OP9/NP-1 stromal cells. The expression of NP-1 was evaluated by flow cytometric analysis and Western blotting. GFP positive cells (Fig.27A) expressed about 130Kda NP-1 (Fig.27B).

Next, we examined whether defective vascularity of $NP\text{-}1^{-/-}$ embryo was rescued or not on such OP9/NP-1 stromal cells. Expectedly, defective vascular structure of $NP\text{-}1^{-/-}$ P-Sp culture was rescued by OP9/NP-1 stromal cells as a dose dependent manner of NP-1 expression on OP9 cells (Fig.27C).

Dimerization of neuropilin-1 is important for inducing angiogenesis

Previously we reported that dimer of soluble NP-1 induced the phosphorylation of VEGFR-2 and enhances vascular development, although monomer of soluble NP-1 did not.[85] It is known that "a" and "c" domains are required for dimerization of neuropilin-1. Then, we constructed L cell

possessed various kind of extracellular domain of NP-1.[80] In addition of L cells possessed only "a" domain (data not shown) or "a" and "b" domains (Fig.28a, b, c) to NP-1$^{-/-}$ P-Sp culture (2x10^{3}cells/well) after four days of starting culture, defective vascularity was not rescued at both adherent (Fig.28b) and non-adherent area of L cells (Fig.28c). On the other hand, in addition of L cells possessed "a", "b", and "c" domains (Fig.28d, e, f) to NP-1$^{-/-}$ P-Sp culture (2x10^{3}cells/well) at the same schedule as above, defective vascularity was rescued at adherent area of L cells (Fig.28e), however, it was not rescued at non-adherent area of L cells (Fig.28f).

These results indicated that dimerization of NP-1 on cell surface is important for inducing angiogenesis at the site where NP-1$^+$ cells and EC co-localize.

Discussion

1. Neuropilin-1 (NP-1) is a receptor for VEGF165 and regulate vascular development

Recently, neuropilin-1 was isolated from non-endothelial cells as a protein that binds $VEGF_{165}$ not binds $VEGF_{121}$.[54] Neuropilin-1 is expressed in endothelial cells (ECs) of capillaries and blood vessels and endocardial cells of the heart as well as developing neurons. When the NP-1 was co-expressed with VEGFR-2, the chemotactic activity and mitogenicity for ECs induced by stimulation of VEGF165 were enhanced (Fig29a, b). Moreover, a murine transgenic model in which native *np-1* cDNA is overexpressed under the transcriptional control of the β-actin promoter revealed an excess of capillaries and blood vessels, dilation of blood vessels, and a malformed heart.[58] These findings indicate that NP-1 is the third VEGF receptor and modulate the vascular development.

We here showed that neuropilin-1 deficiency induces various types of vascular defects. Vascularization in both the central nervous system (CNS) and pericardial portions are severely impaired in the NP-1 mutant embryos. NP-1 mutant embryos also exhibit agenesis of brachial arch-related great vessels and dorsal aorta, transposition of the aortic arch, and insufficient separation of the truncus arteriosus (data not shown). These results indicated that the function of NP-1 in embryonic vessel formation is important for normal vascular development.

2. Soluble NP-1 regulates vasculogenesis and angiogenesis

NP-1 is a transmembrane protein, but recent reports indicate the existence of a soluble monomeric form (Fig.8, ref.57). This soluble NP-1 monomer is expressed in liver hepatocytes and kidney distal and proximal tubules where does not overlap with NP-1 expression. It acts as a competitive inhibitor of $VEGF_{165}$, preventing its stimulation of ECs. On the other hand, our studies show that, NP-1-Fc, which is a dimer type of NP-1, alone can enhance the phosphorylation of VEGFR-2 in $VEGFR\text{-}2^{+}NP\text{-}1^{-}$ ECs from *np-1* mutant embryos in the presence 1ng/ml VEGF. Moreover, the P-Sp culture system, which allows for vasculogenesis and angiogenesis[64, 75], has shown that NP-1-Fc promotes the proliferation of endothelial cells. Furthermore, NP-1-Fc could rescue the suppression of ECs development of $np\text{-}1^{-/-}$ embryo at CNS and pericardium. In neuronal guidance, only the extracellular domain of NP-1 is important for mediation of NP-1 signaling.[73] These results indicate that the cytoplasmic domain of NP-1 is not essential for signal transduction through VEGFR-2. Commonly, soluble receptor proteins bind their ligands with similar affinities as the cognate transmembrane receptors.[86] Most soluble receptors for cytokines and growth factors compete with their membrane bound counterparts for the binding of the ligand and therefore are antagonists. In contrast, as in the case of NP-1-Fc, the soluble receptor works as a signal modulator as it does in hematopoietic cells. IL-6 is one of several molecules that stimulate the proliferation of primitive hematopoietic progenitors.[87, 88] Binding of IL-6 to IL-6R

stimulates the signal transducing molecule gp130.[89] Exogenous soluble IL-6R-IL6 complex has been shown to stimulate gp130 directly on hematopoietic cells.[89] NP-1 is composed with the a/CUB domain, b/FV/VIII domain, c/MAM domain, transmembrane, and the short cytoplasmic domain that does not possess obvious motif. Naturally occurring soluble NP-1 (sNP-1) contains only the a, b domains and does not dimerize. The NP-1 tagged with FLAG epitope (NP-1-FLAG) is a monomeric form and work as a suppressor as observed in the case of naturally occurring sNP-1. On the other hand, NP-1 tagged with Fc part of human IgG1 (NP-1-Fc) is a dimeric form and work as an inducer of vascular development (Fig.29c). Although this NP-1-Fc is engineered protein and does not exist in vivo as a soluble protein, we propose that candidate of dimeric form of soluble NP-1 is hematopoietic cells, because hematopoietic cells can move freely into the vessels and some of them express NP-1 highly. Indeed, we indicated that hematopoietic cells expressing NP-1 bound $VEGF_{165}$ and phospholyrated VEGFR-2 on ECs. Moreover, we showed that NP-1 on hematopoietic cells together with $VEGF_{165}$ rescued vascular development in vitro *np-1*$^{-/-}$ P-Sp culture and induced angiogenesis in vivo matrigel assay. These results suggest that hematopoietic cells expressing NP-1 act as a dimer type of soluble NP-1.

3. NP-1 enhances vasculo-angiogenesis endogenously and exogenously

In this report, we propose the new model of enhancing vasculo-angiogenesis,

which are endogenous and exogenous effect of NP-1. Our present data suggest that dimerization of NP-1 is important for exogenous effect of NP-1. For address this hypothesis, we generated some sublines of L cell harboring mutated NP-1, such as "a" domain alone, "a" and "b" domains, and "a","b", and "c" domain, then added into P-Sp culture of NP-1 mutant. It is known that "a" and "c" domain are required for dimerization of NP-1. The result is clearly showed that just dimer form of exogenous NP-1 on the cell surface could rescue the defective vasculature of NP-1 mutant. We still do not know whether NP-1 on hematopoietic cells is always making dimer or not, however; we might define that at least dimer form of NP-1 on cell surface can stimulate VEGFR2 on endothelial cells exogenously. It has been reported that mesenchymal cells surrounding ECs[58] and stromal cells[66] also expressed NP-1. Moreover such mesenchymal cells are one of major source of VEGF production. These indicate mesenchymal cells which co-express VEGF and NP-1 enhance the vascular development locally. This hypothesis is supported by two analyses reported by Kitsukawa et al.[58] and present results. When NP-1 was over-expressed under the transcriptional control of actin promoter, excess capillaries and blood vessels were observed in such transgenic mice.[58] Our study also showed that ectopic over-expression of NP-1 on OP9 stromal cells enhanced vascular development. Taken together, mesenchymal cells surrounding blood vessels enhance the vascular development exogenously (Fig29). Tumor cells, neural cells, and other cells expressing NP-1 also might enhance the vascular

development exogenously.

4. The possibility of gene therapy for ischemic diseases using NP-1 gene

Therapeutic angiogenesis, the induction of new vessel growth into ischemic tissue, has become an exciting direction of clinical research. VEGF has been used to promote angiogenesis in conditions of occlusive vascular disease, such as ischemic limb or heart disease.[90, 91] However, some patients treated with VEGF for lower limb ischemia may have transient limb edema, possibly due to the permeability increasing effect of VEGF.[90, 92] Regulation of vascular permeability has clinical ramifications beyond the field of therapeutic angiogenesis. Plasma leakage and edema are features of inflammatory, degenerative, and neoplastic diseases; therefore, inhibition of the leakage could have important therapeutic benefits. One candidate molecule to architect more physiological vessels is Ang1.[93] The analysis of K14-Ang1/VEGF mice suggests that angiogenic action of VEGF can be uncoupled from its leakage-inducing effects by Ang1. Therefore, Ang1 has the beneficial effect of forming nonleaky vessels. Another candidate molecule is NP-1. Our study, which injection of NP-1-Fc into the NP-1 heterozygous pregnant mice, revealed that vascularization in CNS and pericardium in NP-1 mutant embryos was rescued and formed the non-leaky, physiological vessels as well as NP-1 wild-type embryos. These results suggest that NP-1 form the physiological vessels as well as enhance the proliferation of ECs effectively. As NP-1 can enhance the

VEGF signaling exogenously as well as endogenously, induction of NP-1 gene into the ischemic lesion might be a useful and effective gene therapy in concert with VEGF administration. We might establish the physiological and proliferous vessels by combination with VEGF, NP-1, and Ang-1.

5. Neural cells induce arterial cells development

Vascular system and nervous system has several common features in terms of structure, behavior, and expression molecules.[94] We have recently established co-culture system with endothelial cells and neural cells (Fig.30). Using this culture system, we can observe the interaction between endothelial cells and neural cells. We found two interesting phenomenon. In this system, we observed the recapitulation in vivo behavior which both nerves and blood vessels follow similar routes. In fact, neural cells attracted the ephrinB2 positive arterial cells, and they followed similar routes (Fig.31). Another is that neural cells induced ephrinB2 positive arterial cells from vascular bed, which express endothelial markers, such as VEGFR-2 and PECAM-1, in vitro P-Sp culture (Fig.31). The molecular mechanism how neural cells induce arterial cells growth is unelucidated now, however, it is possible that neural cells are one of key regulators for arterial cells development. These findings suggest that we also have to consider the neural cells development in angiogenic therapy.

Conclusion

In this study, we clarified that NP-1 worked as a co-receptor of VEGFR-2 on ECs and had an important role in vascular development using NP-1 mutant embryos and in vitro P-Sp culture system. Moreover, soluble NP-1 (sNP-1) was detected in vivo and we showed that monomer type sNP-1 inhibits $VEGF_{165}$ binding to ECs and prevent its stimulation of ECs, therefore, angiogenesis was inhibited. On the other hand, dimer type of sNP-1 works cooperatively with $VEGF_{165}$ and enhances angiogenesis in vitro and in vivo. Although, the existence of dimer type of sNP-1was unknown, we found that some lineages of hematopoietic cells express NP-1 and showed that these cells worked as a dimer type of sNP-1. Furthermore, we indicated that stromal cells, mesenchymal cells, and tumor cells, which were detected as cells expressing NP-1, enhanced vasculogenesis and angiogenesis exogenously.

Now, vascular regeneration is one of the most important fields in regeneration study, and the most important common factor in this field is VEGF. However, VEGF cause plasma leakage and edema for some patients. Our in vivo study which administration of dimer of soluble NP-1 rescue the vascular defect of NP-1 mutant embryos and formed physiological vessels suggests that induction of NP-1 gene into the ischemic lesion might be useful gene therapy in concert with VEGF administration.

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Figure Legends

Fig.1 Structure of Blood Vessel

Blood vessel consists of common three-layered structure that is intimal, medial and adventitial layer. Intimal layer comprises an endothelial cell supported by connective tissue. The middle layer is separated from intima, fenestrated sheath, and the intimal elastic lamina, mostly composed of elastin. The medial layer contains smooth muscle cells embedded in an extracellular matrix composed mainly of collagen, elastin and proteoglicans. An external elastic lamina separates the medial layer from the advential layer. This contains collagenous tissue supporting fibroblasts and nerves. In large arteries and veins, the adventitial layer contains vasa vasorum, small blood vessels which also penetrate into the outer portion of media and supply the vascular wall with oxygen and nutrients.

Fig.2 Differentiation from hemangioblast into endothelial cells and hematopoietic cells

The yolk sac contains hematopoietic activity as early as 7-8.5 days postcoitum (dpc), these progenitors have limited potential and only produced primitive erythrocytes. Recent evidence suggests that pluripotent HSCs with long-term repopulating potential (LTR-HSC) populate the yolk sac secondarily from a para-aortic splanchnopleural mesoderm (P-Sp) or dorsal aorta, genital ridge/gonads and pro/mesonephros (aorta-gonad-mesonephros, AGM) by 10.5

dpc. HSCs and angioblasts from this region are then presumed to colocalized both the yolk sac and fetal liver, where they give rise to definitive hematopoietic precursors after 12.5 dpc.

Fig.3 Origin of endothelial cells, hematopoietic cells and smooth muscle cells

The blood vessels consist of both endothelial cells and smooth muscle cells or pericytes. Origin of endothelial cells are thought to be lateral plate mesoderm, which produce hemangioblast; a common precursor of endothelial cells and hematopoietic cells. On the other hand, most of origin of smooth muscle cells are lateral mesoderm, however some of them derive from neural crest cells. Other than smooth muscle cells, they differentiate into neural cells, glia, melanocytes and so on.

Fig.4 Process of vasculogenesis and angiogenesis

The early vascular plexus forms from mesoderm by differentiation of angioblasts, which subsequently generate primitive blood vessels. The molecular mechanisms responsible for this process are termed vasculogenesis. In this stage, VEGFs and their cognate receptors are the most important and contributing factors. After the primary vascular plexus is formed, more endothelial cells are generated, which can form new capillaries by sprouting or by splitting from their vessel of origin in a process termed angiogenesis. There are at least two different types: true sprouting of capillaries from pre-existing

vessels, and non-sprouting angiogenesis or intussusception. In this stage, angiopoetin-tie-2 system plays a very important role. The final step of this stage, endothelial cells need do be stabilized and maturated. In this step, PDGF-BB recruits smooth muscle cells and pericytes, whereas angiopoietin-1 and TGF-β 1 stabilize the nascent vessel.

Fig.5 Ligands and RTK families involved in vascular development

Arrows indicate documented interactions among ligands with their receptors. Note that receptor and ligand structure are drawn color coded to indicate their domains of expression; purple represents proteins found expressed on both arteries and veins. Red and blue structures are found on arteries and veins, respectively; yellow denotes expression in lymphatic vessels, and green structures represent receptors or ligands expressed in tissues surrounding blood vessels.

Fig.6 Neuropilin-Plexin system

Sema3A, 3B, and 3F bind NP-1 and Sema3B, 3C, and 3F bind NP-2. NP-1 is the functional receptor for Sema3A, on the other hand, NP-2 is the functional receptor for Sema3B, 3C, and 3F, and both of receptors induce the growth cone collapse. Interestingly, Sema3B and 3C work as anti-collapse factor for Sema3A as well as collapse inducing factor. PlexinA1 is coreceptor for NP-1 and enhances its signaling.

Fig.7 VEGFs and VEGFRs involved in vascular development

The VEGFR family includes VEGFR-1, VEGFR-2 and VEGFR-3 characterized by the presence of seven extracellular immunoglobulin homology domains and split tyrosine kinase intracellular domain. VEGFR tyrosine kinase activity is stimulated by specific ligands of the six-member VEGF family: VEGF, placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and the viral homologues, collectively called VEGF-E. PlGF and VEGF-B bind to VEGFR-1, whereas VEGF interacts with both VEGFR-1 and VEGFR-2. VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3 and VEGF-E binds and activates only VEGFR-2. In addition, neuropilin-1, a transmembrane protein involved in the regulation of axonal guidance in neurons, was described as a co-receptor for VEGF-B, PlGF-2, VEGF-E and the $VEGF_{165}$ isoform of VEGF. The role of neuropilin-1 is to enhance the signaling VEGFR-2 on ECs.

Fig.8 Structure of Neuropilin isoform

Schematic representation of the eight NP isoforms that have been cloned. The length of each isoform is indicated by the number of amino acids on the right side. The C-terminal intron-derived amino acid sequences of s11NP1 (soluble 11NP1), s12NP1, and s9NP2 are indicated in italics. S11NP1 and s12NP1 contain the5'end of NP1 introns 11 and 12, respectively, and s9NP2 contains the 5' end of NP2 intron9. The 5-amino-acid insertion (GENFK) present in the NP2a (22) and NP2b (5) isoforms is indicated.

Fig.9 Phenotype of KO mouse in VEGFs and VEGFRs

Knockouts of VEGF and its receptors have yielded defects primarily in the early process of vasculogenesis, and accordingly these mutant embryos tend to die at early stages of development.

Fig.10&11 Interaction between endothelial cells and neural cells through NP-1 receptor

There are an increasing number of examples where the two organ systems, such as vascular system and nervous system, appear to use related developmental mechanisms. In terms of behavior, both nerves and blood vessels often follow similar routes and modes of migration during embryogenesis. In terms of molecules, observations in mouse and zebrafish have provided increasing evidence that blood vessel architecture is, partly, determined by families of proteins and signaling relays which have first been described in the developing nervous system, such as ephrin/Eph and NP-1 signaling. Therefore, angiogenesis and neurogenesis may have some interactions through the utilization of these same molecular tools

Fig.12 & 13 P-Sp culture system

We explanted P-Sp explant from E9.5 murine embryo on OP9 stromal cells in 10% FCS containing RPMI medium supplemented with IL-6, IL-7, SCF and Epo for 14 days. From the explant, endothelial cells proliferated and migrated on

OP9 stromal cells and formed sheet-like structure, so-called vascular bed, after 4 days of culturing. Subsequently, endothelial cells sprouted on OP9 stromal cells to form network formation, so-called vascular network after 14 days of culturing. Cluster of hematopoietic stem cells expressed TIE2 and c-Kit aggregated on vascular bed.

Fig.14 P-Sp culture system reflects in vivo phenotype of knock out mice

Inhibition of VEGF signaling in this system using soluble VEGFR-2 led to lack of vascular bed and network formation. It corresponds to the phenotype of VEGFR-2 knock out mice. On the other hand, inhibition of Ang1- tie-2 signaling using soluble tie-2 led to lack of vascular network formation. It corresponds to the phenotype of angiopoietin-1 or tie-2 knock out mice. These findings indicated that P-Sp culture system supports and mimics in vivo vasculogenesis and angiogenesis.

Fig.15 Vasculogenesis and angiogenesis in NP-1 mutant embryo

Staining with PECAM-1 mAb revealed that no growth retardation was observed in mutants (Fig.15a, b). However, the vascularity of mutant embryos compared to wild type embryos was impaired in the CNS and pericardial regions (Fig.15d, f). At E12.0, vascular sprouting into the CNS and pericardium was observed in wild-type embryos (Fig.15c, e), however few capillaries and branches were observed in mutants (Fig.15d, f).

Fig.16 The structure of recombinant neuropilin-1 proteins

(A) A schematic representation of the Fc-tagged neuropilin extracellular segment (NP-1-Fc) and FLAG-tagged neuropilin-1 extracellular segment (NP-1-FLAG). (B) SDS-PAGE of the recombinant proteins stained with Coomassie brilliant blue G-250. (C) Immunoblot of the recombinant proteins with the anti-rabbit neuropilin-1 antibody. Lanes 1- 4 correspond to (1) NP-1-FLAG, (2) NP-1-Fc, (3) NP-1-FLAG, or (4) NP-1-Fc recombinant proteins, respectively. Non-reducing condition (1,2) and reducing condition (3,4).

Fig.17 Effect of soluble NP-1 in P-Sp culture.

P-Sp explants derived from E9.5 wild-type embryos (a, c, e, g) and mutant embryos (b, d, f, h) were cultured on OP9 stromal cells. 50μg/ml NP-1-FLAG (c, d, g, h), 50μg/ml anti-FLAG IgG (g, h), 50μg/ml NP-1-Fc (e, f), or the same amount of control-FLAG (a, b) was added to this culture. In wild-type, on addition of NP-1-FLAG, vascular bed (vb) formation was suppressed (c), however, on addition of NP-1-Fc (e) or NP-1-FLAG + anti-FLAG IgG (g), which forms a dimer of NP-1, vascular bed (vb) was expanded compared with that on addition of control FLAG (a). In homozygous mutants, on addition of NP-1-FLAG, vascular formation was slightly suppressed (d, arrows) compared with that on addition of control-FLAG (b), however, suppressed EC development was rescued by addition of NP-1-Fc (f) or NP-1-FLAG + anti-FLAG IgG (h).

