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Molecular mechanisms of lymphangiogenesis: role of VEGFR-3 mediated signaling

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Academic dissertation

To be presented, with the permission of the Faculty of Science of the University of Helsinki, for public criticism in the lecture hall 2, Biomedicum Helsinki, Haartmaninkatu 8,Helsinki on June 13th, 2002, at 12 o'clock noon.

Helsinki 2002

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ISBN: 952-91-4702-3 (nid.)
ISBN: 952-10-0580-7 (PDF)
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2002

CONTENTS

ABBREVIATIONS **4**

ORIGINAL PUBLICATIONS **5**

ABSTRACT **6**

REVIEW OF THE LITERATURE **7**

1. Cell signaling by receptor tyrosine kinases 7
   - *1.1. RTK activation* *7*
   - *1.2. Signal transduction* *8*
   - *1.3. Signal down-regulation* *9*
2. Development of the vascular system 10
   - *2.1. Vasculogenesis and angiogenesis* *10*
   - *2.2. Lymphangiogenesis* *11*
   - *2.3. Phylogeny of blood and lymphatic vessels* *12*
   - *2.4. Molecular identity of lymphatic versus blood vascular endothelial cells* *13*
3. Molecular regulation of vascular development 14
   - *3.1. Angiogenic and lymphangiogenic factors* *14*
   - *3.1.1. VEGF family of growth factors* *15*
   - *3.1.2. Angiopoietin family* *17*
   - *3.2. Endothelial cell receptors* *17*
   - *3.2.1. VEGF receptors* *17*
   - *3.2.2. Tie receptors* *19*
   - *3.2.3. Neuropilins* *19*
   - *3.3. VEGFs and VEGFRs in the development of other organ systems* *20*
4. Pathologic conditions involving blood and lymphatic vessels 21
   - *4.1. Tumor development and progression* *21*
   - *4.2. Lymphedema* *22*

AIMS OF THE STUDY **25**

MATERIALS AND METHODS **26**

RESULTS AND DISCUSSION **29**

1. Neuropilin-1 is a receptor for VEGF-B (I) 29
2. VEGFR-3 is required for the development and maintenance of the embryonic lymphatic vasculature (II) 30
3. VEGFR-3 transduces lymphatic endothelial cell growth, survival and migration signals (III) 32
   - *3.1. Isolation of lymphatic endothelial cells* *33*
   - *3.2. VEGFR-3 signal transduction* *33*
4. A genetic program controlling the lymphatic versus blood vascular endothelial cell phenotype (IV) 34
   - *4.1. Gene expression profiling of lymphatic and blood vascular endothelial cells* *34*
   - *4.2. Adhesion molecules in lymphatic and blood vascular endothelial cells* *35*
   - *4.3. Prox-1 as a fate determining factor for lymphatic endothelial cells* *36*

CONCLUDING REMARKS **37**

ACKNOWLEDGEMENTS **38**

REFERENCES **39**

ABBREVIATIONS

Ang	Angiopoietin
BEC	blood vascular endothelial cell
BM	basement membrane
DNA	deoxyribonucleic acid
E	embryonic day
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
Flk1	fetal liver kinase 1 (VEGFR-2)
Flt1	*fms*-like tyrosine kinase 1 (VEGFR-1)
Flt4	*fms*-like tyrosine kinase 4 (VEGFR-3)
GAP	GTPase-activating protein
GTP	guanosine triphosphate
Ig	Immunoglobulin
KDR	kinase insert domain containing receptor (VEGFR-2)
LEC	lymphatic endothelial cell
MAPK	mitogen activated protein kinase
NRP	neuropilin
PDGF	platelet-derived growth factor
PECAM	platelet-endothelial cell adhesion molecule
PI3-K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLCγ	phospholipase C-gamma
PlGF	placenta growth factor
Prox-1	*prospero*-related homeobox protein 1
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SMC	smooth muscle cell
Tek	Tunica interna endothelial cell kinase (Tie-2)
TGF	transforming growth factor
Tie	Tyrosine kinase with immunoglobulin and EGF homolygy domains
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPF	vascular permebility factor (VEGF)
VRF	VEGF related factor (VEGF-B)
VRP	VEGF related protein (VEGF-C)
vWF	von Willebrand Factor

ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Makinen, T.*, Olofsson, B.*, Karpanen, T., Hellman, U., Soker, S., Klagsbrun, M., Eriksson, U. and Alitalo, K. Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms by Neuropilin-1. *Journal of Biological Chemistry* 274, 21217-21222, 1999.

II Mäkinen, T., Jussila, L., Veikkola, T., Karpanen, T., Kettunen, M.I., Pulkkanen, K.J., Kauppinen, R., Jackson, D.G., Kubo, H., Nishikawa, S.-I., Ylä-Herttuala, S. and Alitalo, K. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nature Medicine* 2, 199-205, 2001.

III Mäkinen, T., Veikkola, T., Mustjoki, S., Karpanen, T., Wise, L., Mercer, A., Kerjaschki, D., Catimel, B., Nice, E.C., Stacker, S.A., Achen, M.G. and Alitalo, K. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *The EMBO Journal* 17, 4762-4773, 2001.

IV Petrova, T.V.*, Mäkinen, T.*, Mäkelä, T., Kerjaschki, D., Saarela, J., Ylä-Herttuala, S. and Alitalo K. Prox-1 regulates the genetic program controlling lymphatic versus blood vascular endothelial phenotype. Submitted, 2002.

*) these authors contributed equally to this work

ABSTRACT

Blood and lymphatic vessels form a circulatory system, which allows the transportation of metabolic substances, cells and proteins in the body. A major role of the lymphatic vasculature is to return an excess of the protein-rich interstitial fluid to the blood circulation. Until recently, studies of the lymphatic vessels were hampered due to the lack of specific markers, but to date several such markers have been identified. In addition, recent studies have indicated an important role for lymphatic vessels in certain developmental disorders, such as lymphedema, and as a route for metastasis of tumors. These findings have brought lymphatic biology to the forefront of vascular research.

Two members of the VEGF (vascular endothelial growth factor) family of growth factors, VEGF-C and VEGF-D, have been shown to stimulate the growth of lymphatic vessels via lymphatic endothelial cell receptor, VEGFR-3. Although VEGFR-3 is required for the development of the blood vascular system during early embryonic development, later on its expression becomes restricted to lymphatic endothelial cells and it thus serves as a marker of these cells in adult tissues. Recent studies also demonstrate that VEGFR-3 and its ligands are involved in the development of lymphedema and lymphatic metastasis.

This study was undertaken to obtain more information concerning the molecular mechanisms regulating the development and growth of lymphatic vessels, concentrating on the role of VEGFR-3 in these processes. We showed that VEGFR-3 mediates proliferation, survival and migration of lymphatic endothelial cells and demonstrated that VEGFR-3 function is required for the normal development of lymphatic vasculature. Blocking of VEGFR-3 signaling inhibited specifically lymphangiogenesis without affecting blood vessels, indicating that distinct molecular mechanisms control the development of the two vascular systems. We also analysed the genetic programs which determine the lymphatic and blood vascular endothelial cell identities, and identified the homeobox transcription factor Prox-1 as a fate determining factor for lymphatic endothelial cells. These studies give new insights into the phenotypic diversity of endothelial cells and reveal new potential vascular markers, some of which could provide important targets for the treatment of diseases characterised by abnormal angiogenesis or lymphangiogenesis.

REVIEW OF THE LITERATURE

1. Cell signaling by receptor tyrosine kinases

Coordinated growth and differentiation of cells and their organisation into specific tissues depend on cell-cell signaling. Some of these signals are mediated via secreted growth factors which bind to their receptors on the surface of target cells. The receptor-mediated activation of intracellular signaling cascades usually target transcription factors, modify their activity and reprogram gene expression. Covalent modification of proteins by reversible phosphorylation accomplished by protein kinases is a key mechanism by which intracellular signals are transduced from the cell surface to the nucleus. Growth factor receptors having an intrinsic protein tyrosine kinase activities have been characterised as pehaps the most important regulators of cellular functions. In addition to receptor tyrosine kinases (RTK), other membrane receptors including integrins and guanosine triphosphate-binding protein (G protein)-coupled receptors transduce extracellular signals into the cell and regulate important cellular processes such as cell migration and survival (Lowes *et al.*, 2002; Schwartz, 2001). To increase complexity, many intracellular signaling molecules participate in several signaling cascades. Cell-type specific expression of receptors and effector proteins and the availability of cognate ligands as well as the strength and persistence of the signal all affect how the cell responds to the stimulus. In addition, cellular history defines the developmental competence of the cell and its ability to respond to growth factor stimuli.

1.1. RTK activation

RTKs are divided into distinct subclasses based on their structural characteristics and sequence similarities in their catalytic domain (van der Geer *et al.*, 1994). All RTKs have a ligand-binding extracellular domain, a single hydrophobic transmembrane region and a cytoplasmic portion containing the kinase domain that catalyses the transfer of γ-phosphate from ATP to the acceptor tyrosine residues of target proteins.

RTKs dimerise in response to binding of specific ligands. Presumably the close contacts between receptor monomers lead to phosphorylation of tyrosine residue(s) in the activation loop in the catalytic domain. The phosphorylation induces a conformational change which permits ATP binding into the kinase active site and allows the transphosphorylation to proceed, leading to stimulation of the kinase activity (Hubbard, 1997; Lemmon and Schlessinger, 1994). The phosphorylation of tyrosine residues outside the catalytic domain then creates docking sites for intracellular signaling molecules containing Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains and leads to assembly of intracellular signaling complexes (Schlessinger, 2000).

The most common RTK ligands are secreted growth factors. After being released from the producer cells they usually diffuse relatively short distances in the extracellular matrix until they encounter their target cells. On the other hand, some growth factors bind to the pericellular matrix or are anchored to the cell surface and require close cell-cell contacts in order to bind and activate their receptors on the neighbouring cells. Many of the ligands act as dimers which can form stable receptor dimers by simultaneously binding two receptors. However, certain monomeric bivalent ligands, such as epidermal growth factor (EGF), have one

strong and one weak receptor binding site and may induce receptor activation in a different manner. Even without ligand, EGF receptors have an ability to form dimers, and exist as preformed dimers on the cell surface (Moriki *et al.*, 2001). Rather than causing receptor dimerisation ligand binding induces a rotation of the juxtamembrane and transmembrane regions, which causes the dissociation of the intracellular domains. The catalytic kinase domains then become accessible to their substrate tyrosine residues (Moriki *et al.*, 2001). The activation of preformed receptor dimers via induction of conformational changes rather than through induction of receptor dimerisation may be a more commonly used mechanism in RTK signaling than first believed (Livnah *et al.*, 1999). Another monomeric ligand, fibroblast growth factor (FGF) binds it's receptors monovalently and therefore it requires the assistance of accessory molecules, heparan sulphate proteoglycans, in order to activate the dimerisation and transphosphorylation of the receptors (Schlessinger *et al.*, 2000; Spivak-Kroizman *et al.*, 1994).

1.2. Signal transduction

RTK-induced activation of intracellular signaling molecules is usually accomplished by phosphorylation-induced membrane translocation or conformational changes. Although many signaling cascades ultimately target transcription factors and thus regulate gene expression, cells respond to RTK signaling also by changing their metabolism and cytoskeletal arrangements.