Fig.18 Effect of a dimer of soluble NP-1 on sorted endothelial cells.

(A) Embryos of E12.5 wild-type and *np-1* homozygous mutant were dissociated and stained with PE conjugated anti-PECAM-1 and biotin conjugated anti-VEGFR-2 mAbs. Biotin was developed to avidin-APC. The stained cells were analyzed and sorted by FACSvantage. Approximately 0.3% of cells derived from wild-type embryos and 0.5% of cells derived from mutant embryos were double positive.

(B) Sorted VEGFR-2$^+$PECAM-1$^+$cells from E12.5 wild type (a) and *np-1* homozygous mutant (b, c) were cultured on OP9 cells. Note that the vascular structure was defective in the endothelial cells (ECs) from mutant embryo (b) compared with that of wild-type littermates (a). NP-1-Fc (50μg/ml) or CD4-Fc (50μg/ml) was added to the culture as noted above. Vascular formation was rescued in *np-1* homozygous mutants by addition of NP-1-Fc (c), while the same amount of CD4-Fc did not have any effect (data not shown). Scale bar indicates 100μm.

(C) Cell lysates of VEGFR-2$^+$PECAM-1$^+$ ECs from *np-1*$^{+/+}$ or *np-1*$^{-/-}$ embryos were immnoprecipitated with an anti-VEGFR-2 antibody and then subjected to western blotting using an anti-phosphotyrosine mAb. Lanes: 1 (WT) VEGF 1ng/ml; 2 (mutant) no factor; 3 (mutant) VEGF 1ng/ml; 4 (mutant) VEGF 1ng/ml + NP-1-Fc 50μg/ml. The VEGFR-2$^+$PECAM-1$^+$ ECs from *np-1*$^{+/+}$ or *np-1*$^{-/-}$ embryos were challenged by VEGF with or without NP-1-Fc. In mutant embryos, phosphorylation of VEGFR-2 was induced by addition of a low dose of

VEGF and NP-1-Fc (lane4; arrowhead), while no factor (lane2) or VEGF alone (lane3) did not induce the phosphorylation of VEGFR-2. Lane1 was used as a positive control. Lower panel shows the amount of immunoprecipitated VEGFR-2 confirmed by Western blotting by antiVEGFR-2 mAb.

Fig.19 Synergistic effect of VEGF and dimer of soluble NP-1 in P-Sp culture.

(A) P-Sp explants derived from E9.5 wild-type and *np-1* homozygous mutant embryos littermates were cultured on OP9 stromal cells. Note that vascular bed (vb) and network (vn) formation were defective in mutant embryo explants (b) compared with that of wild-type littermates (a). NP-1-Fc (50μg/ml) and/or VEGF (50ng/ml) were added to this culture system as noted above. Vascular bed (vb) was expanded in *np-1*$^{+/+}$ embryo on addition of VEGF (c) or NP-1-Fc (e). Suppressed vasculature in the culture of *np-1*$^{-/-}$ embryo (b) was partially rescued on addition of 50ng/ml VEGF (d) and was completely rescued on addition of 50μg/ml NP-1-Fc (f). Simultaneous application of VEGF and NP-1-Fc enhance the formation of vascular bed in *np-1*$^{+/+}$ (g) and *np-1*$^{-/-}$ (h) P-Sp culture. Scale bar indicates 1mm. Similar results were obtained in three independent experiments.

(B) Vascular areas in Fig.5A were calculated by NIH image 1.62 software. The area size is as follows. (a) $10.8 \pm 1.48 mm^2$ (b) $1.1 \pm 0.55 mm^2$ (c) $35.2 \pm 2.39 mm^2$ (d) $7.7 \pm 1.68 mm^2$ (e) $18.3 \pm 2.48 mm^2$ (f) $12.1 \pm 3.03 mm^2$ (g) $49.7 \pm 3.91 mm^2$ (h) $33.5 \pm 3.77 mm^2$ The vascular area per explant were obtained from

three independent experiments and expressed as the mean ± S.D.

Fig.20 A dimer of soluble neuropilin-1 induces angiogenesis in vivo

To examine the in vivo effect of NP-1-Fc, we used matrigel assay system which had been already established as a in vivo angiogenic assay. We observed that NP-1-Fc with 20ng/ml VEGF promoted VEGFR-2-positive endothelial cell growth in matrigel, although CD4-Fc with 20ng/ml VEGF did not promote endothelial cell growth in matrigel.

Fig.21 A dimer of soluble NP-1 rescues defective vascularity of *np-1* mutant embryos in vivo.

Injection of CD4-Fc as a control had no effect (Fig.21a, c). On injection of NP-1-Fc, some *np-1*$^{-/-}$ embryos exhibited a significant recovery of vascularity, and interestingly, the caliber of the rescued capillaries (Fig.21b, d) was larger than that of wild-type (Fig.15a and c). Similar results were obtained in three independent experiments. Scale bar indicates 150μm (a and b); 300μm (c and d).

Fig.22 NP-1 expression on hematopoietic cells

(A) RT-PCR analysis of NP-1 and VEGF-A expression in various fractions of hematopoietic cells from the fetal liver and bone marrow. In case of fetal liver, high NP-1 expression was detected in CD45+B220+ and CD45+Mac-1+ cells;

however, very low NP-1 expression was detected in the fraction of stem cells, which is marked as CD45+CD34+c-kit+ cells. In case of adult bone marrow, high NP-1 expression was detected in CD45+CD4+CD8+ cells and Lin-c-kit+Sca-1+ cells; however, low NP-1 expression was detected in CD45+Mac-1+cells.

(B) Analysis of NP-1 expression on hematopoietic cells in an embryo using an anti-NP-1 antibody. NP-1-positive hematopoietic cells were observed in the lumen of a vessel in the pericardium (a, b) and CNS (c, d), and it seemed to adhere to endothelial cells (arrows). (b) and (d) are higher magnifications indicated by the arrow in (a) and (c), respectively. Arrowheads indicate NP-1 positive ECs and endocardiac cells.

Scale bar indicates 50 μm (a, c); 20μm (b, d).

Fig.23 Expression of VEGFRs on B lymphocyte cells

(A), (B) Cells obtained from a E12.5 fetal liver were stained with anti-CD45-PE and anti-B220-APC. Some cells (2.6%) were positively stained by both antibodies (a), and these cells were then stained with VEGF-biotin and streptavidin-FITC. Many of the cells shifted to the right.

Fifty percent (50%) of the cells over the negative gate expressed NP-1 alone among $VEGF_{165}$ receptors.

Fig. 24 CD45 positive hematopoietic cells together with $VEGF_{165}$ phosphorylate

bEND3 endothelial cells

(A) Flow cytometric analysis of VEGFR-2 expression in the bEND3 cell line. The bEND3 cells expressed VEGFR-2 monotonously.

(B) Cell lysates of bEND3 cells that had been stimulated by various factors or cells were immunoprecipitated with anti-VEGFR-2 antibody and then subjected to Western blotting using an anti-phosphotyrosine mAb (anti-PY). The bEND3 cells were incubated with the $CD45^+$ cells from fetal livers of wild (lane 1-5) or NP-1 mutant (lane 6-10) embryos with or without the indicated factors in each lane. No factor (lane 1,6); 2 ng/ml VEGF (lane 2,7); 10 ng/ml VEGF (lane 3,8); 10 ng/ml VEGF + 10 μg/ml NP1-flag (lane 4,9); 10 ng/ml VEGF + 30 μg/ml NP1-flag (lane 5,10). Phosphorylation of VEGFR-2 was induced by the addition of $CD45^+NP1^+$ cells mixed with 2 ng/ml VEGF (lane 2) or 10 ng/ml VEGF (lane 3), and it was specifically blocked by NP1-flag in a dose-dependent manner (lanes 4, 5). The addition of $CD45^+NP1^-$ cells mixed with VEGF barely induced phosphorylation of VEGFR-2 on ECs (lane 7, 8). Wild indicates cells from the wild type and mutant indicates cells from the mutant embryo of NP-1 from the same litter.

Fig. 25 Hematopoietic cells expressing NP-1 rescue vascular defect in NP-1 mutant P-Sp culture

(A) (a) In mutant embryos, formation of the vascular bed and network are defective. (b) The addition of CD45-positive cells from the fetal liver of E12.5

GFP embryo ($5x10^3$ cells/well), which are also positive for NP-1, rescued the defective formation of the vascular bed and network; however, these cells did not differentiate to endothelial cells as confirmed by their morphology (d). (c) The addition of CD45-positive cells from the fetal liver of E12.5 NP-$1^{-/-}$ embryo did not rescue the defective vascular formation.

Scale bar indicates 200 μm.

(B) Comparison of the area of the vascular bed. The area of the vascular beds in the images in Fig. 25A was determined by the NIH image 1.62 software. The vascular area per explants is as follows: (a) 0.3 ± 0.1 mm^2; (b) 20.5 ± 3.2 mm^2; (c) 3.5 ± 0.5 mm^2. Each result was obtained from three independent experiments and expressed as the mean ± S.D.

Fig.26 Hematopoietic cells expressing NP-1 together with VEGF165 induce angiogenesis in vivo

Matrigels containing $CD45^+B220^+$cells from E12.5 fetal liver of wild-type (b, d) or $CD45^+B220^+$cells from E12.5 fetal liver of NP-1 mutants (a, c) were injected subcutaneously near the abdominal midline of 8-week-old C57BL mice. (a, b) Gross appearance of matrigels on day 4. (c, d) Fluorescence analysis of sections from the matrigels. The ECs were visualized as green, and then they were stained with FITC-conjugated PECAM-1 mAb. Scale bar indicates 50 μm (c, d).

Fig.27 Stromal cells expressing NP-1 rescue vascular defect in NP-1 mutant P-Sp culture

NP-1 expression on OP9 stromal cells was not detected at the protein level by FACS analysis (A) or by Western blot analysis (B). Next, we transfected the pMY-IRES-GFP vector with the full-length NP-1 into OP9 stromal cells and generated several lines of OP9/NP-1 stromal cells. NP-1 expression was detected as GFP-positive cells by FACS (A). NP-1 was detected as an approximately 130kDa protein on Western blot analysis (B). MSS31, which has already been established as a cell line possessing properties of endothelial cells, was used as a positive control. (C) P-Sp explants from wild type and NP1 mutant embryos were cultured on OP9 cells and OP9 cells expressing NP1, and the effect of NP1 on the surface of stromal cells on vascular formation was observed. Note that the defective vascular structure of NP-$1^{-/-}$ P-Sp culture was rescued by OP9/NP-1 stromal cells to a much higher greater degree than that by the parental OP9 cells. Scale bar indicates 200 μm.

Fig.28 Dimerization of NP-1 on L cells enhances vascular development exogenously

Upon the addition of L cells that possessed only the "a" domain (data not shown) or "a" and "b" domains of the NP-1 protein (a, b, c) to a NP-$1^{-/-}$ P-Sp culture ($2x10^3$ cells/well) on the fourth P-Sp culture day, the defective vascularity was not rescued at neither the areas that were adherent to the L cells (b) nor the

areas that were not adherent to the L cells (c). (b) and (c) are higher magnifications of the areas indicated by the boxes in (a). On the other hand, upon the addition of L cells that possessed the "a", "b", and "c" domains (d, e, f) to a NP-1$^{-/-}$ P-Sp culture ($2x10^3$ cells/well) on the fourth P-Sp culture day, the defective vascularity was rescued at the areas that were adherent to the L cells (Fig. 4e); however, it was not rescued at the areas that were not adherent to the L cells (f). (e) and (f) are higher magnifications of the areas indicated by the boxes in (d). Scale bar indicates 400 μm (a, d); 200μm (b, c, e, f).

Fig. 29 NP-1 enhances the signaling of VEGFR-2 on ECs endogenously and exogenously

The proliferation and migration of ECs expressing NP-1 together with VEGFR-2 (b) are enhanced in comparison with those of ECs expressing VEGFR-2 alone (a). Soluble clustered NP-1 effectively enhances the signaling of VEGFR-2 (c). These findings suggest that hematopoietic cells, stromal cells, and neuronal cells expressing NP-1 also enhance the signaling of VEGFR-2 on ECs (d).

Fig. 30 P-Sp and DRG co-culture system

E9.5 P-Sp explants were cultured o on OP9 stromal cells in 10% FCS containing RPMI medium supplemented with IL-6, IL-7, SCF and Epo for 10-14 days. From the explant, endothelial cells proliferated and migrated on OP9 stromal cells and formed sheet-like structure, so-called vascular bed, after 4 days of

culturing, subsequently, formed vascular network (vn) after 14 days of culturing. After 7 days of culturing, we added E12.5 dorsal root ganglia (DRG) with NGF to P-Sp culture, then stained with anti-PECAM-1 and anti-neurofilament mAb. PECAM-1 positive endothelial cells growth is promoted by neural cells.

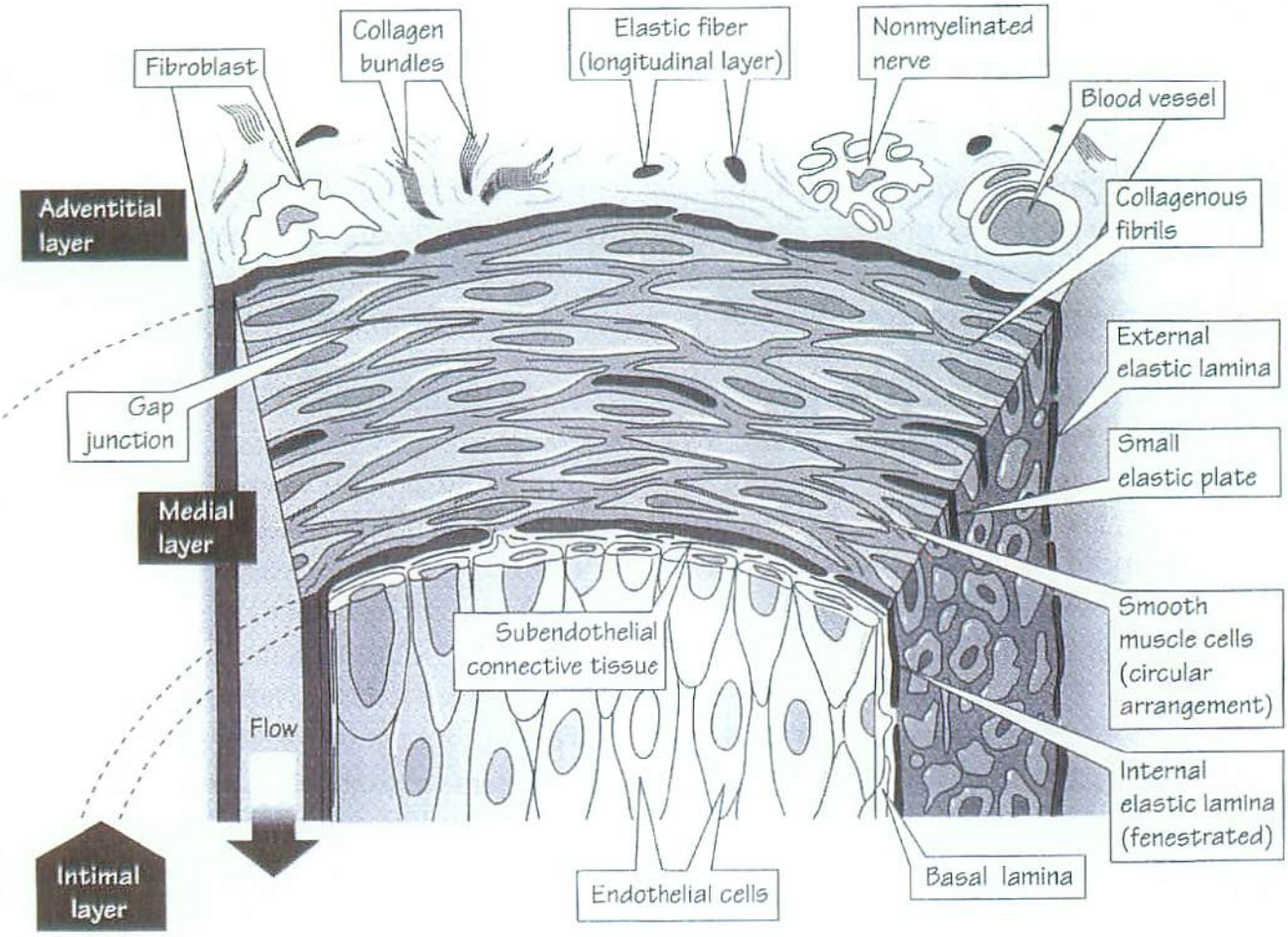
Fig. 31 Neural cells induce arterial cells differentiation and their proliferation and migration

The endothelial cells and neural cells co-culture system revealed two interesting phenomenon. One is that endothelial cells run parallel to the neural cells. The other is that neural cells promoted endothelial cells growth. For further analysis, we used the P-Sp region from ephrinB2-lacZ heterozygous mutant embryos. We found that promoted endothelial cells by neural cells were ephrinB2 positive arterial endothelial cells.

Fig.1 Structure of Blood Vessel

blood vessel

adventitial layer: collagenous tissue, fibloblasts, nerves, capillaries
medial layer: smooth muscle cells, extracellular matrix
(collagen, elastin, proteoglican)
intimal layer: endothelial cells, connective tissue



(The Cardiovascular System at a Glance, Aaronson PI & Ward J-PT)

Fig.2 Differentiation from hemangioblast into endothelial cells and hematopoietic cells

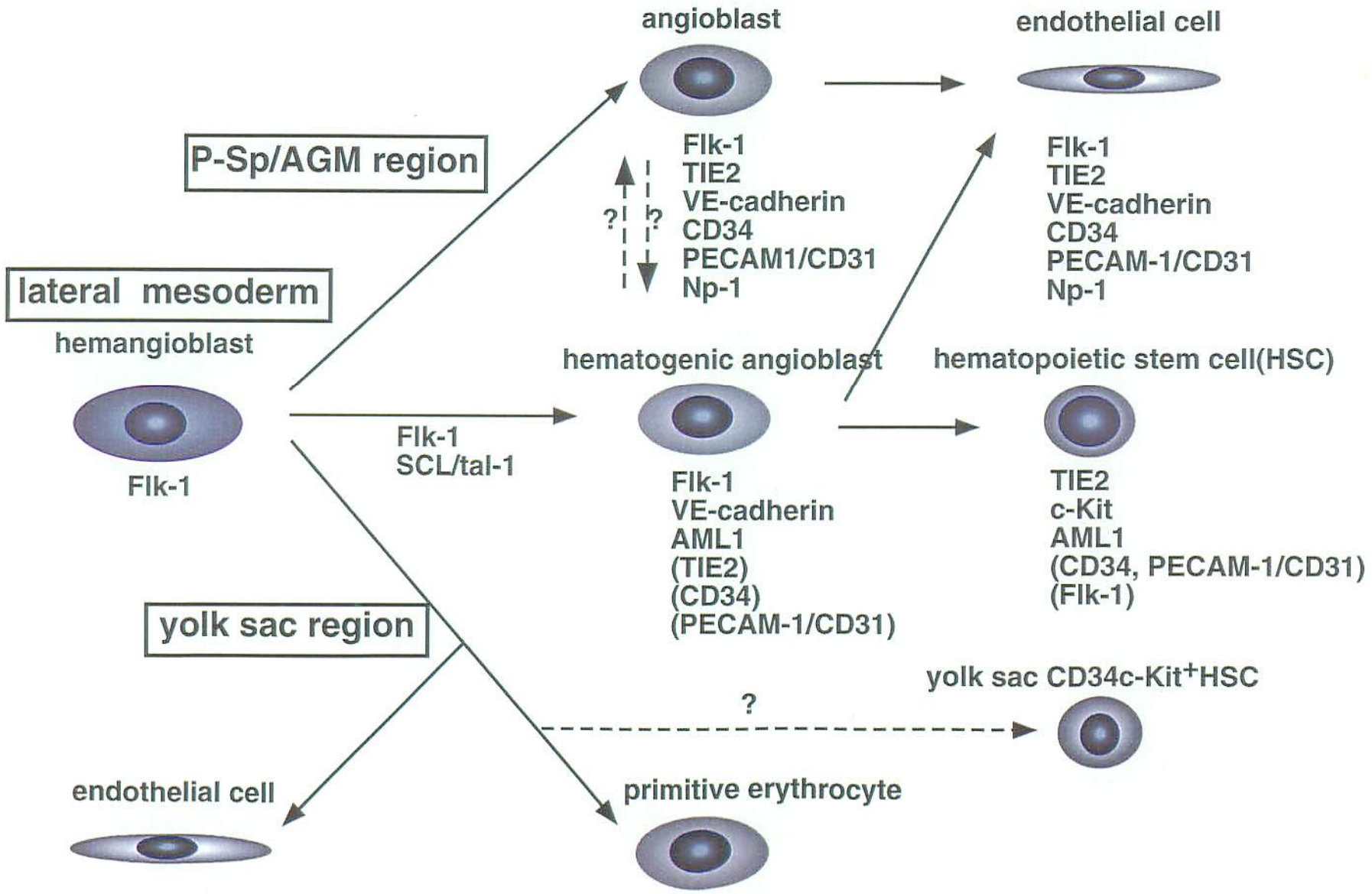


Fig.3 Origin of endothelial and hematopoietic cells, and smooth muscle cells

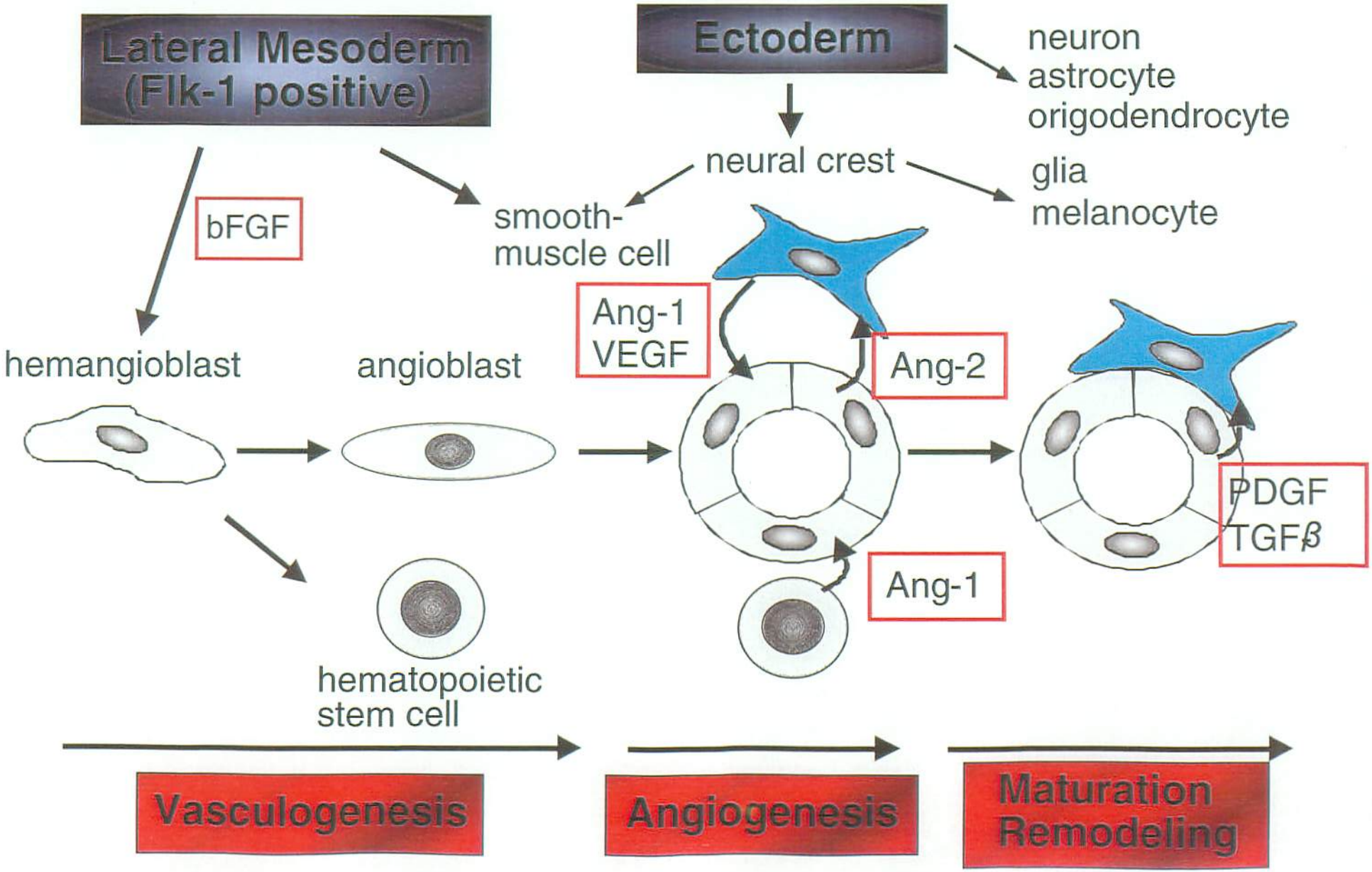


Fig.4 Process of vasculogenesis and angiogenesis

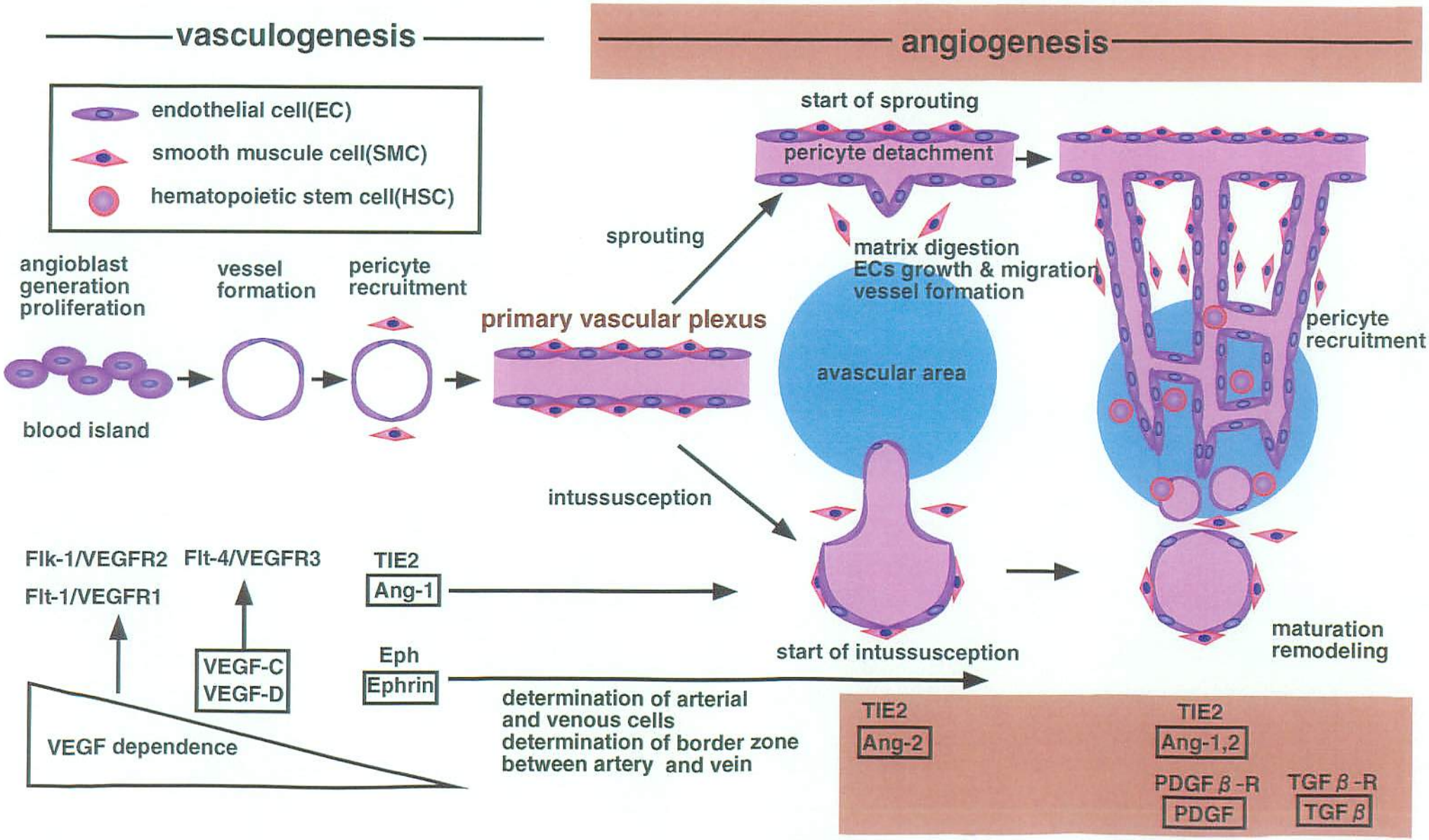


Fig.5 Ligands and RTK families involved in vascular development

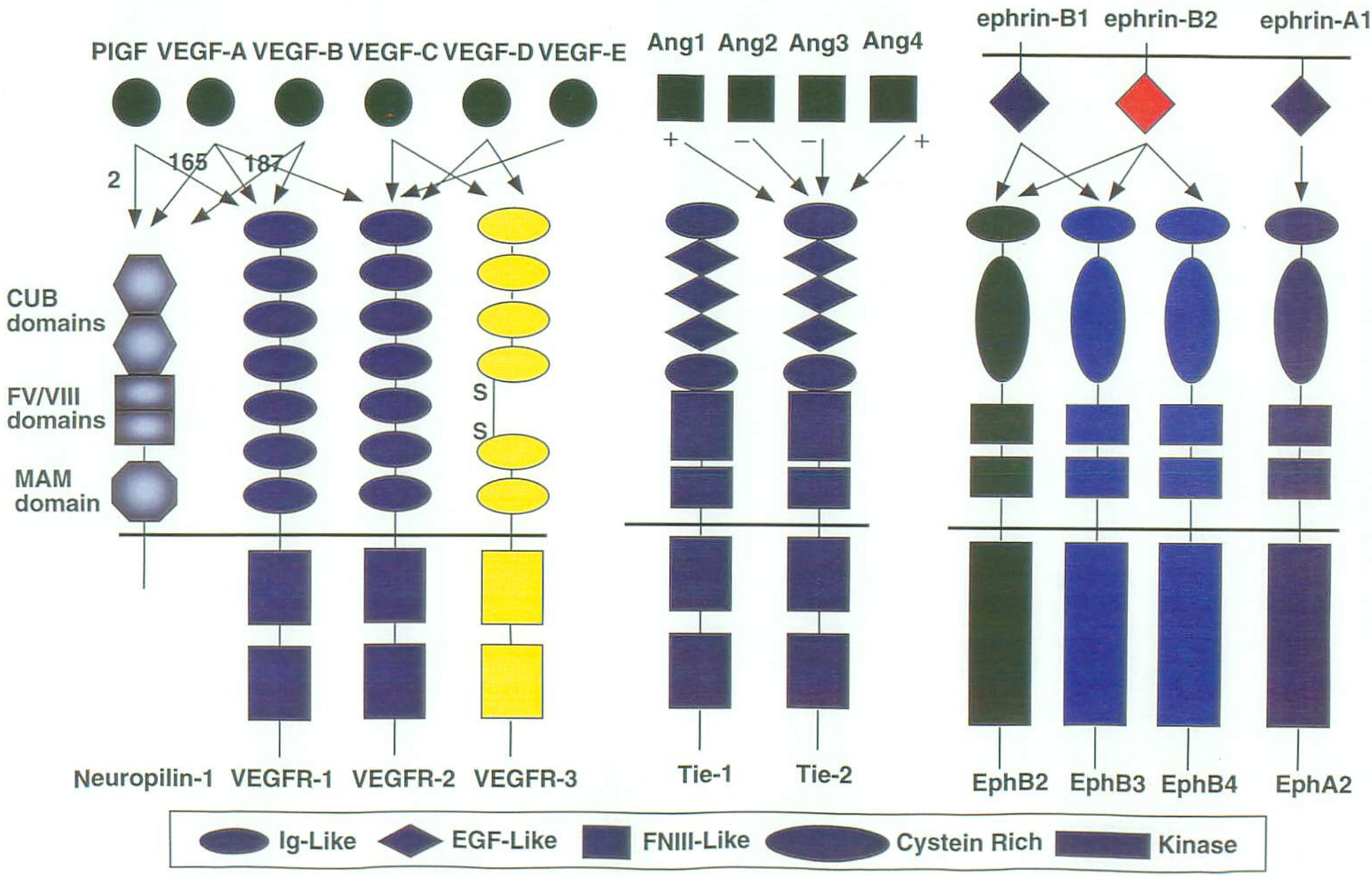


Fig.6 Neuropilin-Plexin System

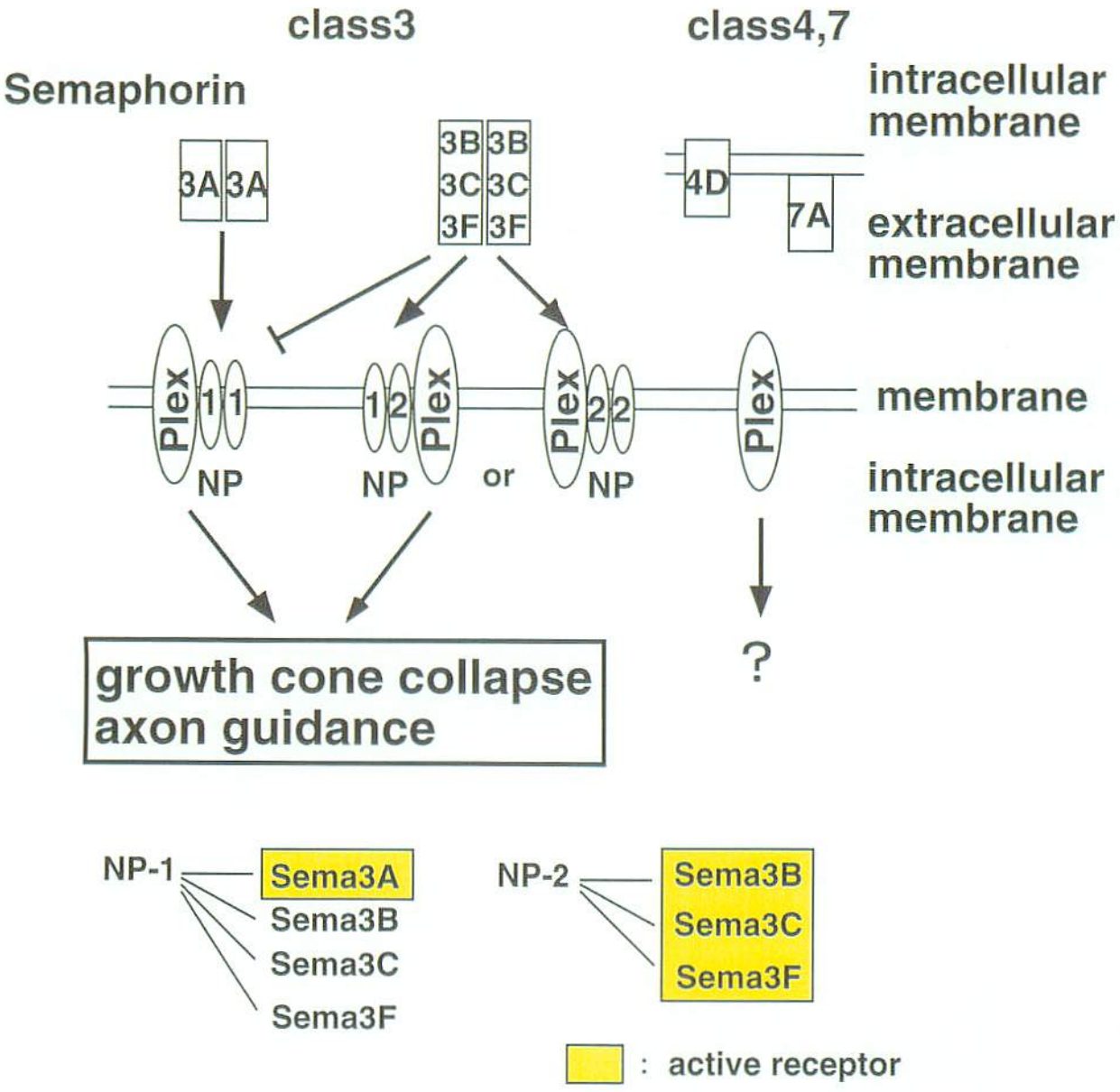


Fig.7 VEGFs and VEGFRs involved in vascular development

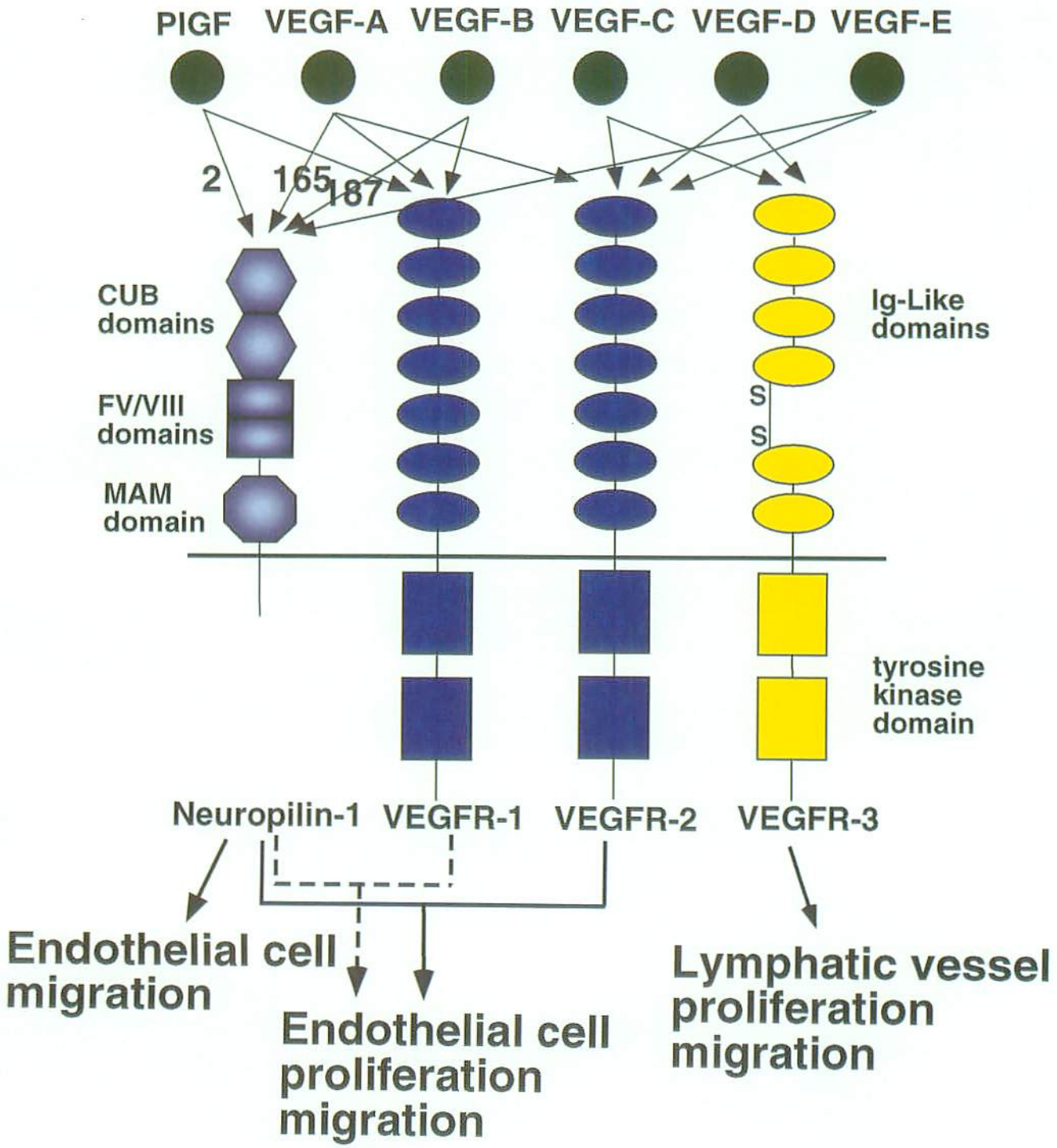


Fig.8 Structure of Neuropilin isoform

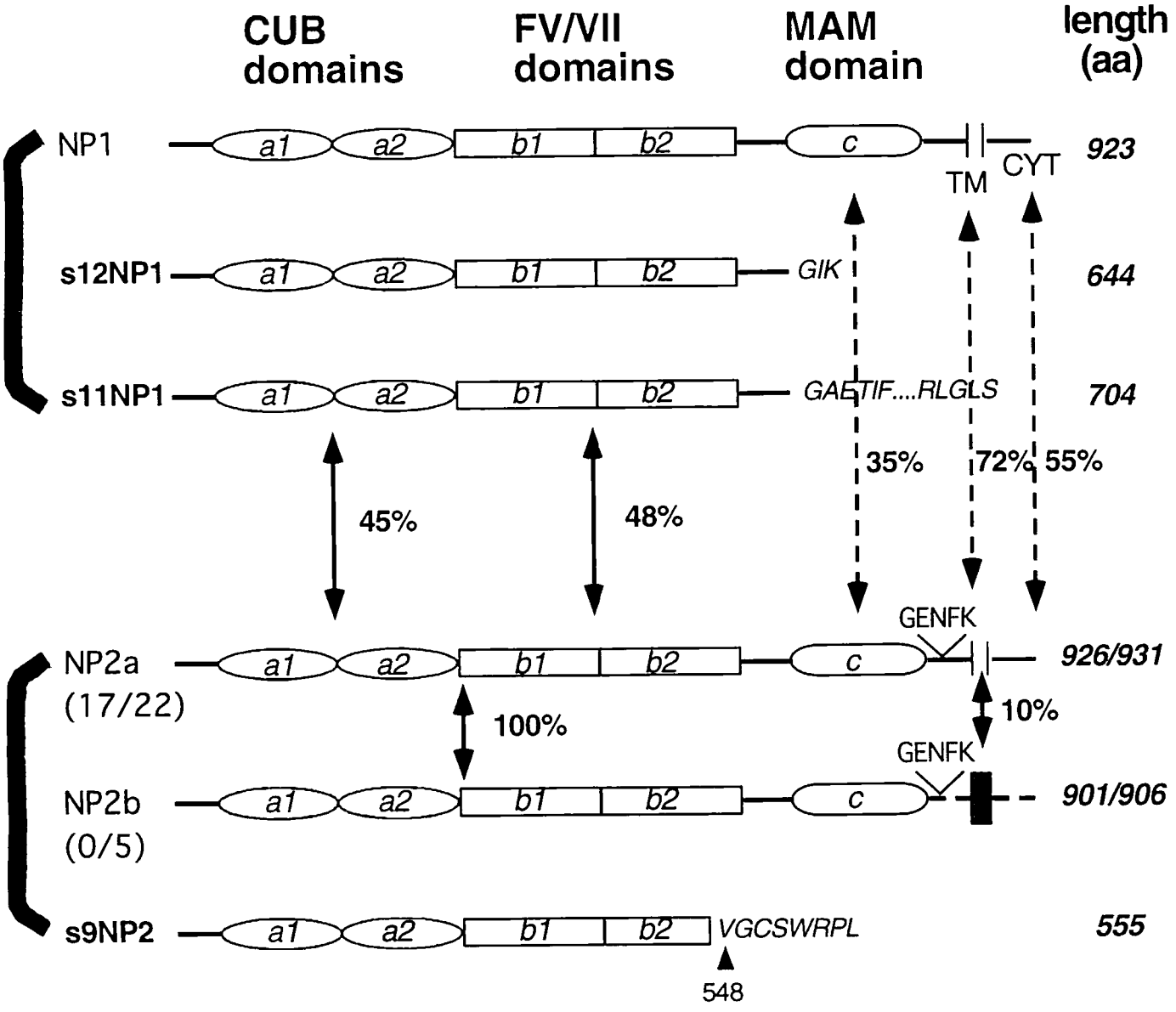


Fig.9 Phenotype of knock out mouse in VEGFs and VEGFRs

Gene KO	Time of death	Causes of lethality