A large family of intracellular signaling molecules possess intrinsic enzymatic activities such as protein kinase, phosphatase, phospholipase activities or GTPase-activating nucleotide exchange activity (Schlessinger, 2000). Other molecules are adaptor proteins which provide links between different signaling molecules and networks. A classical example of the signal transduction patways activated via growth factor signaling is the Ras/MAP (mitogen activated protein) kinase cascade. Ras is a GTPase whose importance in cell proliferation and differentiation is well recognised through many oncogenic forms of Ras that have been found in cancers. Once activated via a guanine nucleotide exchange factor, Sos, the GTP-bound Ras recruits a serine kinase Raf to the plasma membrane. The interaction with Ras is necessary for Raf activation, but possibly an additional event such as phosphorylation is also required. After membrane translocation Raf can be phosphorylated for example by members of the membrane-associated Src family kinases. Activated Raf then starts a kinase cascade including MAPK kinase and MAP kinase (Fig. 1). MAP kinases are a family of enzymes some of which can translocate into the nucleus and phosphorylate and activate transcription factors. In addition to inducing cell proliferation, the highly conserved MAPK cascades regulate metabolic processes, cell survival, differentiation, cell cycle and cell migration in all eukaryotes (Chang and Karin, 2001)

Several RTKs stimulate phosphoinositol metabolism. RTK-activated phospholipase C-γ catalyses the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to generate second messengers diacylglycerol and inositol-1,4,5 triphosphate (IP_3), which then regulates intracellular Ca^{2+} levels (Fig. 1). The phospholipid kinase PI3-kinase activates another signaling cascade. The p85 adaptor subunit of PI3-kinase can bind to the phosphorylated residues in transmembrane receptors and recruit the catalytic p110 unit into the complex at the cell membrane. This activates the enzymatic activity of p110 and leads to phosphorylation of membrane-localized phosphoinositides which in turn act as second messengers. PI3-kinase products activate serine/threonine

kinase Akt which can modify the activity of pro- and anti-apoptotic proteins, transcription factors and their regulators by phosphorylation. Direct Akt targets include for example a pro-apoptotic protein BAD, caspase 9, the NF-κB regulator IKK and some members of the Forkhead family of transcription factors, which are important regulators of cell survival (Datta *et al*., 1999).

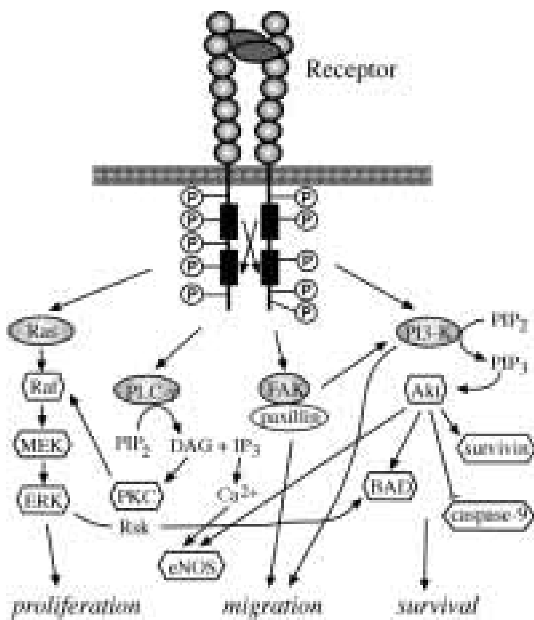


Figure 1. A schematic figure of the receptor tyrosine kinase activated intracellular signal transduction pathways. Ligand binding to the extracellular domain causes the dimerisation of receptor monomers. Mutual transphosphorylation between the catalytic domains (black boxes) activates the kinase activity and phosphorylation of tyrosine residues outside the catalytic domain then creates docking sites (P) for intracellular signaling molecules. Activation of intracellular signaling pathways including Ras, PLC-γ and PI3-kinase cascades then lead to cellular responses such as proliferation, migration or survival.

1.3. Signal down-regulation

There are several mechanisms by which RTK activity and tyrosine phosphorylation are regulated inside the cell. Naturally occurring RTK antagonists and soluble receptor variants provide mechanisms for the inhibition of receptor activation (Kendall and Thomas, 1993; Maisonpierre *et al*., 1997). RTKs may also contain domains with repressor functions which keep them in an inactive state (Gille *et al*., 2000; Tao *et al*., 2001). For example, the juxtamembrane region of vascular endothelial growth factor receptor 1 (VEGFR-1) contains a represssor sequence which inhibits receptor activity (Gille *et al*., 2000).

Phosphorylation by serine/theronine kinases may also regulate RTK activity, enabling unrelated kinases to down-regulate each other. For example, protein kinase C (PKC) can phosphorylate EGF receptor on multiple serine and threonine residues. PKC-induced phosphorylation of EGFR decreases its tyrosine kinase activity and therefore results in the inhibition of EGF signaling (Cochet *et al*., 1984; Schlessinger, 2000). By competing for the binding of the same receptor motif, intracellular inhibitor proteins can block effector protein activation without

modifying RTK activity (Emanuelli *et al.*, 2000). Furthermore, the level of tyrosine phosphorylation is under tight regulation by tyrosine phosphatases (Ostman and Bohmer, 2001). Finally, ligand-induced internalisation of the receptor-ligand complexes usually leads to lysosomal degradation of the receptor.

Since RTKs regulate cellular proliferation, their aberrant expression or dysfunction may lead to uncontrolled growth. For example, several transforming oncogenes encode for constitutive active mutated forms of RTKs or their down-stream effector proteins. Activating or inactivating RTK mutations can also lead to developmental disorders.

2. Development of the vascular system

2.1. Vasculogenesis and angiogenesis

During embryogenesis the cardiovascular system is the first functional organ system and its development and growth are essential for maintaining the growth of the whole embryo. The endothelial cells lining the blood vessels differentiate from mesodermal precursor cells, angioblasts, to form a primitive vascular network in a process called vasculogenesis (Risau, 1997; Risau and Flamme, 1995). The primitive plexus of homogenously sized endothelial tubes is then extensively remodeled into a more mature vessel network consisting of a hierarchy of larger and smaller blood vessels (Fig. 2). This remodeling occurs both by regression of the vessels and by angiogenesis, consisting of sprouting, splitting or fusion of pre-existing vessels to adjust the vascular density according to the metabolic requirements of a certain tissue or organ (Carmeliet, 2000; Risau, 1997). Soon after the formation of the vessels, endothelial cells become surrounded by pericytes or vascular smooth muscle cells (Fig. 2).

Pericytes and endothelial cells share a common basement membrane (BM) which is a thin sheet-like extracellular matrix between the two cell types. The main structural constituents of the BM include fibronectin, laminin, collagens and heparan sulphate proteoglycans. Depending on the type of the vessel and on the tissue environment the composition of the vascular BM is variable. Failure to form a proper BM can lead to decreased pericyte adhesion and migration and to defects in vessel stability, indicating an important role for the BM in normal vascular development (Beltramo *et al.*, 2002; Thyboll *et al.*, 2002).

Already before the onset of blood circulation, the endothelial tubes are specified as two parallel but distinct vascular networks, arteries and veins. The normal sprouting and branching of the maturing vasculature involve repulsive signals which prevent arteries and veins from fusing together. Failure in the establishment of the arterial and venous identity leads to vascular dysplasia (Urness *et al.*, 2000). Recent studies have shed light on the molecular basis for this identity. For example, interaction between EphrinB2 in arteries and its receptor EphB4 in veins is important in defining the boundaries between these vessels (Adams *et al.*, 1999; Wang *et al.*, 1998). Furthermore, signaling via Notch (Lawson *et al.*, 2001; Zhong *et al.*, 2001) and activin receptor-like kinase-1 (Urness *et al.*, 2000) induce the expression of arterial genes and supress venous-specific genes. Therefore these molecules have a crucial role in the proper arterial-venous differentiation.

While vasculogenesis is mainly thought to be restricted to embryonic

development, angiogenesis has an important role throughout adult life in physiological processes such as in the female reproductive cycle and in wound healing. Although the endothelial cells remain quiescent for most of their life in adult organisms, upon angiogenic stimuli they can enter the cell cycle and proliferate rapidly. Endothelial cells are also activated to secrete proteolytic enzymes which degrade the basement membrane, to migrate towards the stimuli and to form capillary sprouts. However, during normal physiological angiogenesis the neovascularisation is short-lived and discontinuation of the stimulus leads to involution and disappearance of the newly formed vessels, as in the case of the granulation tissue of a healing wound. In contrast, in certain pathological conditions, such as in cancer and in chronic inflammatory disorders, the neovascularisation is sustained and excessive angiogenesis contributes to the development of these diseases (Carmeliet and Jain, 2000; Folkman, 1995; Folkman, 1996). Knowledge of the molecular mechanisms by which angiogenesis is controlled may yield strategies for the treatment of diseases characterised by abnormal angiogenesis, and understandably the field has received a lot of research interest during the past decades.

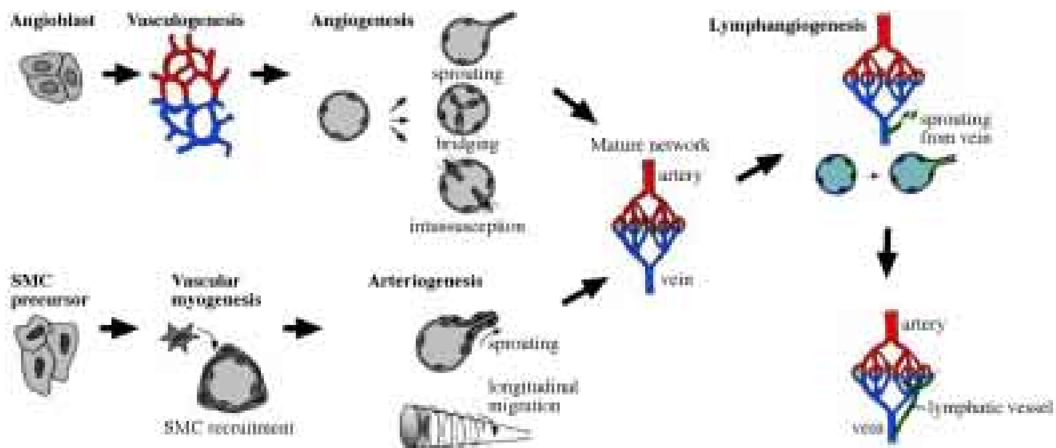


Figure 2. Development of the embryonic blood and lymphatic vessels. During vasculogenesis the endothelial precursor cells, angioblasts, form a primitive capillary plexus which is then further remodelled via angiogenesis. Vessel stabilisation requires the recruitment of smooth muscle cells (SMC). Lymphatic vessels arise by sprouting from the veins. Adapted from (Alitalo and Carmeliet, 2002; Conway *et al.*, 2001).

2.2. Lymphangiogenesis

Due to high pressure inside the blood vessels fluid continuously escapes from the blood capillaries to the surrounding tissue. A major function of the lymphatic system is to transport this extravasated fluid as well as cells and plasma proteins back to the blood circulation. In addition, the lymphatic vasculature is an important part of the immune system, since it filters lymph and its antigens through lymph nodes. Lymphatic vessels also serve as one of the major routes

for absorption of lipids from the gut (Witte *et al.*, 2001).

Lymphatic vessels are characterised by irregular lumen with no red blood cells inside, they have discontinuous basal lamina, overlapping intercellular junctional complexes and anchoring filaments that connect endothelial cells to the extracellular matrix and hold the vessels open when the pressure rises in the interstitial tissue (Witte *et al.*, 1997). Lymphatic vessels possess an intrinsic contractility and larger vessels have smooth muscle cell coverage which helps to pump the lymph fluid forward. The lymph flow is also sustained by arterial pulsation and skeletal muscle movements while the luminal valves prevent backflow (Witte *et al.*, 1997).

During development, the lymphatic vessels arise after the establisment of the blood circulatory system. In the early 20th century, Florence Sabin described the growth of lymphatic vessels by centrifugal sprouting from the lymph sacs which are formed in the vicinity of veins at specific areas in embryos (Sabin, 1902; Sabin, 1909). More recently gathered molecular biological data support Sabin's theory of the venous origin of lymphatic vessels (Dumont *et al.*, 1998; Wigle *et al.*, 2002; Wigle and Oliver, 1999). A homeobox transcription factor Prox-1 has an important role in the formation of the lymph sacs by endothelial cell sprouting from the veins. At the stage when lymphatic development starts Prox-1 is expressed in a subpopulation of endothelial cells which migrate from the anterior cardinal vein and give rise to lymphatic endothelial cells (Wigle and Oliver, 1999; Fig.2). Targeted inactivation of Prox-1 leads to the arrest of this budding and, as a result the Prox-1$^{-/-}$ embryos do not develop lymphatic vessels whereas blood vessel development is not affected (Wigle and Oliver, 1999).