VEGF-A	E10.5	absent dorsal aorta, defective endothelial cell development, failure of endothelial cell formation
VEGF-A$_{165\&189}$	P1-14	bleeding in several organs, impairment of myocardial angiogenesis →ischemic cardiomyopathy(ICM)
VEGFR-1	E8.5-9.5	excess endothelial cells from abnormal vessel structures entering vessel lumens
VEGFR-2	E8.5-9.5	failure of endothelial cell formation
VEGFR-3	E10.5-12	defective vessel remodeling and organization, irregular large vessels with defective lumens
NP-1	E10.5	anomalies in great vessels and heart outflow tracts, impairment in neural and pericardial vascularization

Fig.10 Interaction between endothelial cells and neural cells through NP-1 receptor

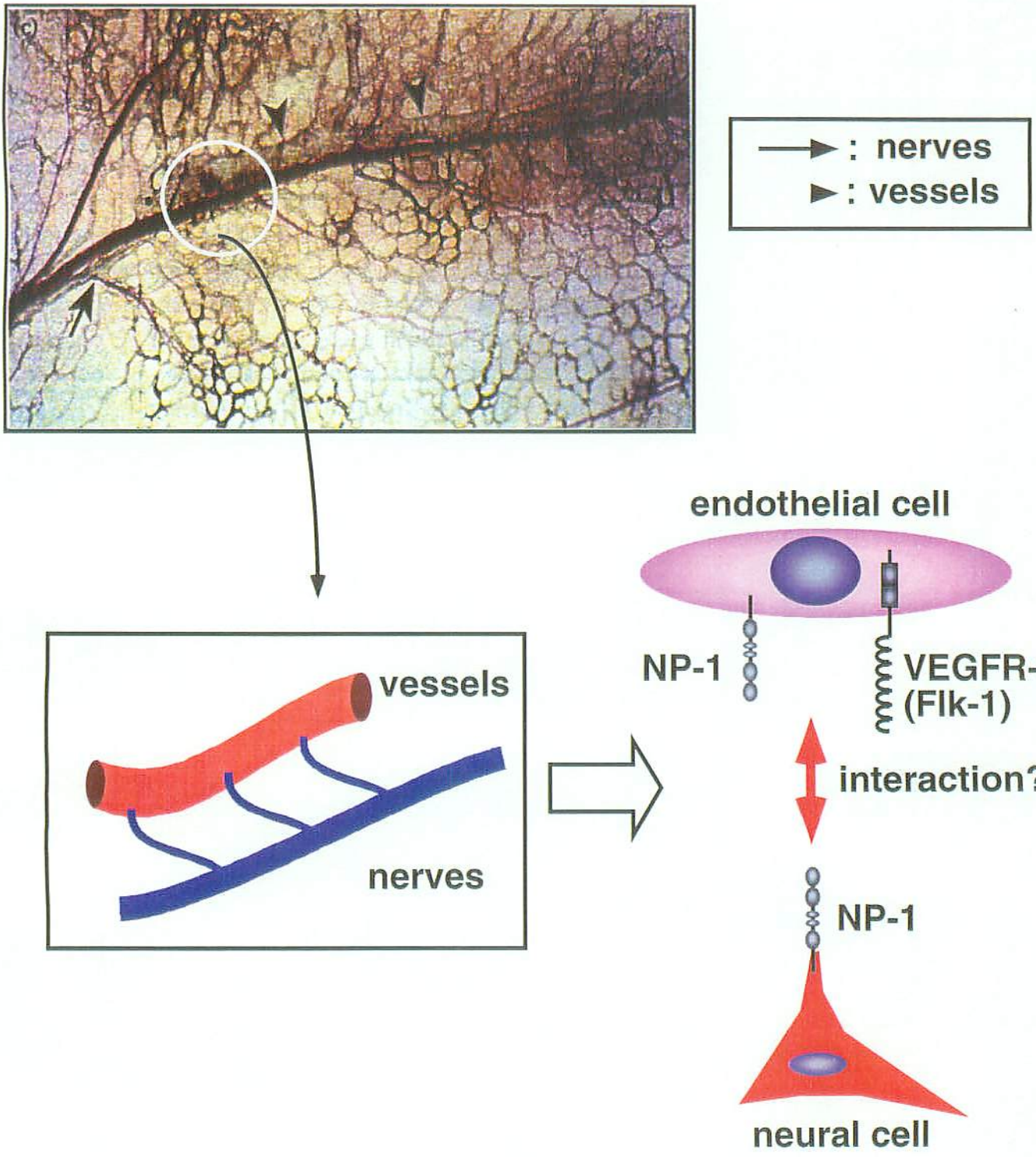


Fig.11 Common molecules in neural cells and endothelial cells

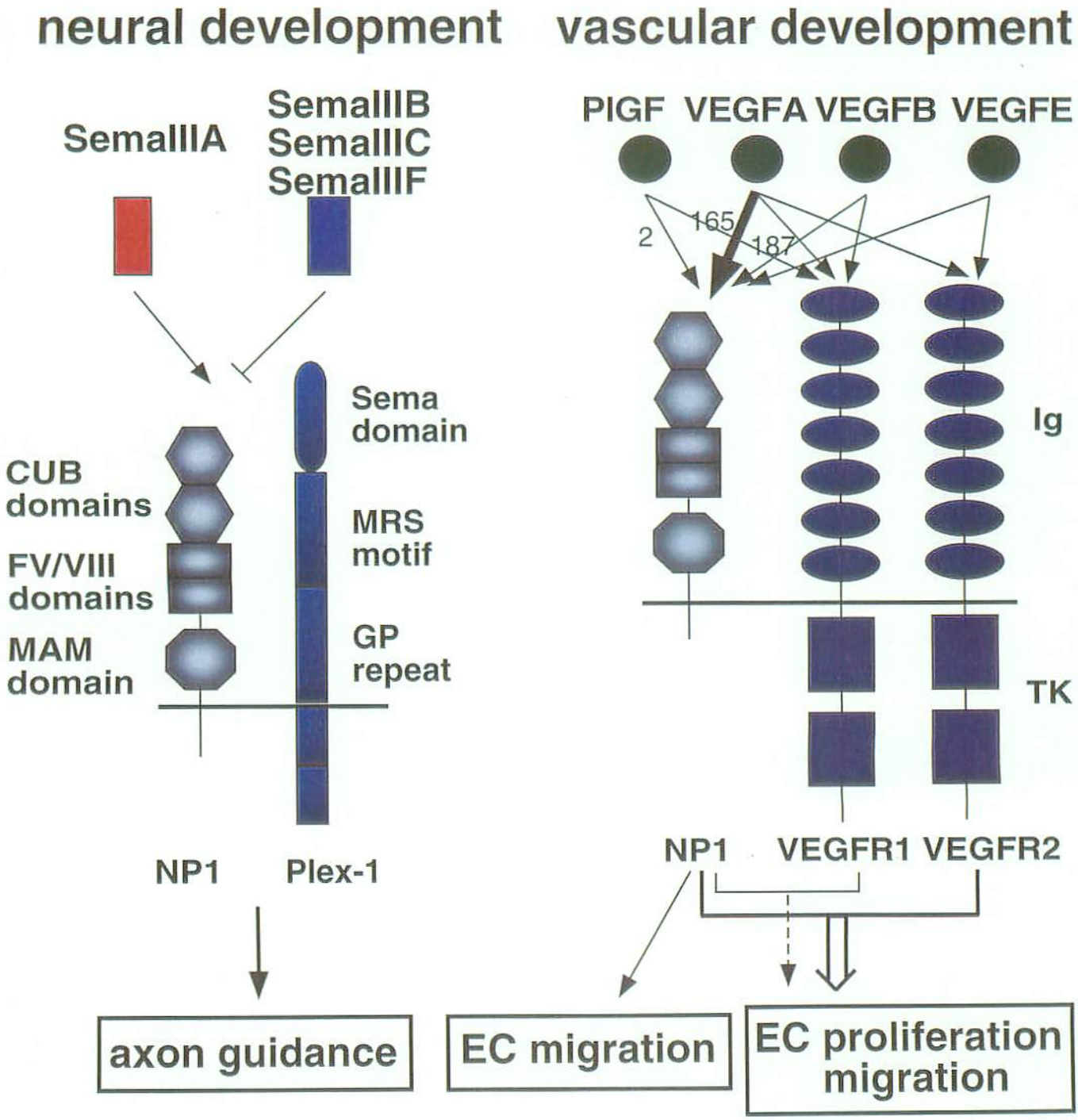


Fig.12
Dissection of P-Sp Region from Murine Embryo

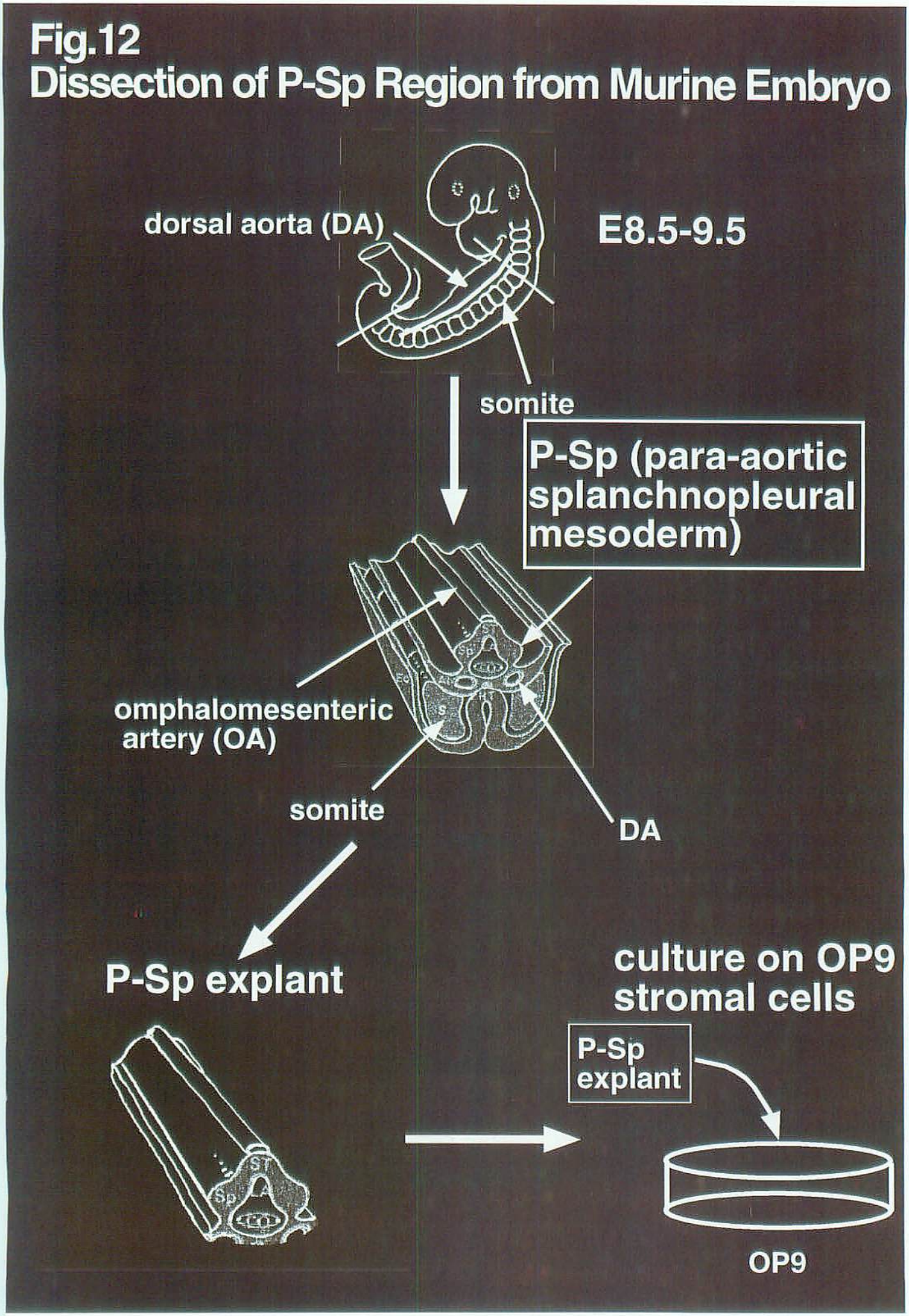


Fig.13 P-Sp Culture System Supports Vasculogenesis, Angiogenesis and Hematopoiesis

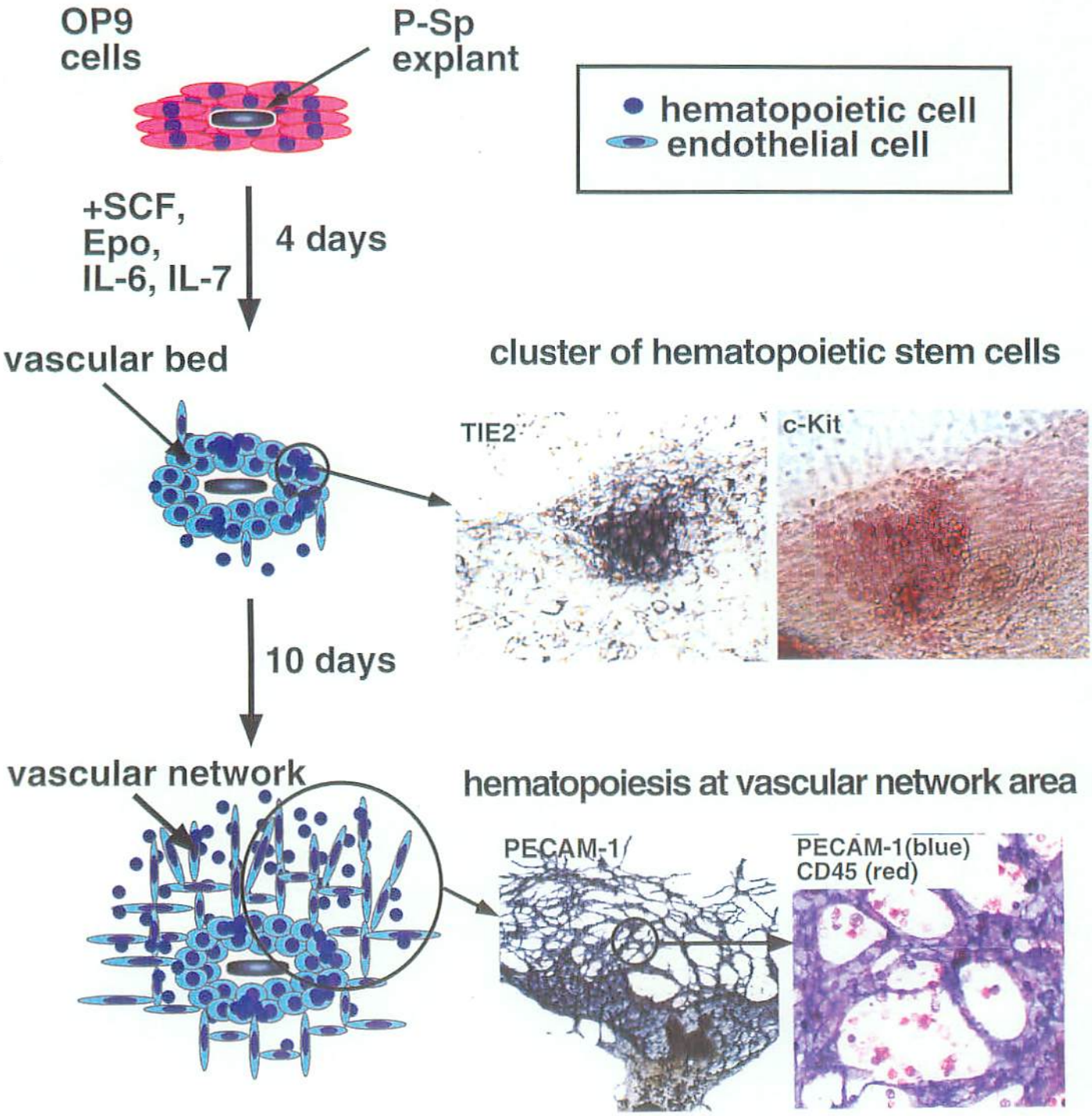
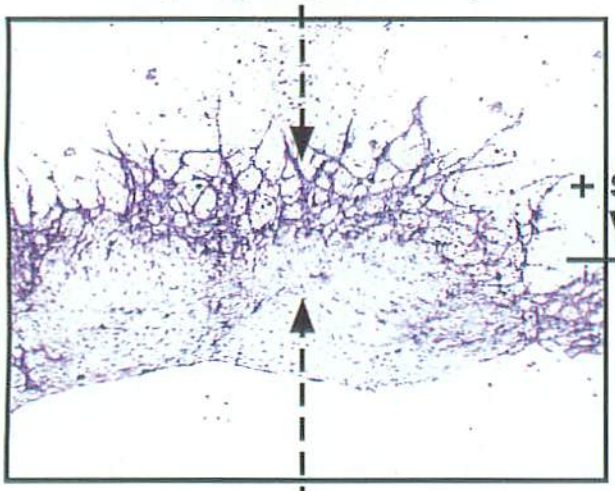