Another theory postulates that the lymphatic endothelial cells are derived from mesenchymal precursor cells (Huntington and McClure, 1908; Kampmeier, 1912). The recent finding of these precursors, lymphangioblasts, in the embryonic mesenchyme of avians supports this theory (Papoutsi *et al.*, 2001; Schneider *et al.*, 1999). Therefore, at least during embryogenesis both sprouting lymphagiogenesis and the differentiation of mesenchymal cells into lymphatic endothelial cells may contribute to the formation of lymphatic vessels.

2.3. Phylogeny of blood and lymphatic vessels

The complexity of the vascular system increases in the phylogeny of animals. Besides being an interesting subject from an evolutionary point of view, knowledge of the differences and similarities between the vascular systems in different animal species may give insights into the mechanisms of embryonic vascular development in mammals.

When the size of organisms increased during evolution, diffusion was no longer sufficient and a blood circulatory system evolved to provide transportation of nutrients, oxygen and waste products. In invertebrates, the endothelial cells are often widely separated from each other and the permeability of vessels is limited by the pericyte layer surrounding the endothelium while the reverse occurs in vertebrates (Casley-Smith, 1987). Also, in the more primitive chordates the endothelial cells of the smallest vessels are separated by gaps and only the larger and the central vessels have continuous endothelium as in mammals. Fenestated endothelium, which allows local exhange of fluid and macromolecules, appears first in hagfish (Casley-Smith, 1987). More commonly the fenestrated endothelium occurs in elasmobrances, where it is responsible for the uptake of lipoproteins in the gut and therefore it carries out some

functions similar to those of the mammalian lymphatic endothelium.

When animals became even larger and more active, increased blood pressure was required to push blood throughout the body. To prevent rupture of the vessels by the high pressure, capillaries became surrounded by a basement membrane and larger blood vessels by elastic tissue and muscle. In parallel with increased blood pressure more protein and fluid leaked out to the tissues and there was a need for the lymphatic vasculature to develop. Teleost fish have two circulatory systems, primary and secondary circulation. The primary and secondary arteries of these fish are directly connected via interarterial anastomoses, which prevent the entry of red blood cells into the secondary circulation but allow the white blood cells and plasma proteins to enter (Steffensen and Lomholt, 1992). While it is not generally accepted whether the secondary circulation of teleost fish can be considered a real lymphatic vasculature, well-developed lymphatic vessels are detected in amphibians (Kotani, 1990; Vogel, 1985). In tailless amphibians, such as frogs, the lymphatic vessels however perform specialised function. The large subcutaneous lymph sacs are involved in maintaining body temperature and act as a water store in certain species. Both in amphibians and reptiles, the aorta runs inside a lymph sinus equivalent to the mammalian thoracic duct (Kotani, 1990). In higher vertebrates, such as birds and mammals, the lymphatic vessels may envelope and run parallel to the blood vessels, but never enclose them.

Interestingly, small venous vessels of the elasmobranchs have openable inter-endothelial structures, a discontinuous basement membrane and connective tissue fibrils attached to the endothelial cells; in other words they resemble lymphatic vessels of higher vertebrates. The functions of the lymphatic vessels is presumably performed in these animals by the openable endothelial intercellular junctions of venous capillaries which are opened by swimming activity (Casley-Smith, 1987). Therefore, similarly to mammalian embryonic development, the veins may have preceeded lymphatic vessels also in evolution.

2.4. Molecular identity of lymphatic versus blood vascular endothelial cells

In addition to neovascularisation, the endothelium has a variety of other important functions e.g. in regulating the vascular tone, maintaining the blood-brain barrier and in inflammatory responses. For example, endothelial cells recruit leukocytes to the inflammatory foci and specialized endothelial cells are responsible for the homing of lymphocytes to the secondary lymphoid organs (Biedermann, 2001). Reflecting the functional diversity, the antigenic profile of the vascular endothelium varies in different types of vessels and in different organs.

In clinical studies pan-endothelial cell markers serve as important tools for the histochemical identification of vessels. For example, the platelet-endothelial cell adhesion molecule (PECAM-1/CD31) and von Willebrand Factor are molecules which are relatively commonly expressed in all endothelial cells (Sleeman *et al.*, 2001; Table I). In studies of a number of physiological as well as pathological conditions it is also important to discriminate between blood and lymphatic vessels. Only quite recently, lymphatic endothelial cell markers have been identified. These lymphatic endothelium specific molecules include VEGFR-3 receptor tyrosine kinase, the Prox-1 transcription factor and the membrane mucoprotein podoplanin (Breiteneder-Geleff *et al.*, 1999; Jussila *et al.*, 1998; Wigle and Oliver, 1999; Table I). None of these molecules is specific for endothelial cells and on the other hand, they may be expressed

only in a subset of the lymphatic vessels. In addition, the function of many of these molecules in lymphatic endothelial cell biology remains to be characterised. However, identification of the lymphatic markers has enabled the detailed studies of lymphatic vessels and provided important information of the functional properties of lymphatic endothelial cells and of the coordinated development and growth of the blood and lymphatic vasculatures.

Table I. Examples of endothelial cell markers

Antigen	EC[1]	Function/molecular identity	Reference
PECAM-1/CD31	P	adhesion molecule	
CD34	B,(L)	adhesion molecule	
von Willebrand Factor	B,(L)	platelet adhesion	
PAL-E	B	antigen unknown	(Schlingemann *et al.*, 1985)
VEGFR-3	L	receptor tyrosine kinase	(Jussila *et al.*, 1998)
LYVE-1	L	lymphatic hyaluronan receptor	(Banerji *et al.*, 1999)
Prox-1	L	homeobox transcription factor	(Wigle and Oliver, 1999)
Desmoplakin I and II	L	intercellular adherens junction molecule	(Ebata *et al.*, 2001)
Podoplanin	L	integral membrane protein	(Breiteneder-Geleff *et al.*, 1999)
D6	L	β-chemokine receptor	(Nibbs *et al.*, 2001)
Macrophage mannose receptor I	L	endocytotic/phagocytotic receptor in macrophages	(Irjala *et al.*, 2001)

[1] Endothelial cell (EC) expression; P=panendothelial, B=blood vessel endothelial, L=lymphatic endothelial

3. Molecular regulation of vascular development

3.1. Angiogenic and lymphangiogenic factors

Angiogenesis is a multistep process controlled by an interplay between stimulators and inhibitors. Many angiogenic factors have been identified by a variety of *in vivo* systems, including chorioallantoic membrane and rabbit corneal assays, and subsequently their effects on the different steps of angiogenesis have been dissected using *in vitro* systems. Angiogenic factors can act directly by stimulating endothelial cells and/or indirectly by stimulating accessory cells such as macrophages and stromal cells. For example, transforming growth factor-ß (TGF-ß) and EGF mediate angiogenic effects indirectly by stimulating other cells to produce angiogenic factors (Goldman *et al.*, 1993; Pertovaara *et al.*, 1994). Due to the indirect action, candidate angiogenic factors may have opposite actions *in vitro* to that expected from *in vivo* experiments and vice versa. For example, TGF-ß inhibits endothelial cell growth *in vitro* but stimulates angiogenesis *in vivo*. Many of the important polypeptides involved in vascular development have mitogenic and chemotactic activity towards several cell types. These factors include for example

platelet-derived growth factor (PDGF) and one of the most potent angiogenic factor, FGF. However, some factors, such as VEGF and Angiopoietins target endothelial cells since their receptors are expressed relatively specifically on these cells.

3.1.1. VEGF family of growth factors

VEGFs form a family of secreted glycoproteins which have the characteristic cystine knot growth factor domain common in the PDGF/VEGF superfamily. VEGFs are disulphide-linked or non-covalent dimers in an antiparallel orientation. Presently, five known members of the VEGF-family have been identified: VEGF, placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D (reviewed in Veikkola *et al.*, 2000). All VEGFs have unique but overlapping patterns of expression and binding to the VEGF receptor tyrosine kinases and neuropilin co-receptors (Fig. 3).

VEGF is one of the most important regulators of both physiological and pathological angiogenesis. It was first isolated from tumor cells due to its ability to induce vascular permeability (Senger *et al.*, 1983). Subsequently, VEGF has been shown to stimulate endothelial cell proliferation, migration and survival (reviewed in (Ferrara, 1999). VEGF is indispensable for embryonic development, since inactivation of even one *VEGF* allele results in embryonic lethality at E11-12 in mice due to failure to establish a normal blood vascular system (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Even during postnatal growth and development, VEGF continues to be critical and its withdrawal results in endothelial cell apoptosis and inhibition of angiogenesis leading to blood vessel regression (Aiello *et al.*, 1995; Ferrara *et al.*, 1998; Gerber *et al.*, 1999a). However, after blood vessel maturation via the recruitment of pericytes endothelial cells are no longer dependent on VEGF for their survival (Benjamin *et al.*, 1998). VEGF is expressed as several different splice-isoforms, which appear to have unique biological properties and tissue-specific expression patterns (Carmeliet *et al.*, 1999b; Robinson and Stringer, 2001). The activity of VEGF is mediated via two high-affinity receptor tyrosine kinases, VEGFR-1 and VEGFR-2, which are expressed primarily on endothelial cells. However, six splice-isoforms of VEGF display differential binding to various coreceptors including heparan sulphate proteoglycans and neuropilin-1 and –2, which modulate their biological activities (Gluzman-Poltorak *et al.*, 2000; Keyt *et al.*, 1996; Soker *et al.*, 1998).

Placenta growth factor (PlGF) and VEGF-B (or VEGF-related factor, VRF) are both expressed as two splice-isoforms (Hauser and Weich, 1993; Maglione *et al.*, 1993; Olofsson *et al.*, 1996b). Both PlGF and VEGF-B bind to VEGFR-1 (Olofsson *et al.*, 1998; Terman *et al.*, 1994) and NRP-1 (Makinen *et al.*, 1999; Migdal *et al.*, 1998), and they can form heterodimers with VEGF (DiSalvo *et al.*, 1995; Olofsson *et al.*, 1996a). The biological functions of PlGF and VEGF-B have remained largely unknown and there is some controversy regarding whether they are angiogenic or not. Both have been reported to stimulate mitogenicity of endothelial cells (Landgren *et al.*, 1998; Maglione *et al.*, 1991; Olofsson *et al.*, 1996a; Sawano *et al.*, 1996). Furthermore, in some studies PlGF was shown to be angiogenic *in vivo* (Ziche *et al.*, 1997), while in others no such effects were detected (Cao *et al.*, 1996; Oh *et al.*, 1997). Several laboratories have also found that PlGF induces migration of VEGFR-1 expressing cells, including monocytes and endothelial cells (Clauss *et al.*, 1996; Migdal *et al.*, 1998; Ziche *et al.*, 1997). PlGF and VEGF-B are not required for the normal vascular development of mouse embryos (Aase *et al.*, 2001; Carmeliet *et al.*, 2001). However, the absence of PlGF

reduced angiogenesis and collateral growth in pathological conditions (Carmeliet *et al.*, 2001). Furthermore, VEGF-B deficient mice may have impaired recovery from cardiac ischemia (Bellomo *et al.*, 2000). These results suggest that both factors may have an important role in regulating angiogenesis in pathological situations.

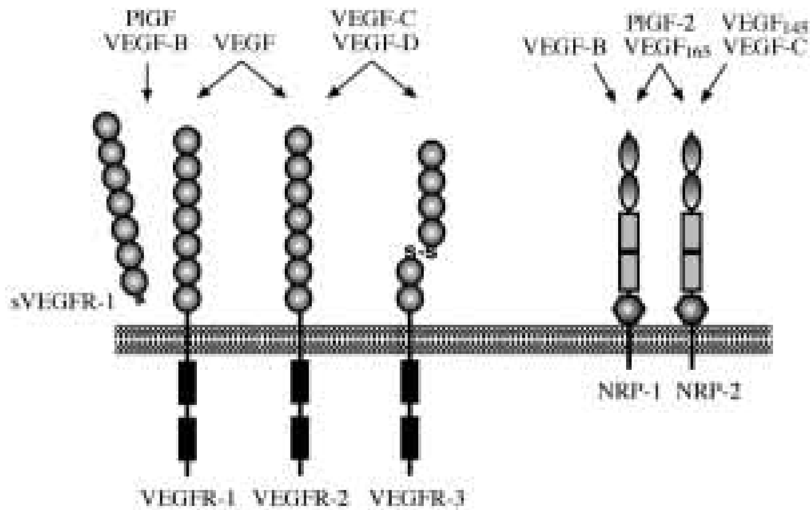


Figure 3. VEGF family of growth factors and their binding to VEGF receptor tyrosine kinases (VEGFRs) and Neuropilins (NRPs). Circle = immunoglobulin homology domain; black box=tyrosine kinase domain; S-S= disulphide bond; oval=CUB (complement binding) domain; gray box=coagulation factor V/VIII homology domain; hexagon= MAM (meprin, A5) domain.

VEGF-C (or VEGF-related protein, VRP) and VEGF-D (or *c-fos*-induced growth factor, FIGF) are expressed as prepro-proteins which are proteolytically processed into polypeptides with increasing affinity towards VEGFR-3, and only their fully processed forms can bind to and activate VEGFR-2 (Joukov *et al.*, 1997; Stacker *et al.*, 1999). However, although human VEGF-D is a ligand for both VEGFR-2 and VEGFR-3, mouse VEGF-D binds selectively to VEGFR-3 (Baldwin *et al.*, 2001). During embryonic development VEGF-C is expressed in the mesenchyme surrounding the developing lymphatic vessels, which suggested its possible role in the regulation of lymphangiogenesis (Kukk *et al.*, 1996). Subsequently, both VEGF-C and VEGF-D were shown to induce lymphatic growth *in vivo* (Jeltsch *et al.*, 1997; Oh *et al.*, 1997; Veikkola *et al.*, 2001) and induce proliferation and migration of lymphatic endothelial cells *in vitro* (Marconcini *et al.*, 1999; Mäkinen *et al.*, 2001b). At higher concentrations and under specific conditions, these factors can also stimulate the proliferation and migration of blood vascular endothelial cells, increase vascular permeability and angiogenesis (Joukov *et al.*, 1997; Mäkinen *et al.*, 2001b; Oh *et al.*, 1997; Witzenbichler *et al.*, 1998). The VEGF-C/D induced effects on blood vessel endothelium are presumably mediated via VEGFR-2 activation, suggesting an important role for specific proteolytic enzymes in regulating the

lymphangiogenic versus angiogenic potential of VEGF-C and VEGF-D.

Additional VEGF-like molecules have been isolated from Orf-virus (Lyttle *et al.*, 1994) and from snake venom (Gasmi *et al.*, 2000; Komori *et al.*, 1999). The double-stranded poxvirus Orf infects sheep and goats and induces proliferation of vascular endothelial cells and dilation of blood vessels (Lyttle *et al.*, 1994). The VEGF-like polypeptides encoded by the genomes of three viral strains, NZ2, NZ7 and D1701, were commonly named as VEGF-E, and these polypeptides were found to induce angiogenesis and microvessel permeability by activating mammalian VEGFR-2 (Meyer *et al.*, 1999; Ogawa *et al.*, 1998; Wise *et al.*, 1999). Most probably the VEGF-like sequences found in the genomes of these viruses was acquired from the host and the gene product is responsible for the angiogenic stimulus associated with the infection.

3.1.2. Angiopoietin family

Angiopoietins form another family of growth factors which specifically target endothelial cells. Angiopoietin-1 (Ang-1) and Ang-2 are ligands for the endothelial cell receptor tyrosine kinase Tie-2/Tek (Davis *et al.*, 1997; Maisonpierre *et al.*, 1997). In addition, mouse Ang-3 and human Ang-4, which represent interspecies orthologues, bind to Tie-2 (Valenzuela *et al.*, 1999). Interestingly, these ligands have opposing actions in Tie-2 function; Ang-1 and Ang-4 function as activating ligands for Tie-2 while Ang-2 and Ang-3 act as competitive antagonists. The data from several mouse models with altered Tie-2 signaling suggest that Tie-2 stimulation by Ang-1 is required for the stabilisation of the vessels and for the maintenance of the interactions between the endothelium and surrounding extracellular matrix and mesenchyme (Sato *et al.*, 1995; Suri *et al.*, 1997; Suri *et al.*, 1998). In contrast, inhibition of Tie-2 signaling by Ang-2 destabilises the vessels by loosening the endothelial cell-matrix contacts and making the cells accessible and more responsive towards angiogenic stimuli (Maisonpierre *et al.*, 1997). Consistent with these observations, Ang-2 expression in adult organisms is found in tissues which undergo angiogenesis; e.g. in placenta and in tumors (Etoh *et al.*, 2001; Goldman-Wohl *et al.*, 2000; Koga *et al.*, 2001).

Recent studies indicate a significant interplay between Angiopoietins and VEGFs in the formation of both blood and lymphatic vessels. Low VEGF levels combined with high expression of Ang-2 lead to vascular regression, while Ang-1 and VEGF may cooperate in promoting angiogenesis. Interestingly, Ang-1 protects the vasculature against VEGF-induced plasma leakage (Thurston *et al.*, 2000; Thurston *et al.*, 1999). On the other hand, Ang-2 may be involved in the regulation of lymphangiogenesis, since inactivation of Ang-2 leads to non-functional and disconnected lymphatic vessels in mice (G. Thurston, pers. comm.). In addition, both VEGF and VEGF-C increase Ang-2 mRNA in endothelial cells (Mandriota and Pepper, 1998; Oh *et al.*, 1999; Veikkola *et al.*, unpublished observations).

3.2. Endothelial cell receptors

3.2.1. VEGF receptors

The three members of the family of VEGF receptor tyrosine kinases (VEGFR-1-3) are key regulators of the development and maintenance of the vertebrate vascular system. All VEGFRs have an extracellular ligand-binding domain consisting of seven immunoglobulin homology domains and an intracellular kinase domain split by a kinase insert (See Fig. 3).

VEGFR-1 (or Flt-1) functions as a receptor for VEGF, PlGF and VEGF-B. In addition to the full-length receptor, alternative splicing of the *VEGFR-1* mRNA generates a soluble form of the receptor, which may antagonise VEGF function (Kendall and Thomas, 1993). During embryogenesis, targeted inactivation of *VEGFR-1* results in disorganization of blood vessels due to increased production of endothelial cells and death of mouse embryos *in utero* at E8.5 (Fong *et al*., 1999; Fong *et al*., 1995). In contrast, mice expressing VEGFR-1 lacking the tyrosine kinase domain survive and develop a normal vascular system (Hiratsuka *et al*., 1998). These studies suggest that during development VEGFR-1 may function only as a non-signaling regulator of VEGF bioavailability (Shibuya, 2001). However, recent studies have provided evidence that during adult life VEGFR-1 signaling may play an important role in pathological angiogenesis and inflammation (Carmeliet *et al*., 2001; P. Carmeliet, pers. comm.). VEGFR-1 specific ligand PlGF can stimulate angiogenesis and collateral growth in ischemic heart and limb muscle. Furthermore, inhibition of VEGFR-1 signaling impairs infiltration of VEGFR-1 expressing leucocytes in inflamed tissues and suppresses mobilisation of bone-marrow derived myeloid progenitor cells, resulting in reduced bone and cartilage destruction in arthritis (P. Carmeliet, pers. comm.). Recruitment and migration of endothelial progenitors and hematopoietic stem cells from the bone marrow has been previously shown to promote pathological angiogenesis (Lyden *et al*., 2001).

VEGFR-1 has also been suggested to regulate VEGFR-2 signaling by forming heterodimers with it, although it seems to be unclear whether VEGFR-1 acts by antagonising VEGFR-2 functions (Rahimi *et al*., 2000) or by mediating positive signals by such heterodimers (Huang *et al*., 2001; P. Carmeliet, pers. comm.).

VEGFR-2 (or KDR/Flk-1) is considered to be the main signal transducing VEGF receptor for angiogenesis and for mitogenesis of endothelial cells. In addition to VEGF, the proteolytically processed mature forms of VEGF-C and VEGF-D bind to and activate VEGFR-2 (Achen *et al*., 1998; Joukov *et al*., 1997). Activation of VEGFR-2 stimulates endothelial cell proliferation, migration and survival as well as blood vessel permeability. Several intracellular signal transduction pathways are activated via VEGFR-2, including the MAPK and the PI3-kinase pathways (reviewed in Matsumoto and Claesson-Welsh, 2001; Petrova *et al*., 1999). Mouse embryos deficient of VEGFR-2 die *in utero* by E8.5-9.5 due to defective development of both endothelial and hematopoietic cells (Shalaby *et al*., 1995). Although the hematopoietic/endothelial progenitor cells are found in VEGFR-2 deficient embryos at E7.5, these cells fail to migrate from the primitive streak to their correct location in the yolk sac, where further development into the hematopoietic and endothelial cell lineages takes place (Shalaby *et al*., 1997). In addition, VEGFR-2$^{-/-}$ embryonic stem cells retain the potential to form blood islands and to differentiate into hematopoietic and endothelial cells *in vitro* (Schuh *et al*., 1999; Shalaby *et al*., 1997). Therefore it seems that the formation of hematopoietic/endothelial cell precursors occurs in the absense of VEGFR-2, but the proper migration of these cells is dependent on VEGFR-2-mediated signaling (Schuh *et al*., 1999; Shalaby *et al*., 1997).

VEGFR-3 (or Flt4) is a receptor for VEGF-C and VEGF-D (Achen *et al*., 1998; Joukov *et al*., 1997). Human VEGFR-3 is expressed as two isoforms generated by differential splicing to the long terminal repeat of an integrated endogenous retrovirus between the last two exons (Hughes, 2001).

The shorter transcript encodes a protein lacking 65 amino acids in the C-terminus, including three putative phosphorylated tyrosine residues which may be important for VEGFR-3 mediated signaling (Pajusola *et al*., 1993). During early vascular development, *VEGFR-3* is expressed in developing blood vessels and it is required for normal vascular development. In the absense of VEGFR-3, the embryos undergo normal vasculogenesis, but the remodelling and maturation of primary vascular networks is defective, which leads to death *in utero* around E10 of mouse development (Dumont *et al*., 1998). However, after the formation of lymphatic vessels VEGFR-3 is detected almost exclusively on lymphatic endothelium (Dumont *et al*., 1998; Kaipainen *et al*., 1995) and it regulates the development and growth of lymphatic vessels (Jeltsch *et al*., 1997; Mäkinen *et al*., 2001a; Veikkola *et al*., 2001). Besides lymphatic endothelia, in adult tissues VEGFR-3 expression occurs also in fenestrated endothelia (Partanen *et al*., 2000), and in the endothelia of angiogenic blood vessels in tumors (Partanen *et al*., 1999; Valtola *et al*., 1999).

3.2.2. Tie receptors

Tie-1/Tie and Tie-2/Tek form another family of endothelial cell specific receptor tyrosine kinases. These receptors have a large extracellular domain consisting of epidermal growth factor homology domains, immunoglobulin-homology domains and fibronectin type III repeats. While several Angiopoietin ligands have been identified for Tie-2, Tie-1 is still an orphan receptor with no known ligands.