Fig.14 P-Sp culture system reflects in vivo phenotype of knock out mice

network formation = vn
(angiogenesis)



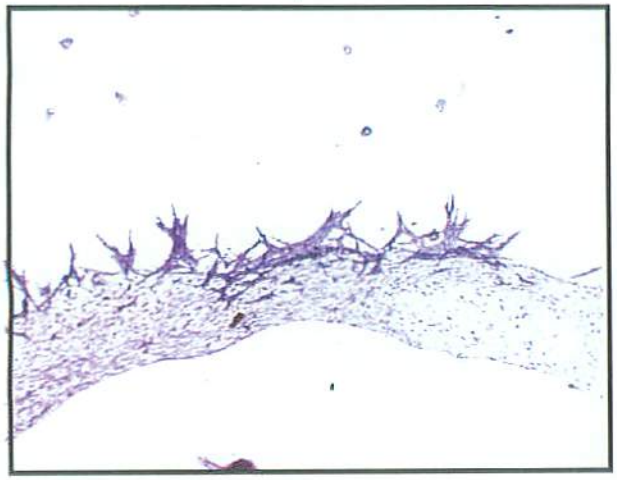
+ soluble
VEGFR-2

vn ↓↓↓
vb ↓↓↓

VEGFR-2 KO mice

vascular bed formation = vb
(vasculogenesis)

+ soluble TIE2



vb →
vn ↓↓

Tie-2 KO mice ⟹

VEGF-VEGFR-2 system is required for both vasculogenesis and angiogenesis stage

This culture system supports and mimics in vivo vasculo-genesis and angiogenesis

Angiopoietins-Tie-2 system is required for angiogenesis stage

Fig.15 Vasculogenesis and angiogenesis in NP-1 mutant embryo

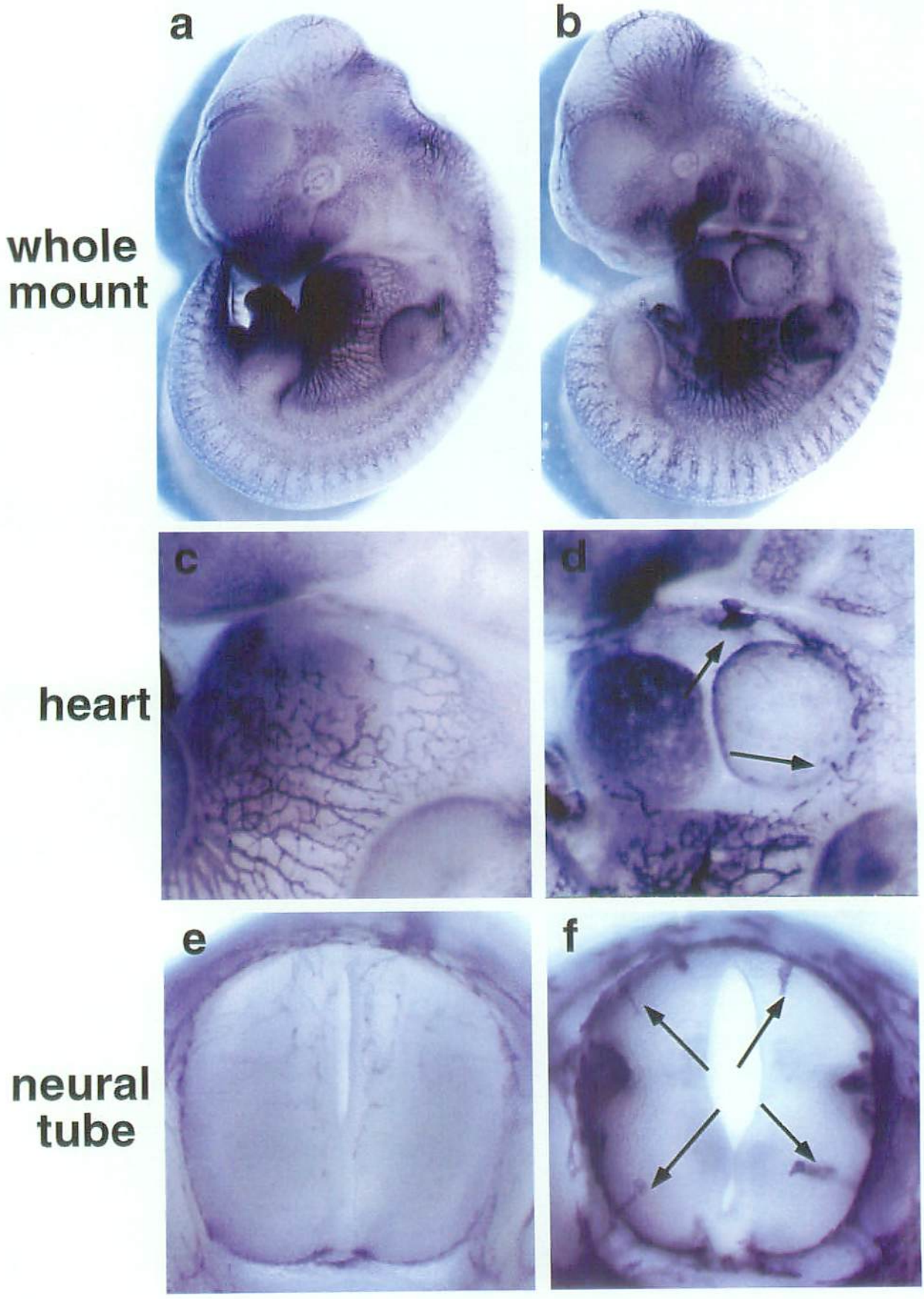


Fig.16 The structure of recombinant NP-1 proteins

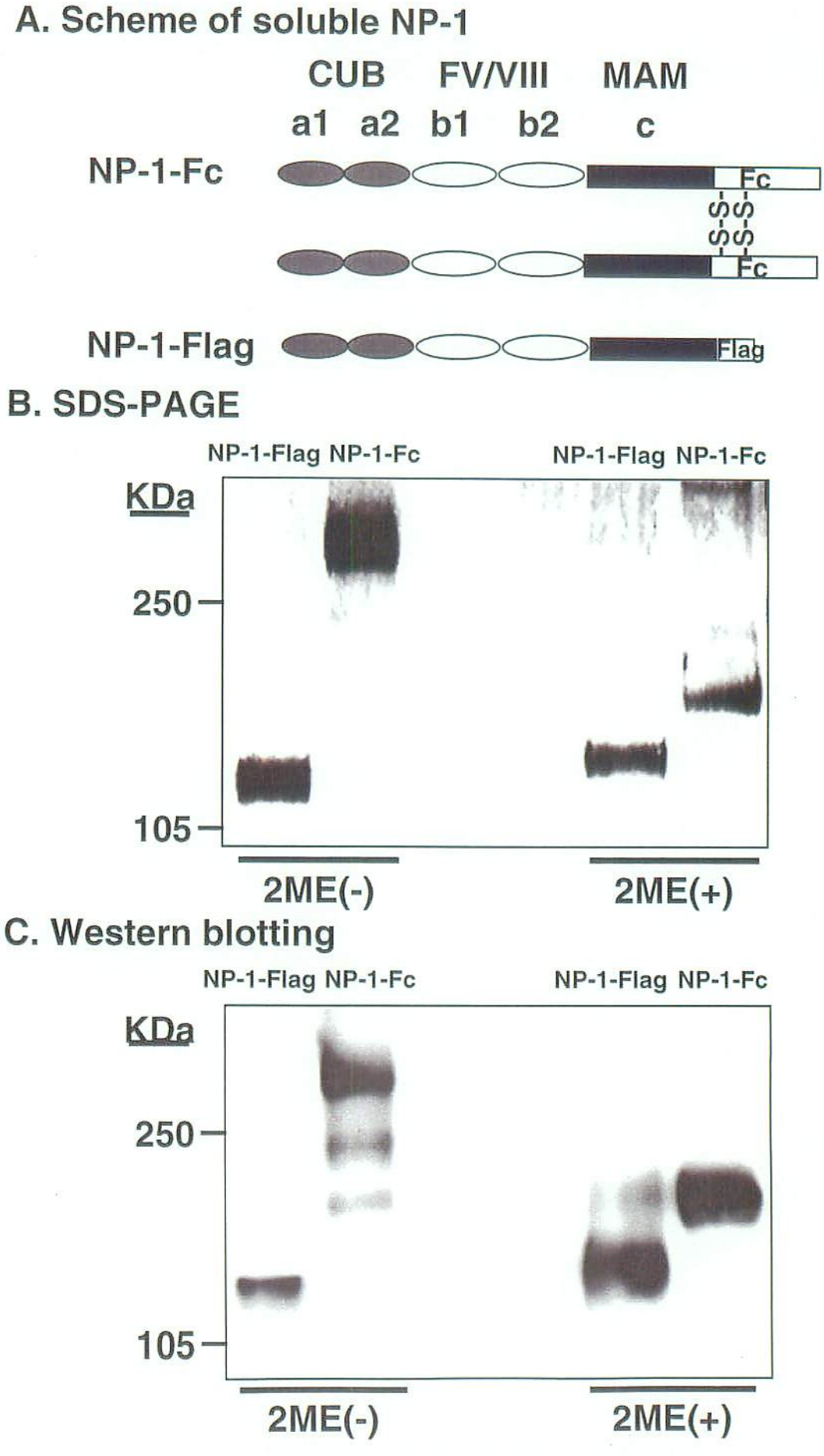
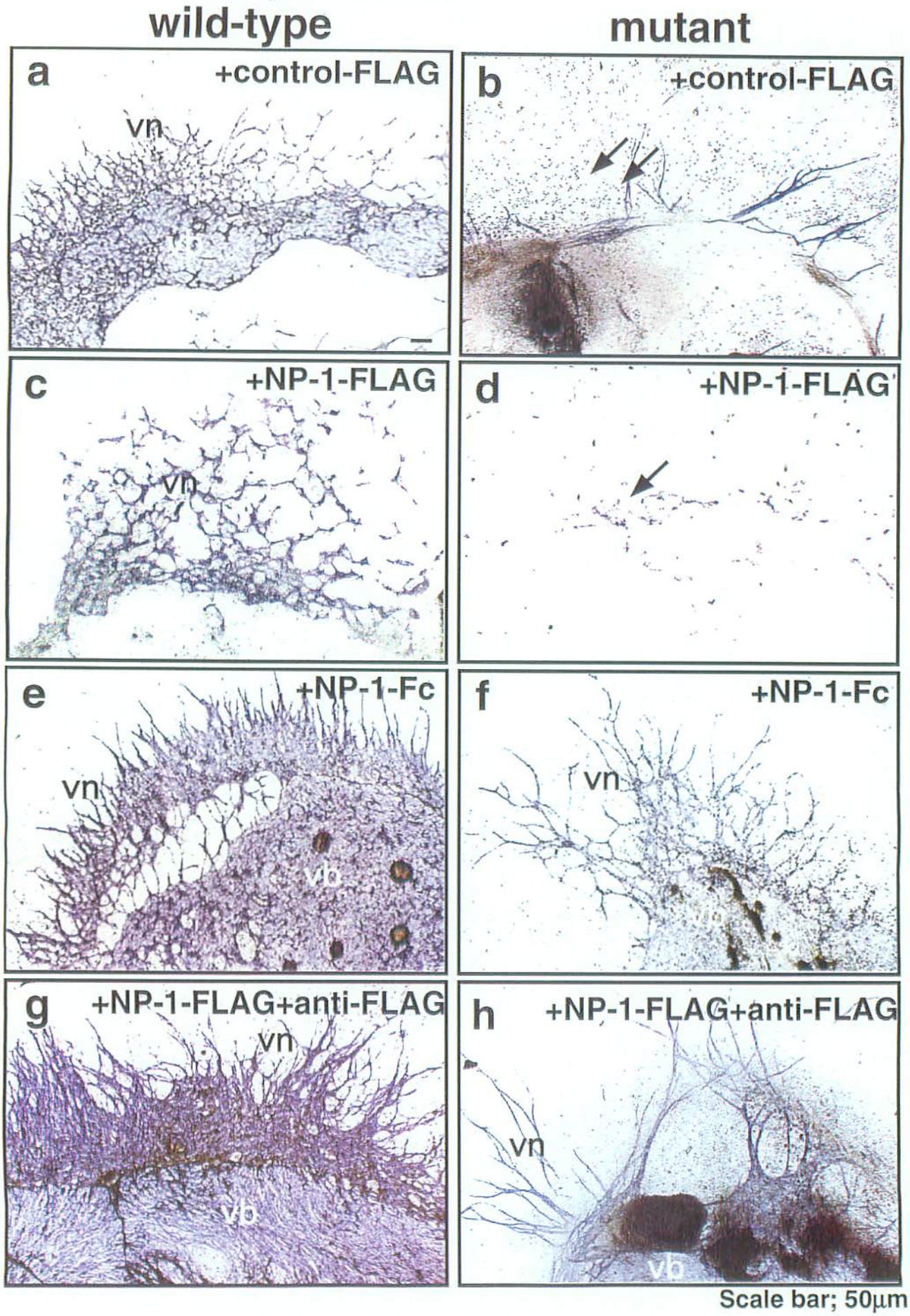


Fig.17 Effect of soluble NP-1 in NP-1 P-Sp culture



Scale bar; 50μm

Fig.18 Effect of a dimer of soluble NP-1 on sorted endothelial cells

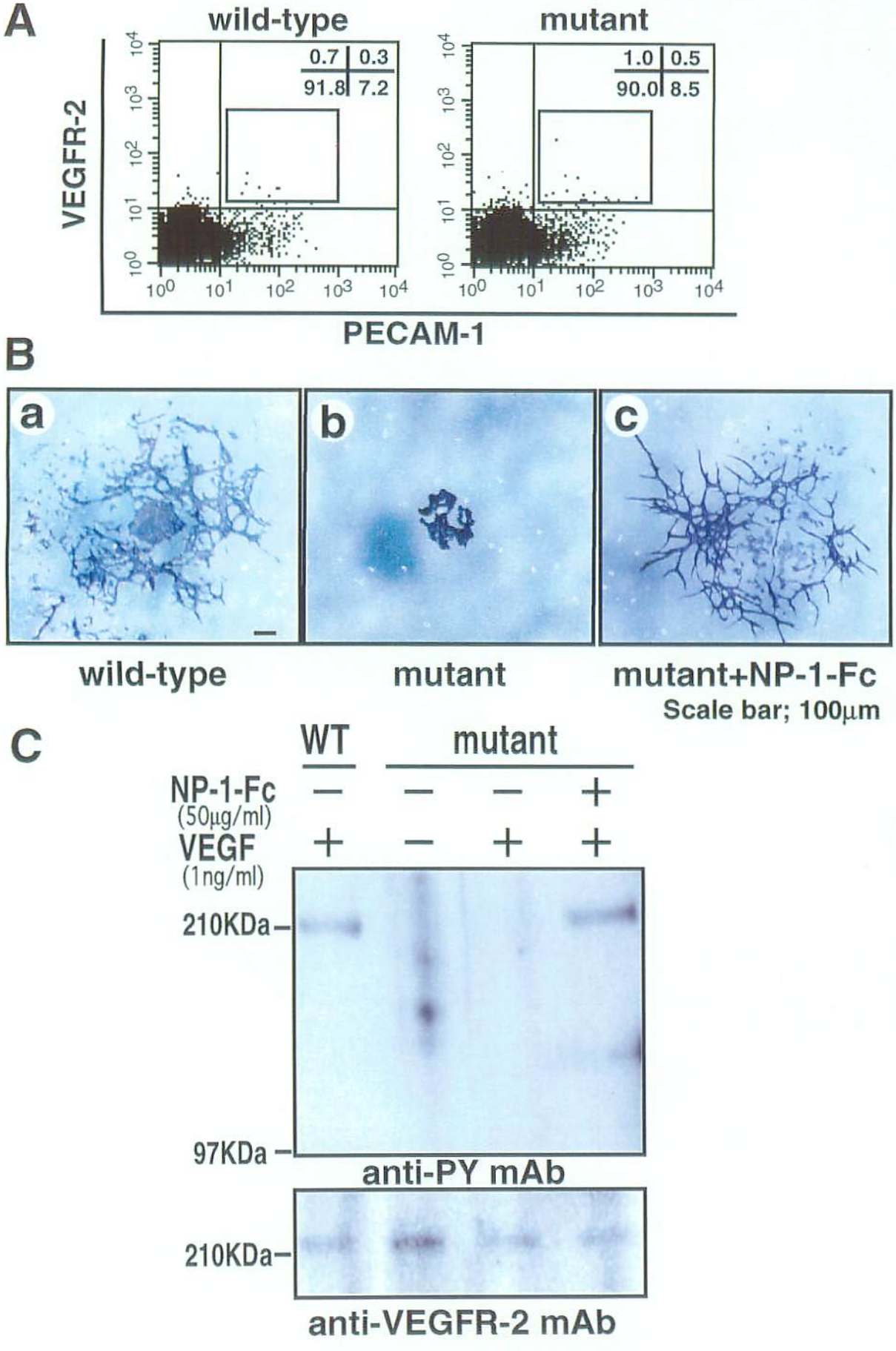
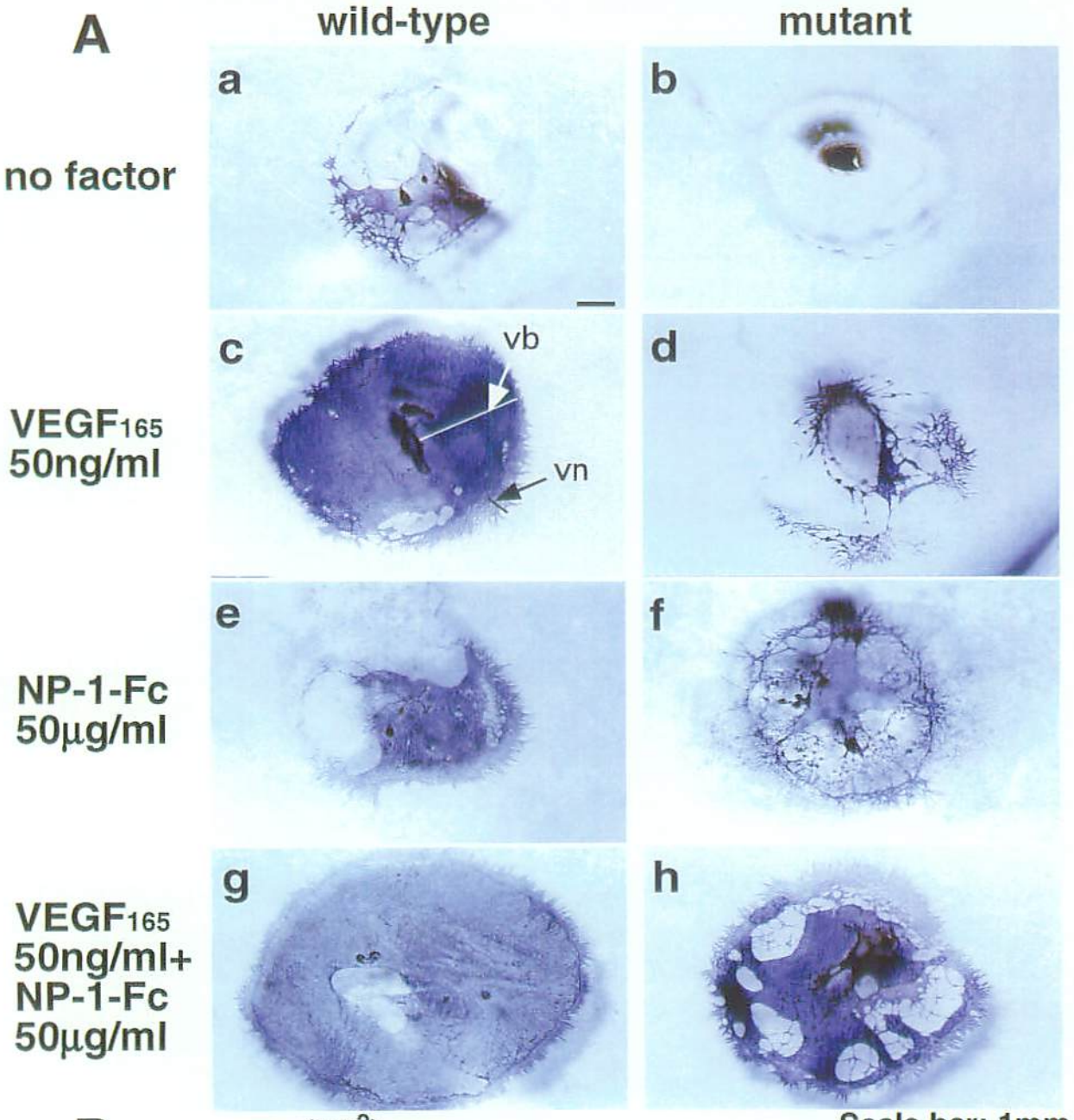