Gene inactivation studies have indicated that both Tie receptors have a critical function in the formation of embryonic blood vasculature. Mouse embryos deficient of *Tie-1* gene die between E13.5–P0 due to failure in the establisment of endothelial cell integrity which leads to edema and haemorrages (Puri *et al*., 1995; Sato *et al*., 1995). On the other hand, *Tie-2* knock-out embryos die due to cardiovascular defects and to defects in the expansion and maintenance of the primitive capillary plexus (Sato *et al*., 1995). The genetic studies suggest that Tie-1 and Tie-2 are required for the survival of endothelial cells starting from late gestation throughout adult life (Jones *et al*., 2001; Partanen *et al*., 1996; Puri *et al*., 1999). Consistent with these findings, both Tie-1 and Tie-2 can activate signal transduction pathways which promote cell survival (Hayes *et al*., 1999; Kontos *et al*., 2002; Kontos *et al*., 1998; Papapetropoulos *et al*., 2000).

Angiopoietin-Tie-2 signaling may also have a role in the regulation of endothelial cell-pericyte interactions. Activating mutations of Tie-2 have been detected in human patients having venous malformations (Vikkula *et al*., 1996). These lesions are characterised by enlarged blood vessels with a lack of supporting smooth muscle cells (Vikkula *et al*., 1996).

3.2.3. Neuropilins

Neuropilin-1 (NRP-1) and NRP-2 are transmembrane non-tyrosine kinase receptors expressed in the tips of growing axons (Chen *et al*., 1997; Kawakami *et al*., 1996; Takagi *et al*., 1995). NRP-1 interacts with receptors belonging to the plexin family and these receptor complexes mediate repulsive axon guidance by binding semaphorins/collapsins (Tamagnone *et al*., 1999). Both NRP-1 and NRP-2 are, however, expressed also in endothelial cells (Herzog *et al*., 2001; Kitsukawa *et al*., 1995; Soker *et al*., 1998). While NRP-1 is expressed mainly in arterial endothelium (Moyon *et al*., 2001), NRP-2 appeares to be expressed in the endothelia of embryonic veins and in adult tissues in some

lymphatic vessels (Herzog *et al.*, 2001; Karkkainen *et al.*, 2001).

Overexpression or inactivation of NRP-1 in mouse embryos lead to lethal abnormalities in both nervous and vascular tissues (Kawasaki *et al.*, 1999; Kitsukawa *et al.*, 1997; Kitsukawa *et al.*, 1995). In contrast, NRP-2 deficient mice are viable but they have an abnormal organization and fasciculation of several cranial and spinal nerves (Chen *et al.*, 2000; Giger *et al.*, 2000). Both NRP-1 and NRP-2 act as receptors for several members of the VEGF family (Neufeld *et al.*, 2002; Fig. 3), suggesting that the vascular defects in the NRP-1 mutant mice may result from modulation of the VEGF-induced angiogenesis by abnormal NRP-1 levels. It seems that NRPs regulate signal transduction by VEGFRs through formation of receptor complexes (Fuh *et al.*, 2000; Gluzman-Poltorak *et al.*, 2001; Whitaker *et al.*, 2001). It was also shown that NRP-1 enhances the VEGF-induced mitogenic signal of VEGFR-2 (Soker *et al.*, 1998) and that VEGFR-2 is not able to efficiently transduce VEGF-mediated signals in the absence of NRP-1 (Yamada *et al.*, 2001).

In neurons, NRP-1 ligand Sema3A induces growth cone collapse and repulsion of neurites, and during prolonged exposure, it promotes apoptosis. Interestingly, in neuroectodermal progenitor cells the repulsive signals of Sema3A depended on the expression of VEGFR-1 (Bagnard *et al.*, 2001). Furthermore, Sema3A-induced apoptosis of neuroectodermal progenitor cells was antagonised by $VEGF_{165}$ which promoted cell survival, migration, and proliferation (Bagnard *et al.*, 2001). Similarly in endothelial cells Sema3A acts as a competitive inhibitor of VEGF-induced motility and capillary sprouting (Miao *et al.*, 1999). These studies indicate that the balance between Semaphorins and VEGFs can modulate the migration, survival and proliferation of neural progenitor cells as well as endothelial cells through shared receptors. NRPs may also induce biological responses of VEGFs in cells which do not express VEGFRs. For example, VEGF was shown to induce tumor cell survival via NRP-1 interaction (Bachelder *et al.*, 2001).

3.3. VEGFs and VEGFRs in the development of other organ systems

Several studies have pointed out striking similarities in the molecular mechanisms which control the development of the vascular and nervous systems. Several axon guidance molecules have proved to be also regulators of angiogenesis. These molecules include for example Neuropilins and Semaphorins. In addition, Eph receptors and their ligands, Ephrins, are expressed both in neural and endothelial cells and some of these molecules are indispensable for the development of both vascular and nervous systems (Adams *et al.*, 1999). These observations raise questions whether the angiogenesis regulators VEGFs and VEGFRs might also have a role in the development of the nervous system.

To date, little data is available on the involvement of VEGFs and VEGFRs in neural development. Both VEGFR-1 and VEGFR-2 have been reported to occur in neural cells, including retinal cells, Schwann cells, dorsal root ganglia and astrocytes in the central nervous system (Jin *et al.*, 2000; Krum and Rosenstein, 1998; Sondell *et al.*, 1999; Suzuma *et al.*, 1998). In addition, during embryonic development VEGFR-3 is expressed in the notochord (Partanen *et al.*, 2000; Wilting *et al.*, 1997). Furthermore, VEGFR-3 deficient embryos appear to have a neuronal phenotype which may be caused by impaired survival of cells in the developing neural tube (unpublished observations). Recently, VEGF was reported to be a survival factor for neuronal cells (Jin *et al.*,

2000; Oosthuyse *et al.*, 2001; Sondell *et al.*, 1999; Sondell *et al.*, 2000) and VEGF-C may possess a similar function (Olofsson *et al.*, submitted). Although these functions were suggested to be at least partly due to NRP-1 interactions, involvement of VEGFRs was also proposed.

VEGF and its receptors are involved also in the regulation of endochondral ossification. Blocking of VEGF function inhibits blood vessel invasion into the hypertrophic zone of long bone growth plates and results in impaired bone formation (Gerber *et al.*, 1999b). VEGF may, however, regulate bone formation also through a direct effect on osteoblasts, which express both VEGFR-1 and VEGFR-2 (Deckers *et al.*, 2000). VEGF induces the migration and increases alkaline phosphatase activity in cultured osteoblasts *in vitro* (Mayr-wohlfart *et al.*, 2002; Midy and Plouet, 1994). Furthermore, VEGF appears to be required for normal chondrocyte maturation and regulation of osteoblastic activities *in vivo* (Zelzer *et al.*, 2002).

Indirectly, VEGFs and their receptors may control organ morphogenesis. Recent studies indicated that prior to their vascular function endothelial cells and nascent vessels can guide the early development of the liver and pancreas (Lammert *et al.*, 2001; Matsumoto *et al.*, 2001). In the absence of endothelial cells the outgrowth of the liver bud was inhibited (Matsumoto *et al.*, 2001). On the other hand, endothelial cells secrete factors which induce embryonic endoderm to differentiate into pancreas, suggesting that vessels can provide inductive signals for organ development (Lammert *et al.*, 2001).

4. Pathologic conditions involving blood and lymphatic vessels

The pathophysiology of several diseases is affected by insufficient or excessive angiogenesis and/or lymphagiogenesis. For example, the chronic inflammation in rheumatoid arthritis is sustained due to excessesive angiogenesis, caused by production of angiogenic factors from infiltrating macrophages and inflammatory cells. On the other hand, duodenal ulcers are devoid of vessels and stimulation of angiogenesis might be a strategy to accelerate their healing. Since blood and/or lympatic vessels have a central role in a number of pathological conditions, the ability to control their growth could provide strategies for the treatment of such diseases.

4.1. Tumor development and progression

Tumor cells are often undifferentiated cells which overexpress several growth factors and growth factor receptors and can stimulate their own growth. Tumor cells also secrete angiogenic factors, such as VEGF, and thus they can induce the growth of blood vessels. In order to grow larger than a few millimeters in diameter the newly formed vessels are needed to provide nutrients and oxygen for tumor cells (Folkman, 1996). Therefore, the tumor-induced stimulation of blood vessel growth is considered to play a critical role in controlling tumor growth.

Metastasis of tumor cells by direct invasion or via blood or lymphatic vessels is one of the leading causes of death in cancer patients (Plate, 2001). Although hematogenous metastasis via blood vessels is well recognised, for many tumors the most common pathway for metastatic spread is via lymphatic vessels. In addition, the lymphatic spread of tumor cells to regional lymph nodes is an indicator of tumor aggressiveness. In general, it is thought that high interstitial pressure inside the tumor prevents the growth

of lymphatic vessels into the tumor cell mass (Carmeliet and Jain, 2000). However, the studies of the involvement of the lymphatic vessels in tumor growth were hampered for a long time due to absence of specific markers of lymphatic endothelium.

Several experimental studies have recently demonstrated an important role for lymphatic vessels as a route for tumor metastasis. Overexpression of the VEGFR-3 ligands, VEGF-C or VEGF-D, by tumor cells induced lymphangiogenesis in the tumor periphery, but also intralymphatic tumor growth and metastasis. These studies were done using transfected cells (Karpanen *et al.*, 2001; Skobe *et al.*, 2001; Stacker *et al.*, 2001) or transgenic mice (Mandriota *et al.*, 2001), but correlation of VEGF-C expression with increased lymphatic metastasis has also been reported in several human cancers (Ohta *et al.*, 1999; Tsurusaki *et al.*, 1999; Yonemura *et al.*, 1999). In addition, when highly metastatic tumor cells were selected from a human cancer cell line, those cell clones which displayed high metastatic potential expressed VEGF-C although the parental cell line did not (He *et al.*, 2002). These studies suggest that VEGF-C or VEGF-D secreting tumors can activate the growth of lymphatic vessels which may then facilitate metastasis of tumor cells.

Inhibition of tumor angiogenesis is considered to be one of the most promising therapeutic strategies to inhibit tumor growth. Presumably anti-angiogenic therapies can act on any tumor type, do not induce tumor cell resistance and should not affect normal tissues (Plate, 2001). Since VEGFs and their receptors are major regulators of both angiogenesis and lymphangiogenesis, they are excellent targets for cancer therapy. Dominant-negative VEGFR-2, neutralising VEGF antibodies or soluble VEGFR-1 or VEGFR-2 proteins have been successfully used in the inhibition of tumor angiogenesis and growth in mouse models (Goldman *et al.*, 1998; Kong *et al.*, 1998; Lin *et al.*, 1998a; Millauer *et al.*, 1994; Takayama *et al.*, 2000). On the other hand, tumor lymphagiogenesis and metastasis were inhibited by blocking VEGF-C or VEGF-D actions with neutralising antibodies or soluble VEGFR-3 protein (He *et al.*, 2002; Karpanen *et al.*, 2001; Stacker *et al.*, 2001).

Another promising strategy for cancer therapy is to target drugs specifically to tumors. Selective expression of certain molecules in tumor vasculature has allowed the isolation of peptides that specifically recognise the tumor blood vessels. When linked to chemoterapeutic drugs these peptides can facilitate efficient drug delivery into the tumor and thus reduce the toxicity of the drug to other tissues (Arap *et al.*, 1998). The screening of phage-displayed peptide libraries has also allowed the isolation of peptides which selectively home in on the vasculature of individual organs and tissues (Arap *et al.*, 2002; Essler and Ruoslahti, 2002). These peptides may facilitate organ-specific drug targeting in a variety of pathological conditions, including cancer.

4.2. Lymphedema

Lymphedema is a disorder characterised by insufficiency of the lymphatic system which leads to accumulation of protein-rich fluid in the tissues and to a disfiguring and disabling swelling of the extremities. Ultimately, the patients also suffer from tissue fibrosis and adipose degeneration, from impaired wound healing and have higher susceptibility to infections (Witte *et al.*, 2001). Noninherited secondary or aquired lymphedema develops when the lymphatic vessels are damaged for example by surgery or radiation therapy or obstructed by filarial infection. In contrast, inherited primary lymphedema is usually due to hypoplasia or aplasia of the superficial or subcutaneous lympatic vessels.

In some families of hereditary lymphedema heterozygous missense mutations were found in *VEGFR-3* gene. All the lymphedema-associated mutations inactivated the tyrosine kinase domain and therefore led to insufficient VEGFR-3 signaling (Irrthum *et al*., 2000; Karkkainen *et al*., 2000). However, primary lymphedemas are a heterogenous group of disorders which are also sometimes associated with additional malformations of other organs. In lymphedema distichiasis, characterised by congenital lymphedema, double row of eyelashes and other complications, mutations were reported in the *FOXC2* gene. FOXC2 is a forkhead transcription factor, which regulates cardiovascular development and somitogenesis in mice (Winnier *et al*., 1997; Winnier *et al*., 1999). Most of the lymphedema-linked FOXC2 mutations cause premature termination in the protein and are likely to produce haploinsufficiency (Bell *et al*., 2001; Fang *et al*., 2000; Finegold *et al*., 2001).

Genetically modified mouse mutants have provided important tools for the studies of molecular mechanisms involved in lymphatic growth and development of lymphedema (Table II). A mouse model for human lymphedema was obtained using N-ethyl-N-nitrosourea-induced chemical mutagenesis. Like in human lymphedema, heterozygous mice carrying an inactivating missense mutation in *VEGFR-3* gene had hypoplastic dermal lymphatic vessels (Karkkainen *et al*., 2001). In addition, the mice had leaky intestinal lymphatics, which resulted in accumulation of intraperitoneal chylous fluid after suckling. Since the mice carrying only one functional *VEGFR-3* gene allele appeared normal (Dumont *et al*., 1998) while tyrosine kinase inactivating mutation caused lymphedema, the phenotype was suggested to arise due to a "dominant-negative" effect by the kinase inactive receptor. Despite this, gene therapy using adenoviral VEGF-C induced lymphangiogenesis in the lymphedema mice, suggesting a therapeutic application for human disease (Karkkainen *et al*., 2001).

We have also evidence that in different mouse backgrounds the reduction of the *VEGFR-3* gene dosage is sufficient to cause a lymphedema-like phenotype (unpublished observations). The dependence of the phenotype on the genetic background resembles the observed variability in the penetrance of human lymphedema, and suggests that genes modulating either VEGFR-3 activated or other yet uncharacterised signaling pathways may significantly contribute to the development of the disease. Similar background-dependent lymphatic phenotypes, determined by accumulation of chylous ascites, have been previously observed in mice deficient of Prox-1 (Wigle and Oliver, 1999), Ang-2 (G. Thurston, pers. comm.) or transcription factor Sox18 (Pennisi *et al*., 2000a; Pennisi *et al*., 2000b; Table II). In endothelial cells, Sox18 interacts with a MADS-family transcription factor MEF2C which potentiates its transcriptional activity (Hosking *et al*., 2001). Interestingly, targeted mutagenesis of MEF2C leads to a similar phenotype as observed in VEGFR-3 deficient mice. MEF2C deficient embryos die at E9.5-10 due to defects in the remodeling of the primary vasculature and abnormal endocardiogenesis (Bi *et al*., 1999; Lin *et al*., 1998b). While the endocardial defects may be caused by significant decrease in Ang-1 and VEGF expression in the myocardium (Bi *et al*., 1999), decreased VEGFR-3 expression may contribute to the failure in vascular remodeling. In line with this hypothesis, VEGFR-3 promoter region contains a conserved binding motif for MEF2 (Iljin *et al*., 2001). In conclusion, both Sox18 and MEF2C may participate in a common transcriptional program involved in the development of the lymphatic vasculature.

Table II. Mouse mutants having a lymphatic phenotype

Gene	Mouse model[1]	Lymphatic phenotype	Lethality[2]	Reference
Prox-1	KO	Defective lymphatic sprouting and differentiation (-/-) Accumulation of chylous ascites (+/-)	E14.5 P2-3	Wigle and Oliver, 1999
Integrin α9	KO	Chylothorax (-/-)	P6-12	Huang *et al*., 2000
Ang-2	KO	Disconnected lymphatic vessels, accumulation of chylous ascites		G. Thurston, pers. comm.
VEGFR-3	Mut (kinase dead)	Accumulation of chylous ascites, hypoplasia of dermal lymphatic vessels (+/mut)	viable	Karkkainen *et al*., 2001
Net transcription factor	Mut (loss of the Ets DNA-binding domain)	Chylothorax (mut/mut)	P2	Ayadi *et al*., 2001
Sox18 transcription factor	Mut (truncated transactivation domain)	Accumulation of chylous ascites (mut/mut)	before weaning	Pennisi *et al*., 2000b
VEGF-C	TG (K14 promoter)	Hyperplasia of dermal lymphatic vessels	viable	Jeltsch *et al*., 1997
	(Insulin promoter)	Hyperplasia of lymphatic vessels around the islets of Langerhans	viable	Madriota *et al*., 2001
VEGF-D	TG (K14 promoter)	Hyperplasia of dermal lymphatic vessels	viable	Veikkola *et al*., 2001
VEGFR-3-Ig (VEGF-C/D "trap")	TG (K14 promoter)	Lack of dermal lymphatic vessels	viable	Mäkinen *et al*., 2001a

[1] KO = knock-out (gene inactivation), Mut = mutagenesis (chemically induced (VEGFR-3) / gene-targeted (Net) / spontaneous (Sox18)), TG = transgenic (gene overexpression)
[2] E = embryonic day, P = postnatal day

AIMS OF THE STUDY

This study was undertaken to obtain more information concerning the mechanisms of VEGF/VEGFR interactions involved in the regulation of the growth and development of blood and lymphatic vessels. We wanted especially to elucidate the role of VEGFR-3 and its ligands, VEGF-C and VEGF-D, in these processes. To achieve these goals we studied:

- VEGF-B binding to the isoform-specific VEGF receptor, neuropilin-1 (I)

- the role of VEGFR-3 signaling in the development of embryonic blood and lymphatic vessels by using a competitive inhibitor of VEGFR-3-ligand interactions (II)

- VEGFR-3 mediated signal transduction in lymphatic endothelial cells (III)

- the genetic program controlling lymphatic versus blood vascular endothelial phenotype (IV)

MATERIALS AND METHODS

The materials and methods used are described in detail in the respective publications.

Cell line	Description	Source or reference	Used in
293T, 293EBNA	human fibroblast	ATCC	I
Ba/F3	mouse pre-B-lymphocytes		II, III
HMVEC	human dermal microvascular EC	PromoCell	III
HUVEC	human umbilical vein EC	PromoCell	III
PAE	porcine aortic EC	Dr. L. Claesson-Welsh	III
S2	*Drosophila* Schneider S2 cells	Invitrogen	I

ATCC – American Type Culture Collection

Growth factor	Description	Source or reference	Used in
bFGF	human basic FGF, FGF-2	R&D Systems	III
PlGF-1	human PlGF-1 splice-isoform	Dr. G. Persico	III
$VEGF_{121}$	human VEGF 121 splice-isoform	R&D Systems	I
$VEGF_{165}$	human VEGF 165 splice-isoform	R&D Systems	III
$VEGF\text{-}B_{167}$	mouse VEGF-B 167 splice-isoform	Dr. B. Olofsson	I
VEGF-C	mature human VEGF-C (residues Thr103-Leu215)	(Joukov *et al*., 1997)	II, III
VEGF-C156S	Cys156->Ser mutation, mature human VEGF-C (residues Thr103-Ile225)	(Joukov *et al*., 1998)	III
VEGF-D	mature human VEGF-D (residues Phe93-Ser201)	R&D Systems	III
VEGF-E (ORFV2-VEGF)	orf-viral VEGF homolog, NZ2 isoform	Dr. L. Wise (Wise *et al*., 1999)	III

Antigen	Description	Source or reference	Used in
Akt	rabbit antiserum against Akt/protein kinase B (aff)	New England Biolabs	III
CD31/PECAM-1	mouse mAb against human CD31	Dako	III, IV
	rat mAb against mouse PECAM-1	Pharmingen	II
CREB	rabbit antiserum against cAMP response element binding protein (aff)	New England Biolabs	III
LYVE-1	rabbit antiserum against mouse LYVE-1	Dr. D.G. Jackson	II
	rabbit antiserum against human LYVE-1	Dr. D.G. Jackson	III
MAPK (ERK1/ERK2)	rabbit antiserum against p42/p44 MAPK (aff)	New England Biolabs	III
myc	mouse mAb (9E10) against c-myc epitope	Babco	I
NRP-1	rabbit antiserum against mouse NRP-1 (aff)	Dr. H. Fujisawa	I
PCNA	mouse mAb (PC10) against proliferating cell nuclear antigen	Santa Cruz Biotechnology	III, IV
podoplanin	rabbit antiserum against human podoplanin (aff)	Dr. D. Kerjaschki/ H. Kowalski	III
prox-1	rabbit antiserum agains human prox-1	Dr. Jörg Wilting	IV
VEGF	mAb against human VEGF	R&D Systems	I
VEGF-B	rabbit antiserum against mouse VEGF-B (aff)	Dr. B. Olofsson	I
VEGF-C	rabbit antiserum (882) against human VEGF-C	(Joukov *et al*., 1996)	I
VEGFR-3	mouse mAb against human VEGFR-3		
	9D9F9	(Jussila *et al*., 1998)	III
	7B8F9		II
	2E11D11		III, IV
	rat mAb against mouse VEGFR-3	Dr. H. Kubo (Kubo *et al*., 2000)	II
vWF	mAb against human von Willebrand Factor	Dako	III

Abbreviations: mAb = monoclonal antibody, aff = affinity-purified

Methods	Used in
Adenoviral infection of endothelial cells	IV
Analyses of cell viability and apoptosis	II, III
Annexin-V staining	III
MTT assay	II, III
TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)	II, III
Analysis of cell migration (Boyden chamber assay)	III
Biosensor analysis of receptor-ligand interaction	III
Cell culture	I-IV
DNA cloning and subcloning	I, II, IV
ELISA (enzyme-linked immunosorbent assay)	I-III
FACS (fluorescence activated cell sorting)	III
Generation of transgenic mice	II
Immunofluorescence	III, IV
Immunohistochemistry	II, IV
Immunoprecipitation	I
Isolation of endothelial cells using magnetic cell sorting	III, IV
Lectin perfusion staining	II
Magnetic resonance imaging	II
Metabolic labeling	I
Microarray analysis of gene expression	IV
Northern blotting	II-IV
PCR	I, II, IV
Preparation of mouse tissues	II
Receptor stimulation and ligand binding	I, III
RNA extraction	II-IV
RT-PCR	IV
Southern blotting	II
Transfection	I
Visualisation of lymphatic vessels in mice (by Dextran and Evans blue dye)	II
Western blotting	I-III
X-Gal staining of tissues	II

RESULTS AND DISCUSSION

1. Neuropilin-1 is a receptor for VEGF-B (I)

Neuropilin-1 (NRP-1) controls axon growth and guidance through interactions with Semaphorins. In addition, NRP-1 was shown to act as a receptor for certain splice-isoforms of VEGF and PlGF, and it was speculated to modulate VEGF-induced angiogenesis. Based on the high homology with VEGF and PlGF, we asked whether VEGF-B also binds to NRP-1.