Fig.19 Synergistic effect of soluble NP-1 and $VEGF_{165}$ in NP-1 P-Sp culture system



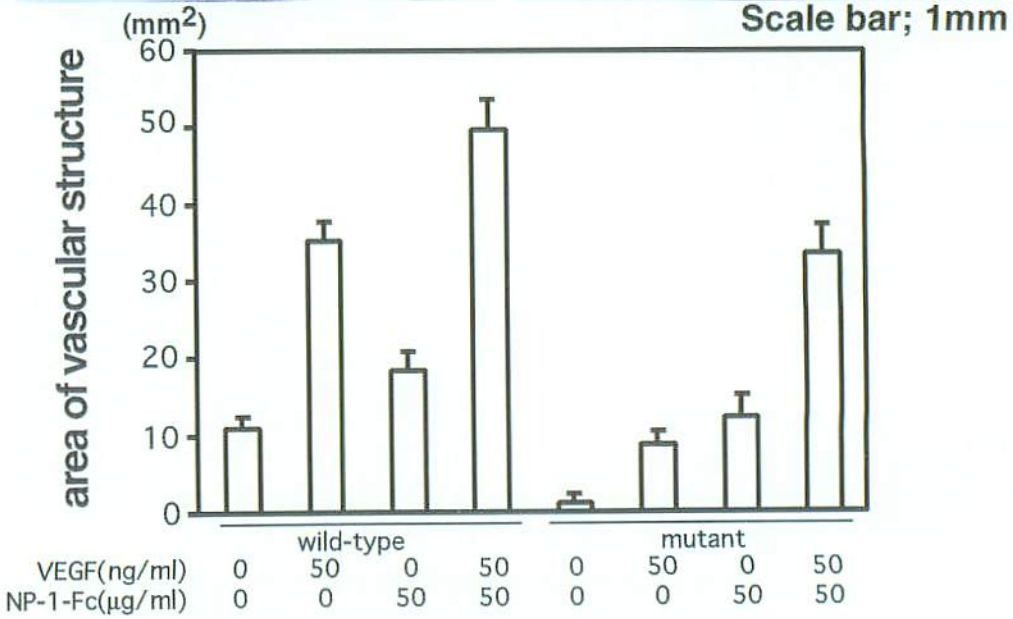
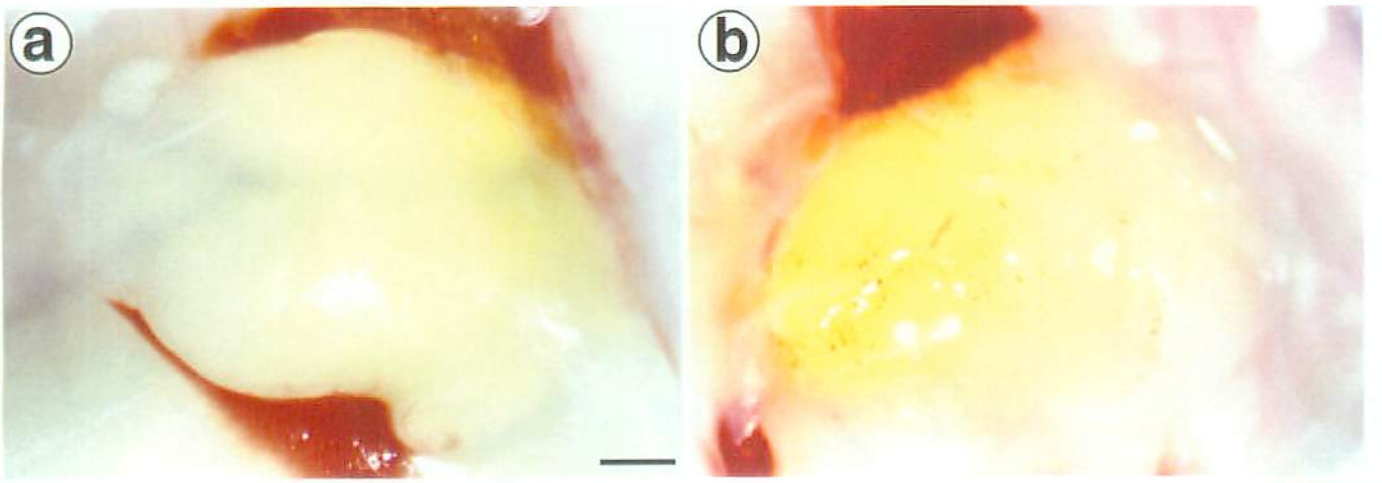


Fig.20 A dimer of soluble Neuropilin-1 induces angiogenesis in vivo

CD4-Fc 50μg/ml
+ VEGF 20ng/ml

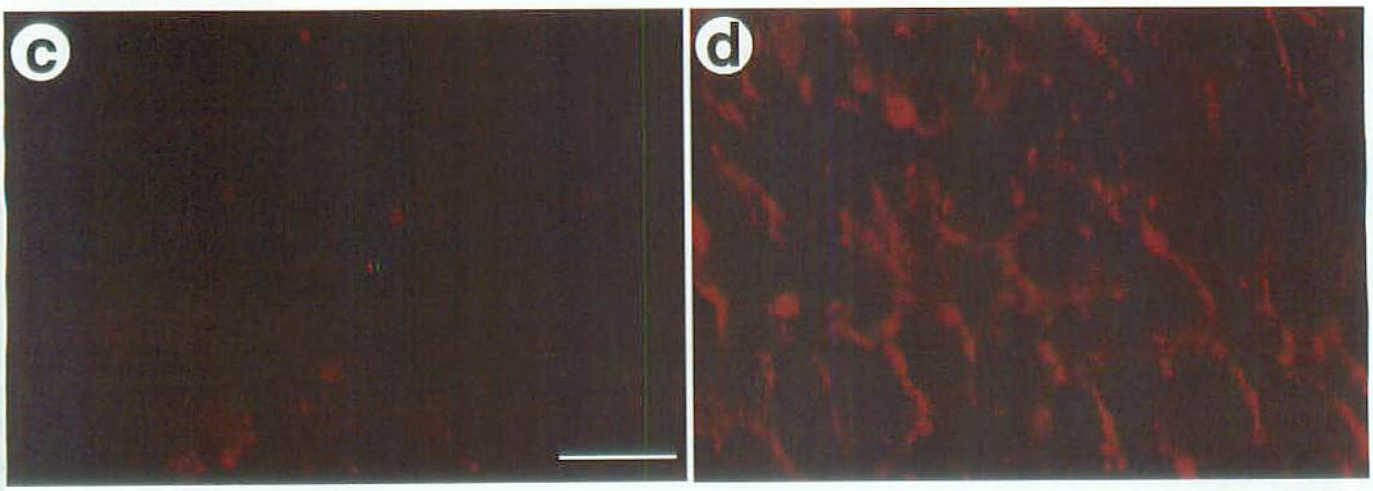
NP-1-Fc 50μg/ml
+ VEGF 20ng/ml

gross appearance



500μm

VEGFR-2-PE

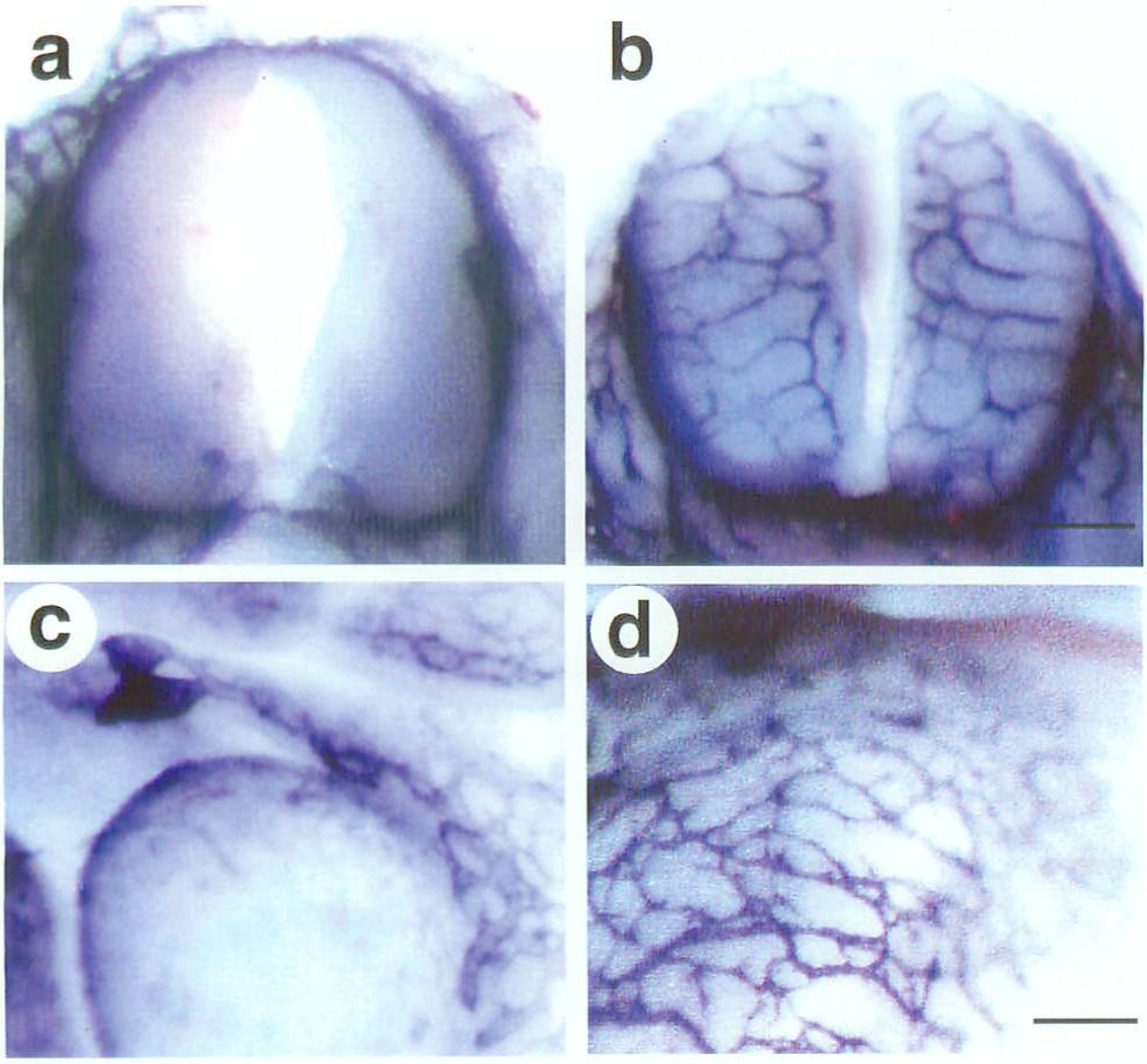


200μm

Fig.21 Rescue of defective vascularity of NP-1 mutant embryos in vivo by soluble NP-1