VEGF-B is expressed as two isoforms, VEGF-B_{167} and VEGF-B_{186}, which are generated by alternative splicing (Olofsson *et al.*, 1996b). VEGF-B_{167} has a basic, heparin binding C-terminus, by which the protein becomes sequestered to the cell surface after secretion. However, in VEGF-B_{186} an alternative splice acceptor site in exon 6 generates an insertion and frameshift in the coding sequence, giving rise to a different C-terminus and to a soluble, O-glycosylated protein (Olofsson *et al.*, 1996b). The common N-terminal cystine knot growth factor domain of VEGF-B (amino acid residues 1-115) is responsible for binding to VEGFR-1 (Olofsson *et al.*, 1998).

We constructed a soluble neuropilin-1 Ig-fusion protein and used it for the binding studies with metabolically labelled growth factors. In these expriments we found that both splice-isoforms of VEGF-B bind to neuropilin-1 (Makinen *et al.*, 1999; I). Interestingly, only the proteolytically cleaved form of VEGF-B_{186} was able to bind NRP-1. The NRP-1 binding epitopes were different from the VEGFR-1 binding epitopes, and the former were located in the C-termini of the two isoforms. We also found that the binding of VEGF-B_{167} to NRP-1 was mediated by the heparin-binding domain encoded by exon 6B. This domain is highly homologous to the NRP-1 binding epitopes of $VEGF_{165}$ and PlGF-2. In contrast, the NRP-1 binding of VEGF-B_{186} was regulated by the exposure of a short C-terminal proline rich peptide upon proteolytic processing, which thus constituted a novel NRP-1 binding epitope. The proteolytic processing site was further mapped to Arg-127 with mass-spectrometry and therefore the NRP-1 binding epitope constituted minimally only 12 amino acid residues.

NRP-1 and VEGF-B are co-expressed for example in the myocardial cells of the developing heart and in the smooth muscle cells surrounding blood vessels (Aase *et al.*, 1999; Kitsukawa *et al.*, 1995). In addition, the juxtacrine relationship between NRP-1 in endothelial cells and VEGF-B in the surronding smooth muscle cells suggests that the molecules are used for paracrine communication between these cells.

Because a NRP-1 deletion mutant lacking the intracellular domain can mediate Sema3A-induced growth cone collapse, it was concluded that rather than having a signaling function of its own, NRP-1 functions as a co-receptor for other signal transducing receptor(s) (Nakamura *et al.*, 1998). Indeed, it was found that NRPs form complexes with receptors of the plexin family, plexin-A1 and plexin-A2, which are responsible for mediating chemorepulsion (Rohm *et al.*, 2000; Takahashi *et al.*, 1999a; Tamagnone *et al.*, 1999). Similarly, NRPs regulate signal transduction by VEGFRs through formation of complexes with them (Fuh *et al.*, 2000; Gluzman-Poltorak *et al.*, 2001; Whitaker *et al.*, 2001).

No obvious protein homology domains have been found in the relatively short intracellular domain of NRP-1, which consists of 40 amino acid residues. However,

the intracellular domain is highly conserved between species, suggesting importance of this part for NRP-1 function. A PDZ-domain containing protein NIP was shown to interact with NRP-1 intracellular domain (Cai and Reed, 1999). NIP may link NRP-1 with other signaling molecules or with the cytoskeleton, suggesting that NRP-1 has also an intrinsic signaling function.

NRP-1 appears to function also as a cell surface adhesion molecule (Takagi *et al.*, 1995). Interaction with another adhesion molecule, L1-CAM, may be involved in the NRP-1 mediated cell adhesion activity. However, in addition to its adhesive function, L1-CAM modulates Semaphorin signals by forming tripartite complexes with plexins and NRPs (Castellani *et al.*, 2000). Interestingly, L1-CAM has been shown to turn Sema3A induced repulsion of neuronal growth cones into attraction (Castellani *et al.*, 2000).

In summary, our studies show that NRP-1 functions as a receptor for both splice-isoforms of VEGF-B. Since the biological function(s) of VEGF-B, as well as the VEGFR-1 mediated signaling have remained largely unknown, it has been difficult to define the role of NRP-1 binding in the biology of VEGF-B. However, analogous to VEGF and Semaphorins, VEGF-B may induce biological responses such as cell migration in cells which express NRP-1, but no VEGFRs. NRP-1 may also potentiate the effects of VEGF-B in cells where VEGFR-1 is expressed. Furthermore, competitive interactions between Semaphorins and VEGFs may regulate the biological responses both in endothelial cells and in neurons.

2. VEGFR-3 is required for the development and maintenance of the embryonic lymphatic vasculature (II)

VEGFR-3 is expressed in lymphatic endothelial cells in adult tissues and its ligands were reported to stimulate lymphangiogenesis (Jeltsch *et al.*, 1997; Oh *et al.*, 1997). However, homozygous disruption of *VEGFR-3* led to death of mouse embryos before the emergence of lymphatic vessels and thus these mice did not provide any information on the role of VEGFR-3 in the development of lymphatic vessels (Dumont *et al.*, 1998). In order to address this question, we used a soluble VEGFR-3 as a competitive inhibitor of VEGF-C/VEGFR-3 signaling (Fig. 4).

According to our *in vitro* experiments, a fusion protein consisting of the ligand binding portion of human VEGFR-3 and human IgG1 Fc domain (VEGFR-3-Ig) efficiently inhibited VEGF-C/D binding to their cell surface receptors and blocked VEGFR-3 signaling. To test the inhibitory effect of VEGFR-3-Ig *in vivo*, we generated transgenic mice expressing the fusion protein under the keratin-14 (K14) promoter which directs the transgene expression to the basal epidermal cells of the skin. The K14-driven transgene expression is turned on after the developmental period when lymphatic vessel development has started and VEGFR-3 expression has been downregulated in blood vessels. The late onset of the transgene expression should eliminate the presumably lethal effects of the inhibition of VEGFR-3 signaling on the blood vasculature.

Transgenic K14-VEGFR-3-Ig mice were devoid of dermal lymphatic vessels as evaluated by histochemistry, immunostaining and lack of lymphatic transport of macromolecules injected into the dermis. In contrast, the blood vasculature remained normal, indicating that the inhibition was

specific for the lymphatic vessels (Mäkinen *et al.*, 2001a; II).

Neutralising levels of circulating soluble VEGFR-3-Ig protein in the sera of the transgenic mice were also associated with a transient loss of lymphatic vessels in several internal organs of neonatal mice. However, after three weeks of age, partial regeneration of the lymphatic vessels was observed, suggesting that alternative pathways may be involved in mediating the growth of the lymphatic vessels. For example, the maturing extracellular matrix may provide signals for the survival and proliferation of lymphatic endothelial cells, and new lymphatic vessels may grow alongside the existing blood vessels and receive proliferative and chemotactic signals from the blood vessel endothelial cells. Similar results of vessel regeneration have been obtained using adenoviral VEGFR-3-Ig. Adenoviral infection of newborn mouse pups results in the regression of lymphatic vessels, but the vessels regenerate in a few weeks in spite of the high serum levels of the VEGFR-3-Ig protein (Karpanen *et al.*, unpublished observations). However, in adult mice AdVEGFR-3-Ig fails to induce the regression of normal lymphatic vessels (He *et al.*, 2002; Karpanen *et al.*, 2001; Karpanen *et al.*, unpublished observations). These results suggest the existence of a mechanism of lymphatic vessel maturation after which the growth and survival of the vessels may no longer be (totally) dependent on VEGF-C. For the blood vessels, pericyte recruitment acts as a signal for vessel maturation, after which the endothelial cells can survive in the absence of VEGF (Benjamin *et al.*, 1998).

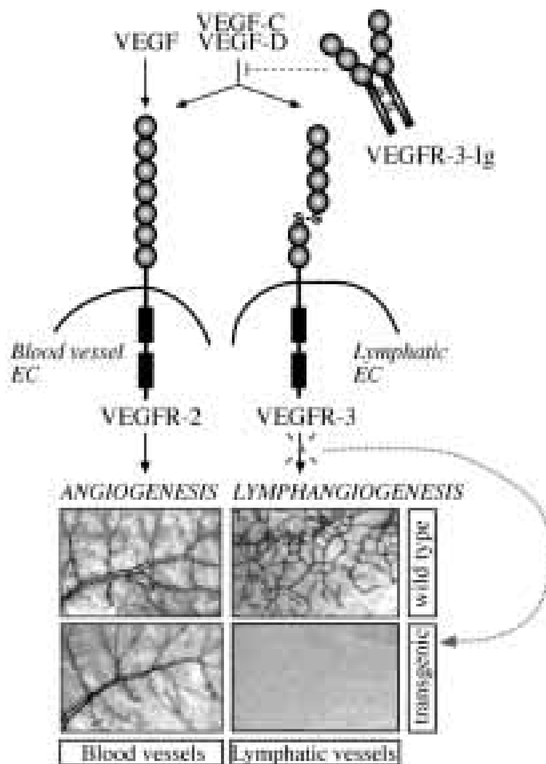


Figure 4. Inhibition of VEGFR-3 signaling by the transgenic expression of a soluble VEGFR-3 Immunoglobulin (Ig)-fusion protein. Visualisation of dermal blood and lymphatic vessels in the wild type and transgenic mice. Inhibition of VEGFR-3 signaling leads to a specific inhibition of lymphangiogenesis.

The absence of dermal lymphatic vessels in the K14-VEGFR-3-Ig mice was associated with fibrosis, increased fluid accumulation in the skin and subcutaneous tissue and swelling of the feet. The phenotype thus resembled human lymphedema, and supported the human studies which demonstrated the deficient VEGFR-3 signaling as the cause of the disease (Irrthum *et al.*, 2000; Karkkainen *et al.*, 2000). Furthermore, as in some cases of lymphedema, the size and appearance of certain lymph nodes varied in the transgenic mice, indicating that lymph flow and a functional lymphatic vasculature are essential for the formation of normal lymph nodes.

The early development of the lymphatic vasculature was not disturbed in the transgenic mice. However, in parallel with significant increase in K14 promoter activity and transgene encoded protein expression, the dermal lymphatic vessels regressed in the transgenic embryos via apoptosis of lymphatic endothelial cells. In conclusion, our results suggest that continuous VEGFR-3 signaling is required not only for the proliferation but also for the survival of the lymphatic endothelial cells and thus for both the development and maintenance of the lymphatic vasculature during embryonic development.

3. VEGFR-3 transduces lymphatic endothelial cell growth, survival and migration signals (III)

As the VEGF receptors are key mediators of angiogenic responses, the signal transduction pathways initiated in endothelial cells upon their activation have been under extensive study. These studies have been mainly carried out using transfected cell lines, but lack of an appropriate cellular background can compromise results obtained from such studies. In addition, little was known of the VEGFR-3 activated signal transduction. In order to clarify these questions, we used a VEGFR-3 specific mutant form of VEGF-C (Joukov *et al.*, 1998) and primary cultures of human dermal microvascular endothelial cells for the studies of VEGFR-3 mediated cellular signaling.

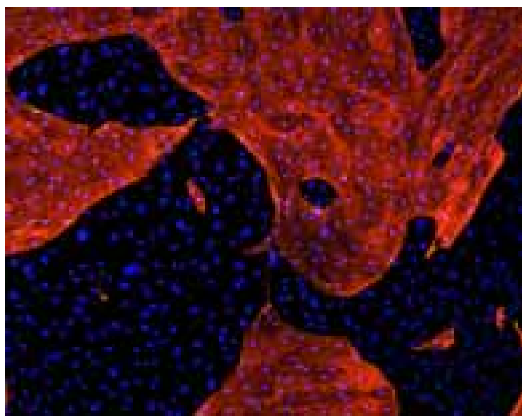


Figure 5. Human dermal microvascular endothelial cells in culture. The lymphatic endothelial cells (stained in red) grow in distinct islands surrounded by blood vascular endothelial cells. The nuclei of all cells have been stained blue.