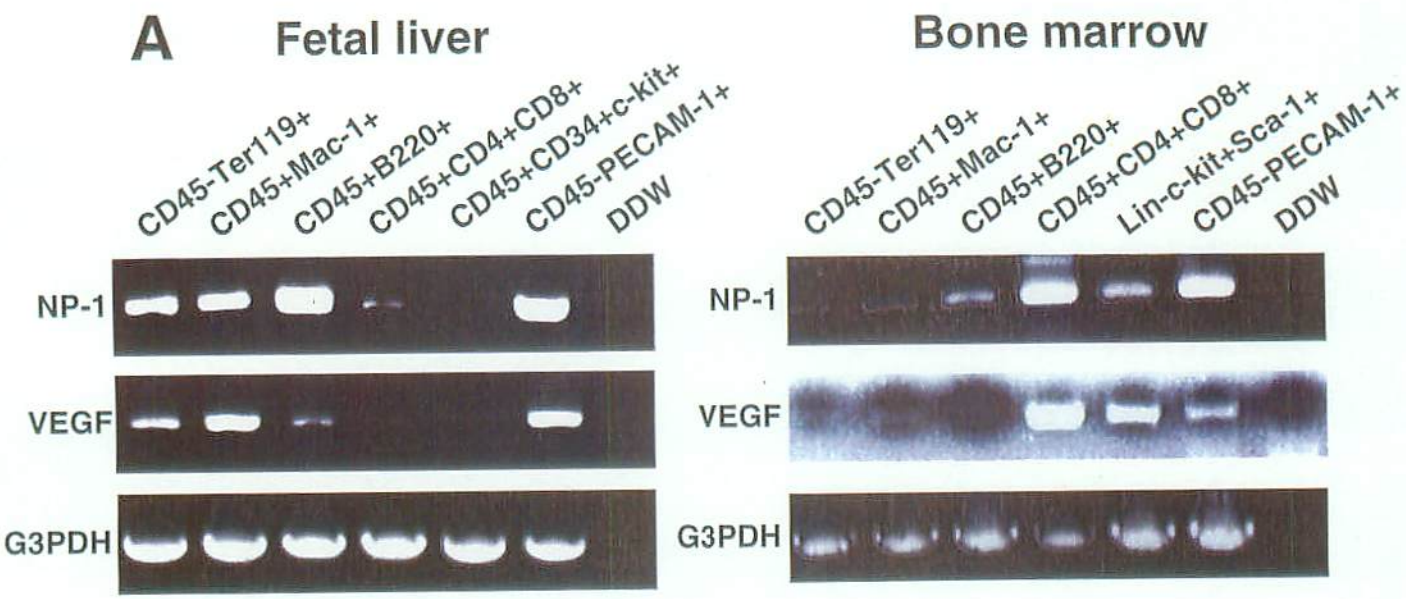
CD4-Fc 300μg/day

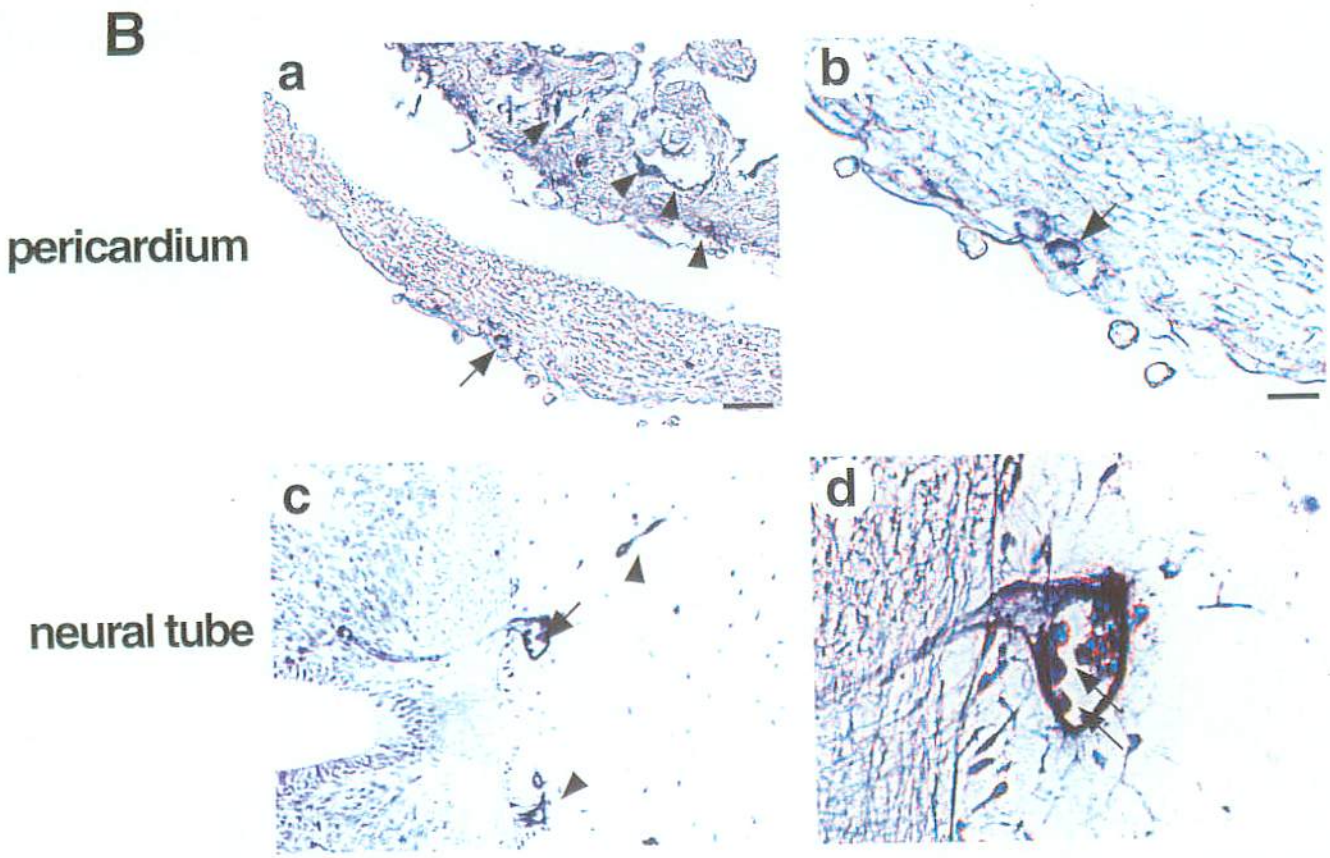
NP-1-Fc 300μg/day



Scale bar; a, b 300μm
c, d 150μm

Fig.22 NP-1 expression on hematopoietic cells





Scale bar; a, c 50μm
b, d 20μm

Fig.23 Expression of VEGF receptors in B lymphocyte cells

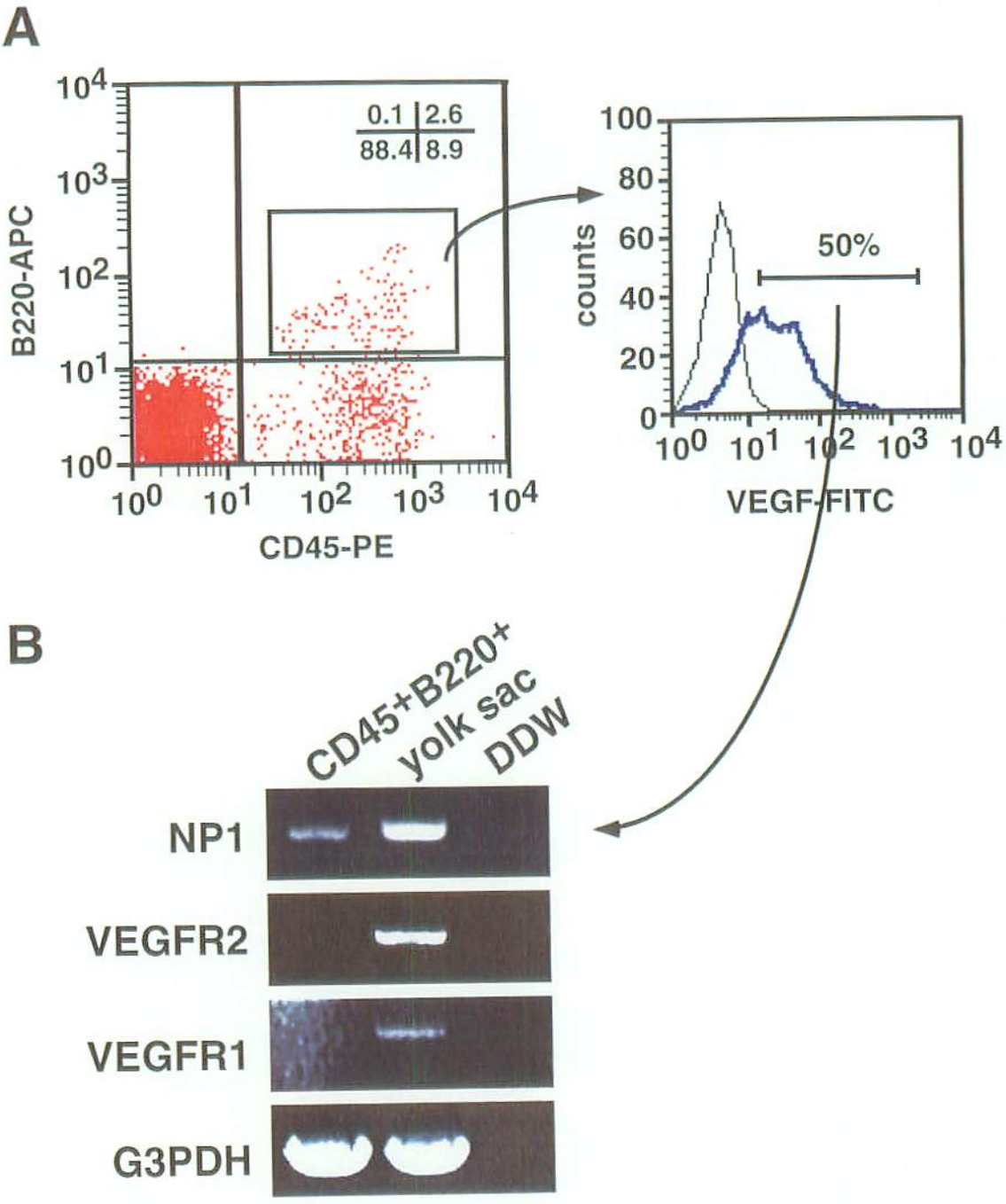
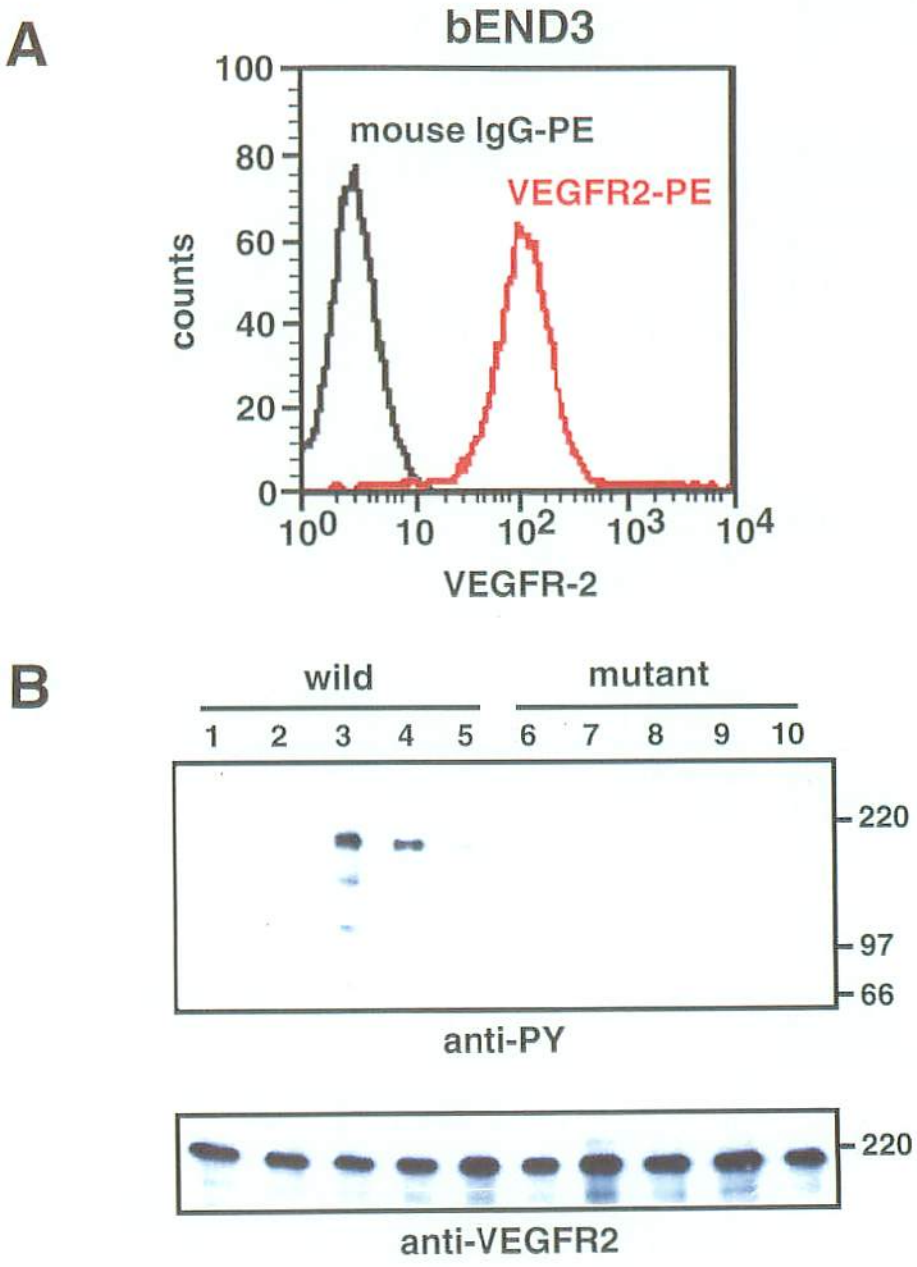
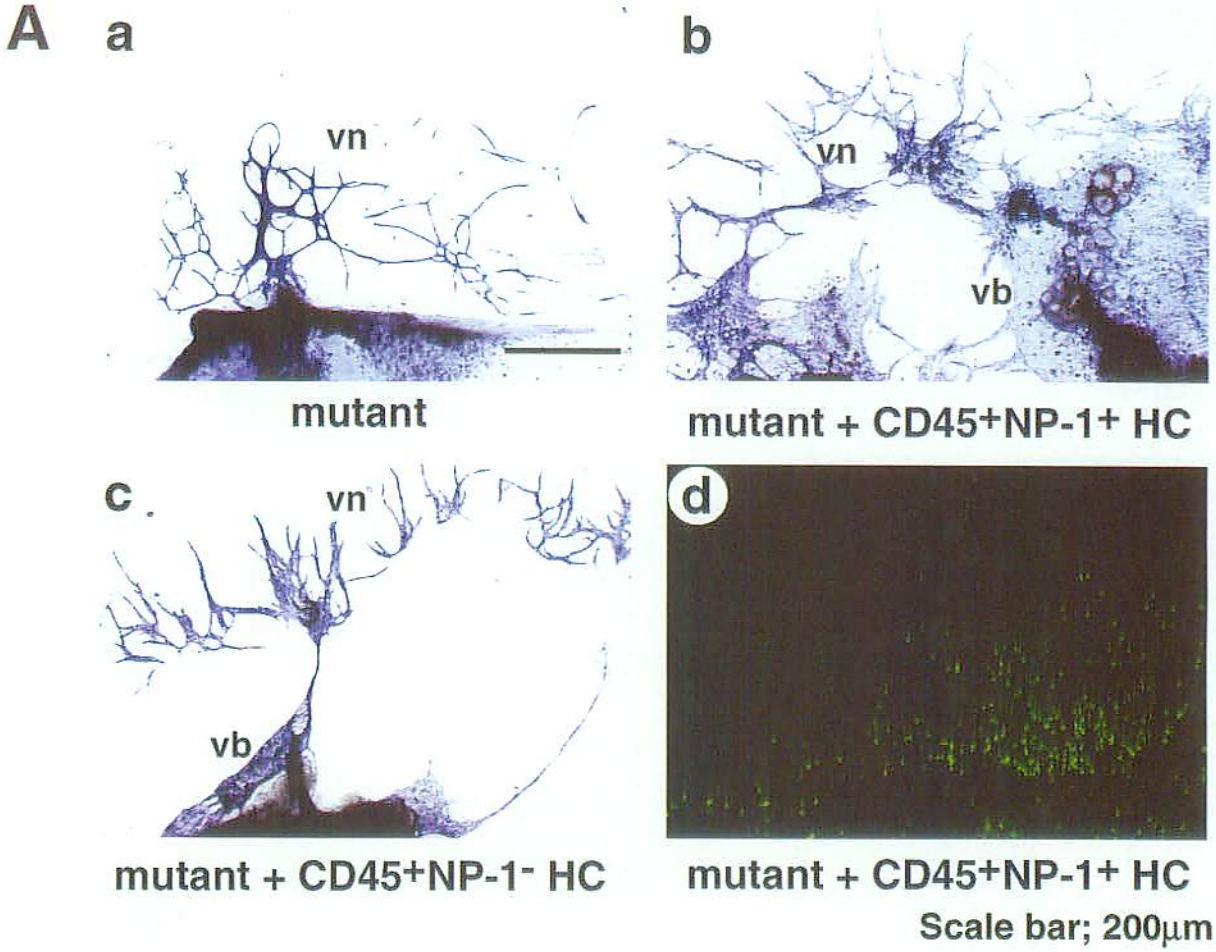


Fig.24 CD45 positive hematopoietic cells together with $VEGF_{165}$ phosphorylate bEND3 cells



lane1-5; $CD45^+$ cells from fetal liver from NP-1 wild-type
lane 6-10; $CD45^+$ cells from fetal liver from NP-1mutant
lane1, 6; no factor
lane2, 7; 2ng/ml VEGF
lane3, 8; 10ng/ml VEGF
lane4, 9; 10ng/ml VEGF+10μg/ml NP1-flag
lane5, 10; 10ng/ml VEGF+30μg/ml NP1-flag

Fig.25 Hematopoietic cells expressing NP-1 rescue vascular defect in NP-1 mutant P-Sp culture



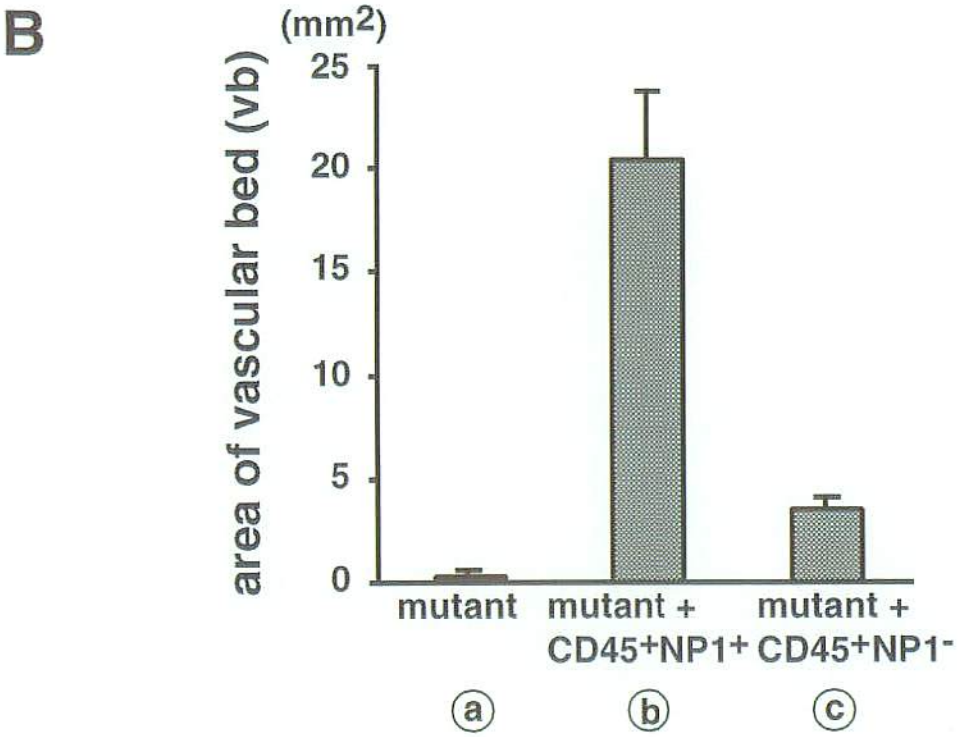


Fig.26 Hematopoietic cells expressing NP-1 together with $VEGF_{165}$ induce angiogenesis in vivo matrigel assay

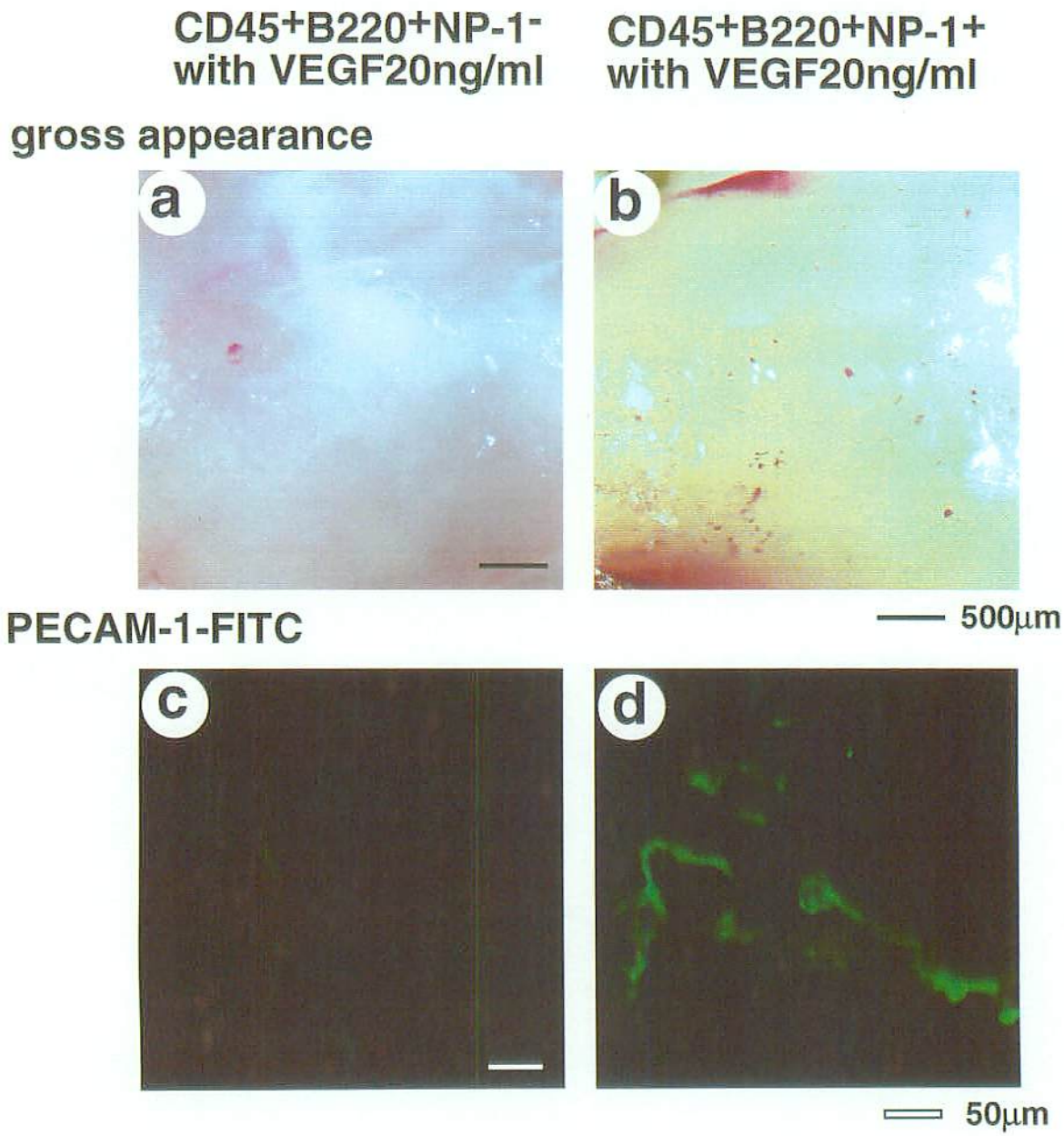
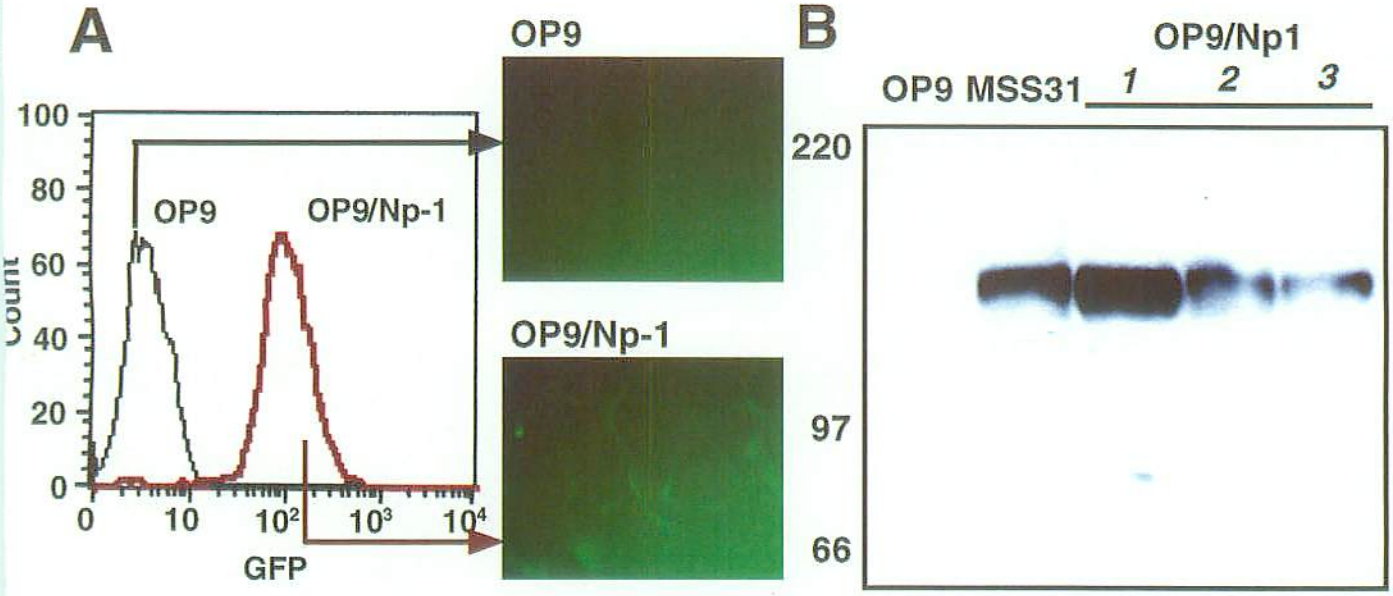
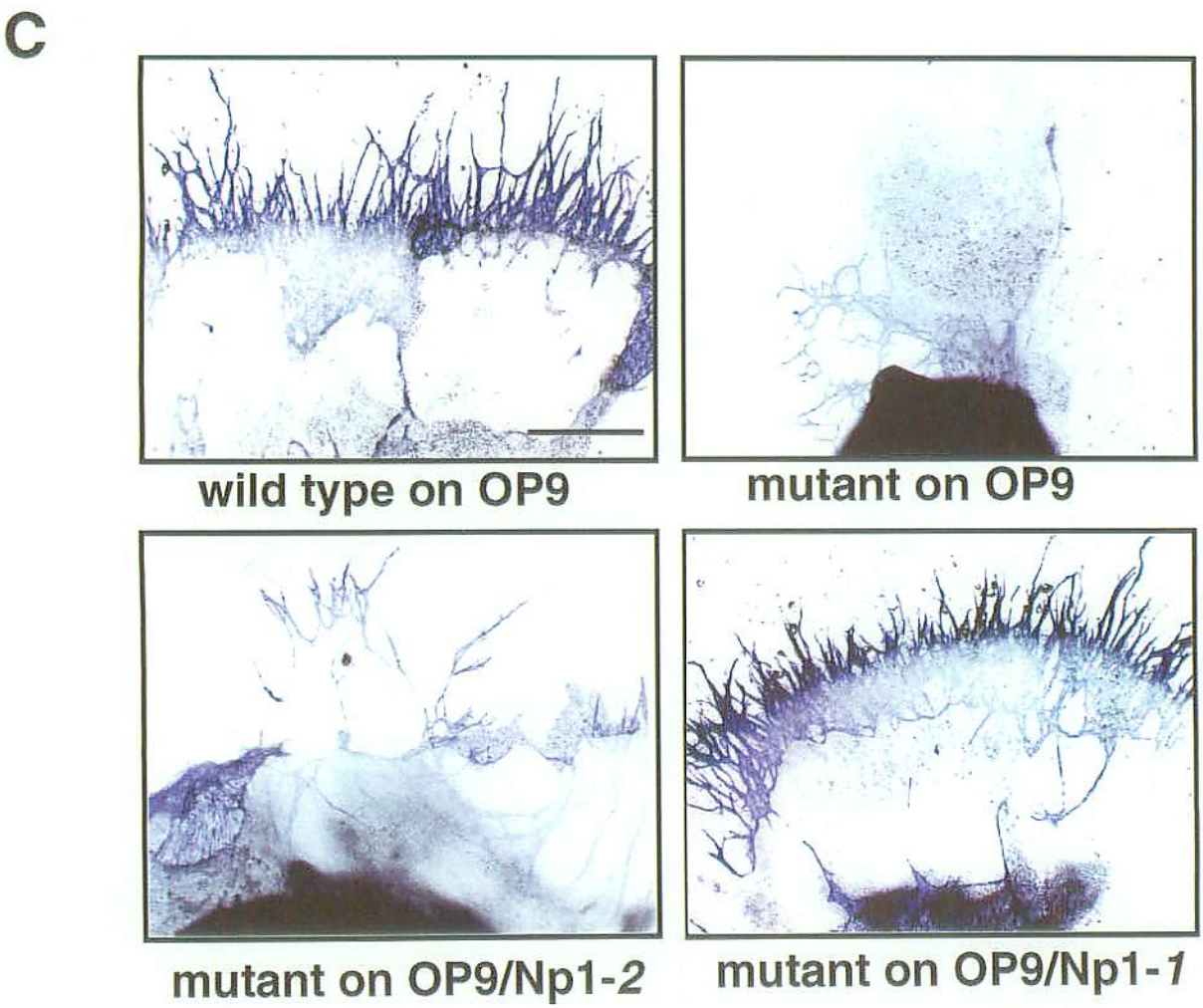


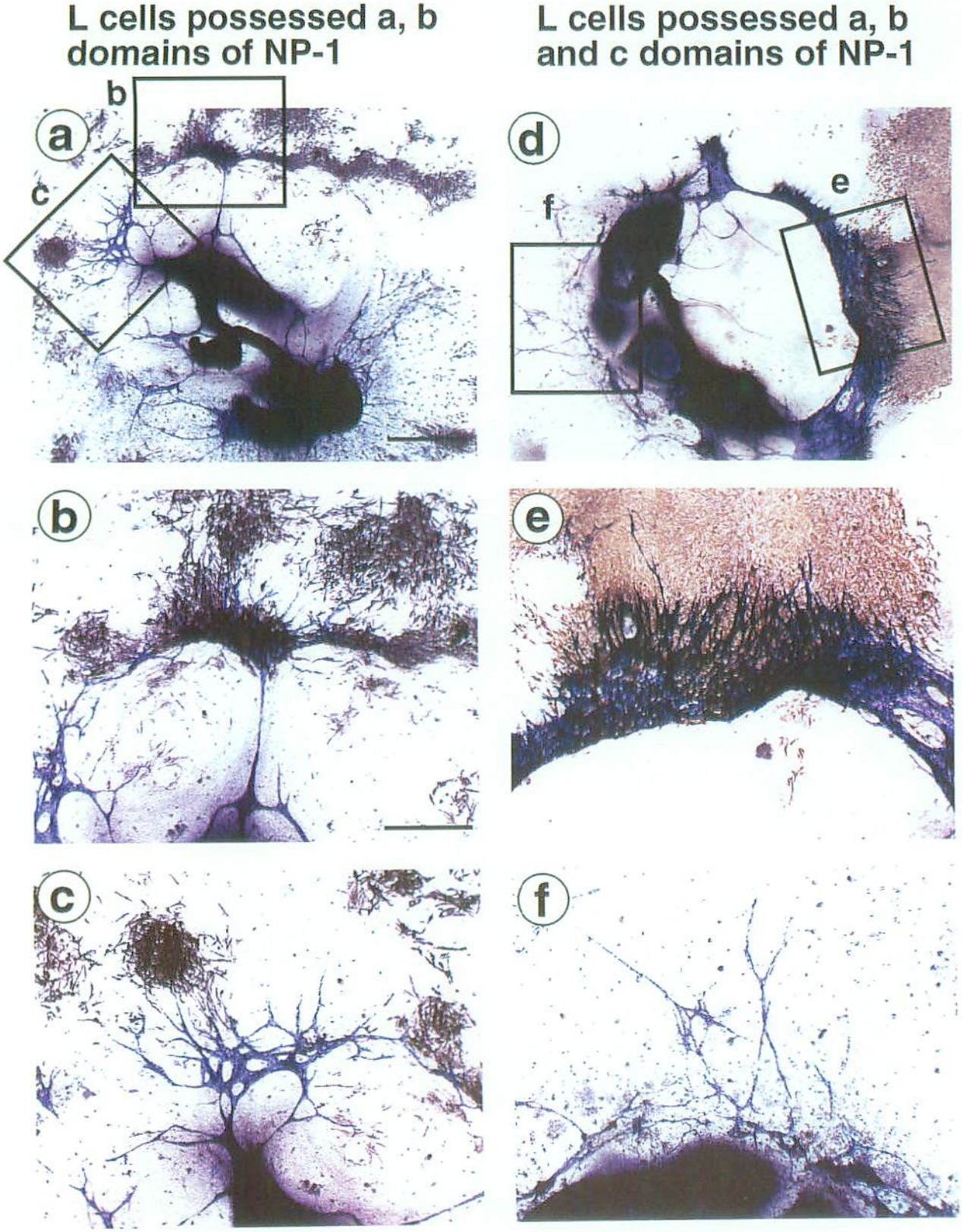
Fig.27 Stromal cells expressing NP-1 rescue vascular defect in NP-1 mutant P-Sp culture





Scale bar; 200μm

Fig.28 Dimerization of NP-1 on L cells enhances vascular development exogenously



Scale bar
a, d 400μm
b, c, e, f 200μm

Fig.29 NP-1 enhances the signaling of VEGFR-2 on ECs endogenously and exogenously

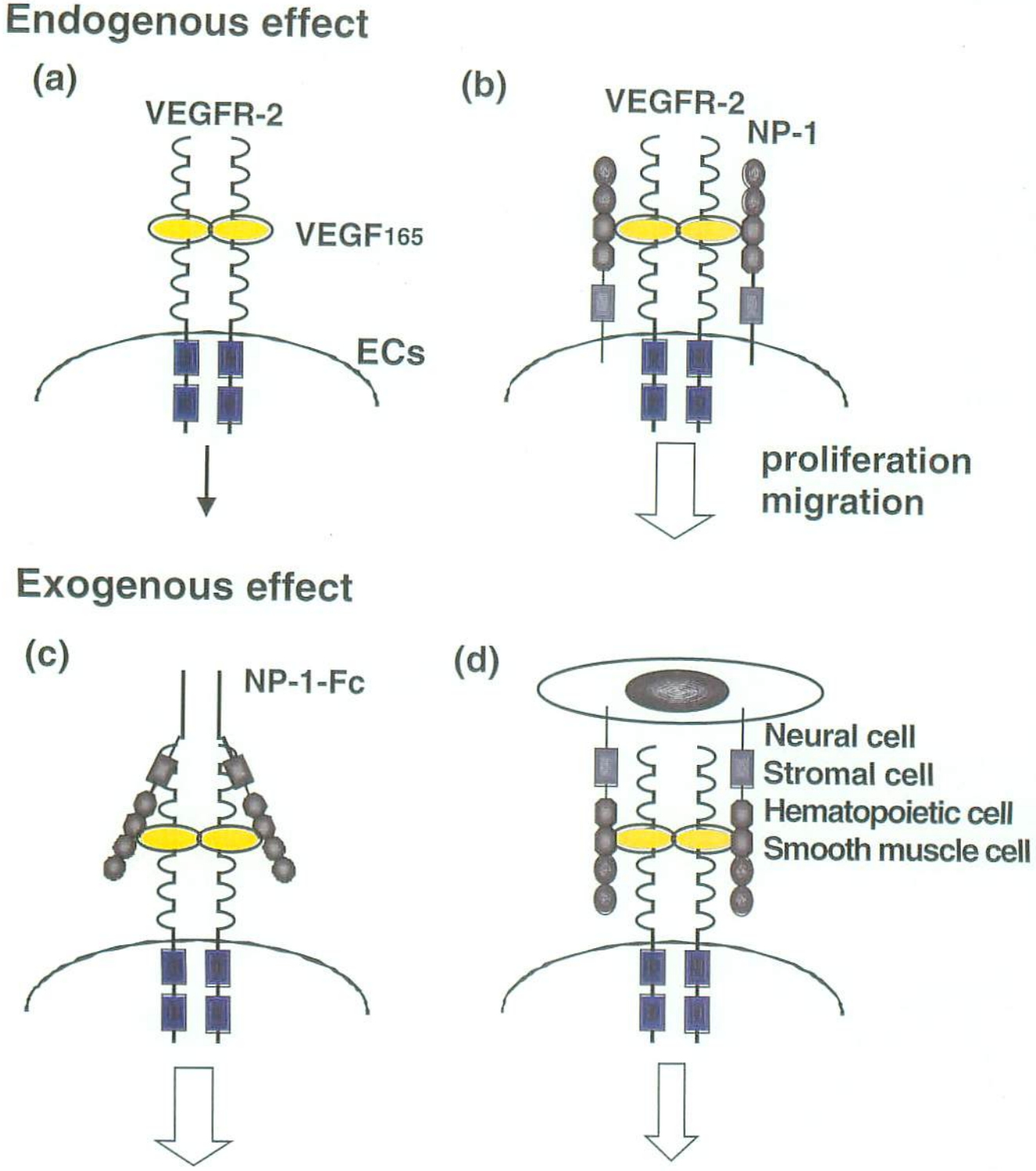


Fig.30 P-Sp and DRG co-culture system

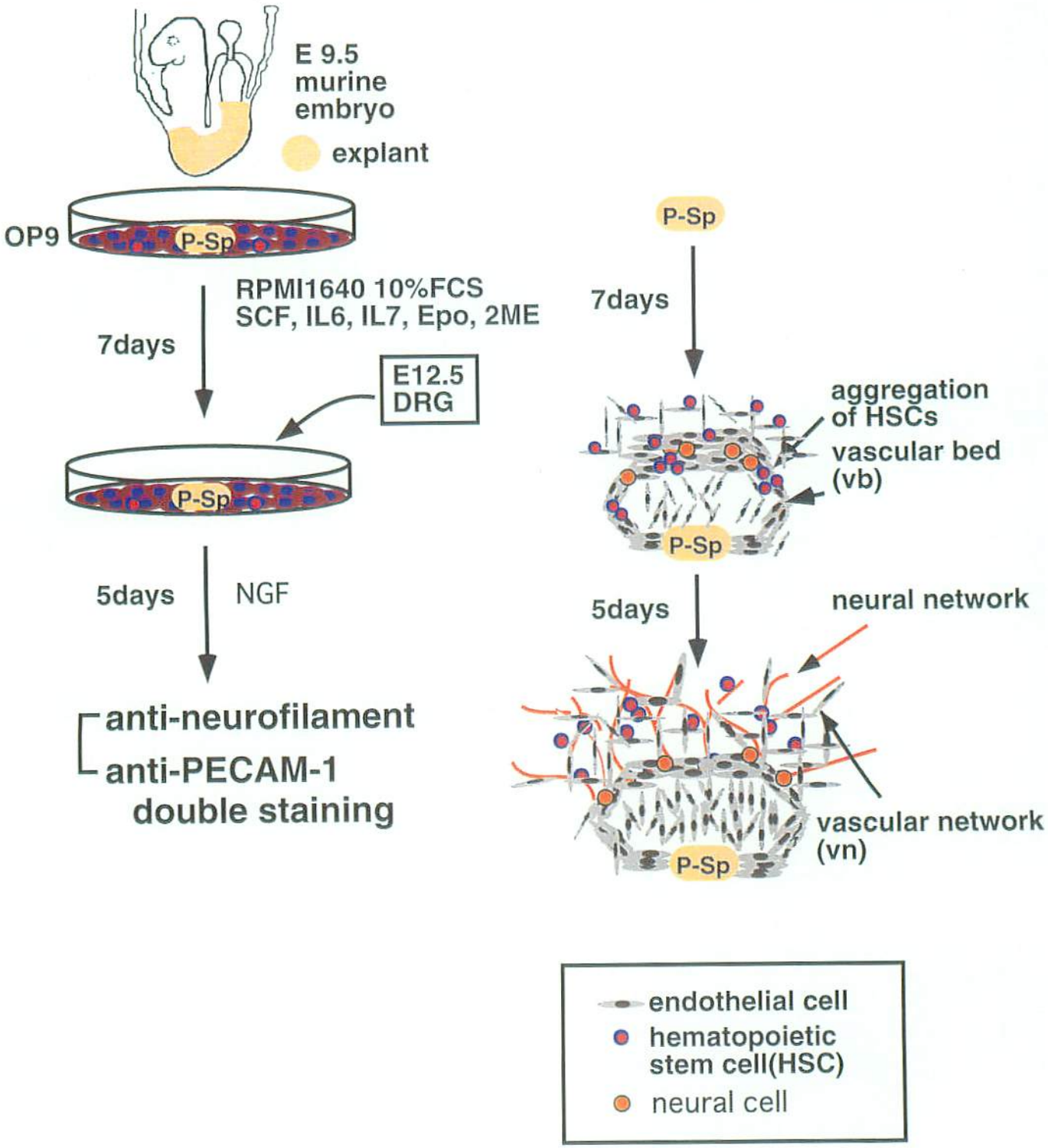
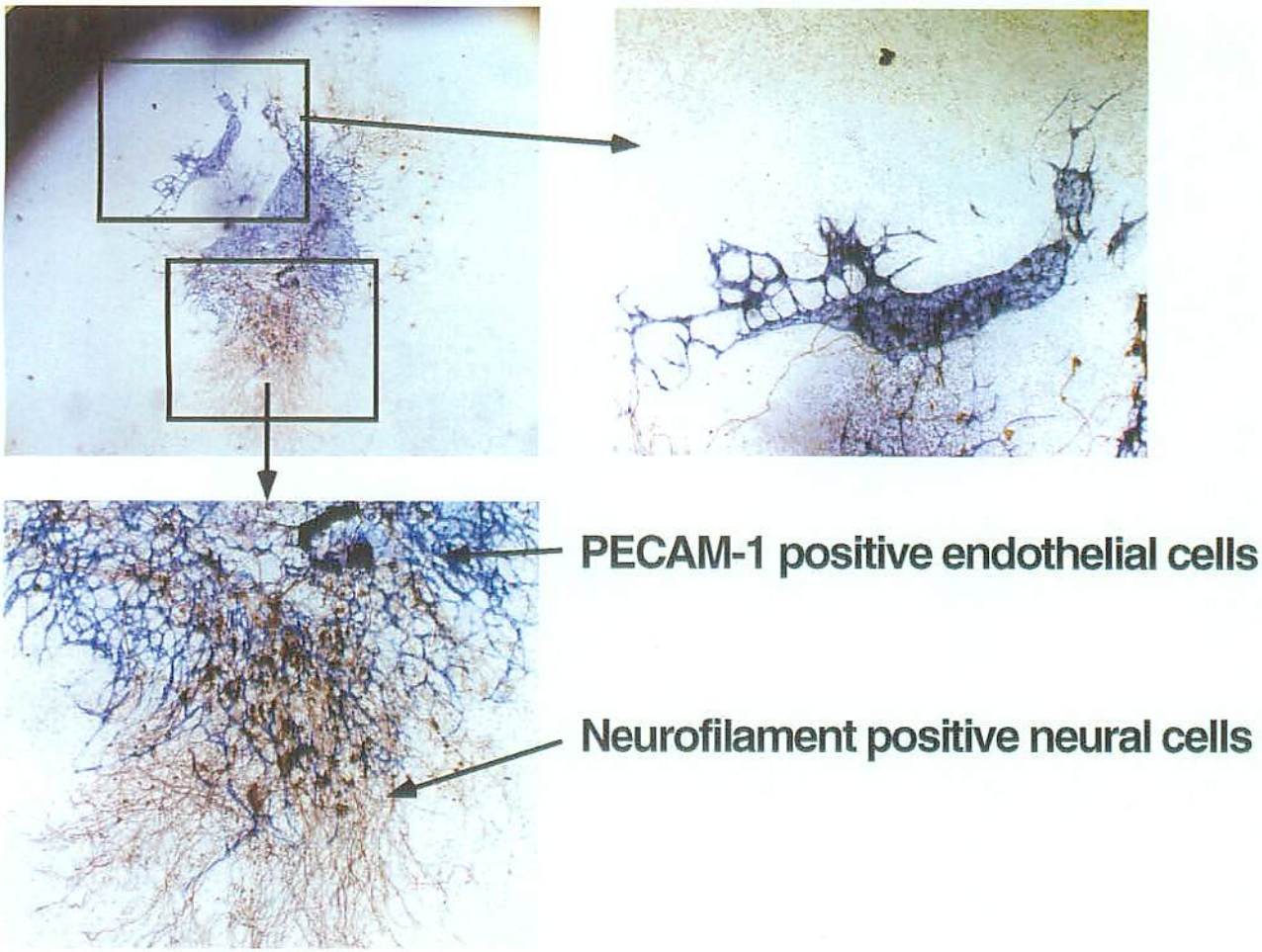
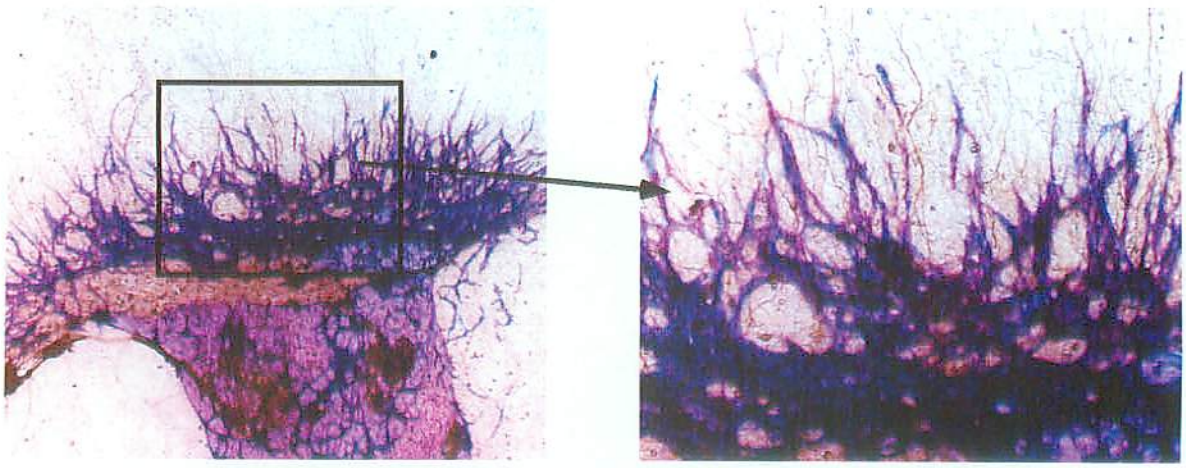


Fig.31 Neural cells induce arterial cells differentiation and their proliferation and migration



further analysis



red : PECAM-1 positive cells (arterial and venous cells)
blue: EphrinB2 positive cells (arterial cells)
brown : Neurofilament positive cells (neural cells)