3.1. Isolation of lymphatic endothelial cells

Based on the expression of three lymphatic endothelial cell specific markers, VEGFR-3, podoplanin and LYVE-1, we found that the microvascular endothelial cell cultures consisted of two lineages of blood vascular and lymphatic endothelial cells (Fig. 5). The lymphatic endothelial cells (LECs) grew in distinct islands surrounded by blood vascular endothelial cells (BECs). We then used antibodies against VEGFR-3 or podoplanin and magnetic microbeads for the separation of BECs and LECs, and maintained the isolated cells in culture (Mäkinen *et al.*, 2001b; III). No interconversion between the cell types was detected in the cultures. In an independent study by Kriehuber *et al.* (2001) BECs and LECs were separated from dermal cell suspension by flow cytometry using antibodies against podoplanin and CD34 (Kriehuber *et al.*, 2001). In agreement with our results their studies demonstrated that LECs and BECs constitute stable and specialized EC lineages.

The ability to isolate and to culture BECs and LECs allowed us to study the signal transduction pathways of different VEGF receptors in these two cell lineages. Both BECs and LECs expressed VEGFR-1 and VEGFR-2 while VEGFR-3 was mainly detected in LECs. On the other hand, BECs produced VEGF-C and extracellular matrix components (III; Kriehuber *et al.*, 2001; unpublished observations), which were required for the growth and survival of the LECs. We found that VEGFR-3 activation induced survival, migration and proliferation of LECs, while VEGFR-2 stimulated these processes in both cell lineages.

3.2. VEGFR-3 signal transduction

In earlier studies with transfected cells VEGFR-3 has been reported to associate with Grb2, Shc and Sos adaptor proteins (Fournier *et al.*, 1995; Pajusola *et al.*, 1994; Wang *et al.*, 1997) and to induce MAPK phosphorylation (Joukov *et al.*, 1998). Shc promotes VEGFR-3 dependent cell proliferation by the activation of the Ras pathway. Other reported substrates for VEGFR-3 include a non-receptor tyrosine kinase Pyk2 (proline-rich kinase 2, also called RAFTK for related adhesion focal tyrosine kinase, CAKß for cell adhesion kinase ß and FAK2 for focal adhesion kinase 2; (Liu *et al.*, 1997; Wang *et al.*, 1997). Pyk2 activates serine kinases ERK and JNK (Jun NH_2-terminal kinase) and phosphorylates the focal adhesion molecule paxillin (Fig. 6). Paxillin phosphorylation then leads to recruitment of actin-anchoring proteins into the focal adhesions and to actin reorganisation which is associated with cell migration. In contrast, Pyk2 induced JNK activation stimulates cellular proliferation via the modulation of transcriptional activation factors such as AP1.

When we studied the basis for the VEGFR-3 transduced survival and migratory signals in primary cultures of endothelial cells, we detected activation of two important signaling molecules which have been associated with cell proliferation and survival; ERK1/ERK2 (p42/p44 MAPK) and Akt.

VEGFR-3 induced MAPK activation was mediated via PKC, not via Ras, similarly to what has been previously shown for VEGFR-2 (Doanes *et al.*, 1999; Takahashi *et al.*, 1999b; Yoshiji *et al.*, 1999). Such a pathway is rather unique among receptor tyrosine kinases since classically PKC-dependent MAPK activation was thought to be employed mainly by certain seven-transmembrane, G protein-coupled receptors. Although the activation of the p42/p44 MAPK cascade is linked in many cells to a proliferation response, this pathway can also lead to increased cell survival (Bonni *et al.*, 1999; Gupta *et al.*, 1999).

VEGFR-3 stimulated phosphorylation of Akt was dependent on PI3-kinase activity. In spite of this, we were not able to detect the association of VEGFR-3 with the regulatory subunit of PI3-kinase, p85. This may indicate that PI3-kinase does not directly bind VEGFR-3 or that the interaction between these molecules is transient.

In addition to VEGFR-3, the LECs also expressed VEGFR-1 and VEGFR-2. Interestingly, VEGFR-2 activation stimulated the proliferation, migration as well as survival of both BECs and LECs, suggesting that VEGF, via VEGFR-2, can regulate signal transduction also in LECs. In addition, even the highest concentrations of the VEGFR-3 specific mutant form of VEGF-C were not as effective as VEGF-C in promoting survival or migration, suggesting that activation of both VEGFR-2 and VEGFR-3 may be required for the VEGF-C-induced maximal responses in LECs. These results suggest that VEGFR-2 may have an important role in the regulation of signal transduction in lymphatic endothelial cells and possibly also in the regulation of lymphangiogenesis *in vivo*.

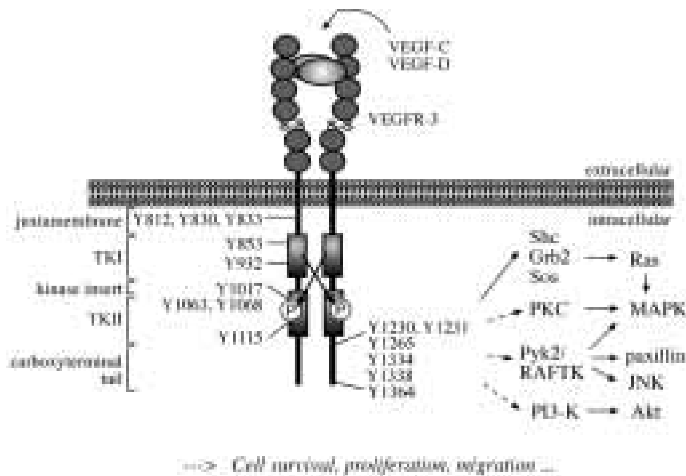


Figure 6. Tyrosine residues (Y) in the cytoplasmic domain of VEGFR-3 and VEGFR-3 activated intracellular signaling molecules. Dashed line: a direct interaction or activation has not been shown.

4. A genetic program controlling the lymphatic versus blood vascular endothelial cell phenotype (IV)

4.1. Gene expression profiling of lymphatic and blood vascular endothelial cells

Identification of differentially expressed genes between BECs and LECs may provide new potential vascular markers, and also give a better understanding of the structural and functional properties of these cells. After establishing the isolation and culture of BECs and LECs, we addressed the question of the genetic program controlling the identity of these two cell lineages using a gene profiling approach.

For the gene expression studies, we used Affymetrix oligonucleotide microarrays which contain sequences from about 12.000

known genes, i.e approximately 30% of the total number of all predicted human transcripts. We identified ~300 genes which were differentially expressed between the cultured BECs and LECs. The most striking differences were detected in genes which encode for proteins involved in cytoskeletal and cell-cell or cell-matrix interactions as well as in the expression of pro-inflammatory cytokines and chemokines (Petrova *et al.*, submitted, IV; Mäkinen *et al.*, manuscript in preparation). We also identified several genes previously unknown in the context of endothelial cell biology, which were differentially expressed in the two cell lineages. Several such genes have been originally cloned from neural tissues. Our gene expression profiling data therefore support the view (Shima and Mailhos, 2000) that the same molecular mechanisms which are involved during neuronal development may also be commonly used in the development of the vascular system as well as in the establishment of the BEC and LEC identity.

4.2. Adhesion molecules in lymphatic and blood vascular endothelial cells

Integrins are a family of membrane receptors which are important mediators of cell adhesion, migration and survival (Giancotti and Ruoslahti, 1999). Integrins consist of two polypeptides, the α and ß subunits. Their ectodomains bind extracellular matrix proteins while the cytoplasmic domains interact with the cytoskeleton and with proteins involved in signal transduction. Integrins can also act synergistically with growth factors and share signaling pathways initiated by RTKs. Integrins can even modulate the activation of RTKs in the absence of their ligands (Miyamoto *et al.*, 1996; Moro *et al.*, 1998; Sundberg and Rubin, 1996). Recently, VEGFR-3 was shown to interact with integrin ß1, and stimulation of ß1 with its ligands fibronectin and collagen induced phosphorylation of VEGFR-3 (Wang *et al.*, 2001). Interaction between VEGFR-3 and integrin ß1 appeared to be also necessary for endothelial cell migration.

In our gene profiling studies we observed a LEC specific expression of integrin α9, which provides a subunit for the receptor for osteopontin and tenascin. Interestingly, integrin α9 has been earlier shown to be important for the normal development of the lymphatic system. Mice lacking integrin α9ß1 develop respiratory failure due to the accumulation of a milky pleural fluid and die after birth (Huang *et al.*, 2000). Therefore, integrin α9ß1 seems to regulate the development of the lymphatic vasculature, possibly via the interaction with VEGFR-3.

Cadherins are another family of transmembrane adhesion molecules, which mediate the formation of stable cell-cell junctions via homophilic cell adhesion. We found that both BECs and LECs expressed VE-cadherin whereas N-cadherin was expressed exclusively in the BECs. VE-cadherin is an important regulator of endothelial cell survival (Carmeliet *et al.*, 1999a). It forms complexes with VEGFR-2 and PI-3 kinase and transmits the VEGF-induced Akt activation (Carmeliet *et al.*, 1999a). Also, association of VEGFR-3 and VE-cadherin was observed in these studies (Carmeliet *et al.*, 1999a). On the other hand, N-cadherin has an important role in mediating endothelial cell-SMC interactions (Gerhardt *et al.*, 2000). Our finding of the BEC-specific expression of N-cadherin is in line with the *in vivo* situation; blood but not lymphatic capillaries are covered by SMCs/pericytes, whose proliferation and migration alongside the blood capillaries is essential for the maturation of the vessels during angiogenic stimulation.

4.3. Prox-1 as a fate determining factor for lymphatic endothelial cells

Homeodomain transcription factors are important regulators of cellular fates and development. Gene targeting studies have demonstrated that Prox-1 homeobox transcription factor has a crucial role in the development of the lymphatic vasculature (Wigle and Oliver, 1999). Therefore we wanted to study the role of Prox-1 in the regulation of BEC and LEC phenotypes.

Prox-1 was found to be expressed specifically in the cultured LECs. However, when introduced into the BECs using adenoviral gene transfer, Prox-1 induced the expression of certain lymphatic specific genes, including VEGFR-3 (IV). Interestingly, Prox-1 overexpression also resulted in the suppression of approximately 40% of the genes characteristic for the BECs. These results suggest the role of Prox-1 as one of the fate determining factors for the LECs. Prox-1 was also capable of inducing the expression of genes associated with S-phase progression, which indicates that Prox-1 may have a function in the regulation of cell proliferation.

These results may help to explain the phenotype observed in the Prox-1 null mice. Targeted disruption of Prox-1 results in the arrest of lymphatic vessel development, whereas development of blood vessels is not affected (Wigle and Oliver, 1999). The first lymphatic sprouts normally bud from the anterior cardinal vein at E10.5, but this budding is arrested in *Prox-1*$^{-/-}$ embryos at E11.5. Our results suggest that the default endothelial cell differentiation state in the absence of Prox-1 corresponds to the blood endothelial phenotype and that Prox-1 is required for the establishment of lymphatic endothelial cell identity. Similar conclusions were drawn from a recent study in which the phenotype of *Prox-1*$^{-/-}$ embryos was analysed in more detail (Wigle *et al.*, 2002). Wigle *et al.* showed that in contrast to the wild type embryos, in *Prox-1*$^{-/-}$ embryos the endothelial cells that bud off from the cardinal vein at E11.5-E12 did not express lymphatic endothelial cell markers VEGFR-3, LYVE-1 or SLC chemokine. Instead, the mutant cells expressed blood vascular markers laminin and CD34, suggesting that these cells were not committed to the lymphatic endothelial cell lineage in the absence of Prox-1 (Wigle *et al.*, 2002).

Gene targeting also revealed that Prox-1 haploinsufficiency was associated with accumulation of lymphatic chylous fluid and neonatal death, suggesting that the level of Prox-1 is critical for proper lymphatic development (Wigle and Oliver, 1999). We found that Prox-1 upregulates the expression of genes associated with a proliferative phenotype, suggesting that after the differentiation of lymphatic endothelial cells, Prox-1 may be also essential for the maintenance of their proliferation during the development.

CONCLUDING REMARKS

Lymphatic vessels are essential for the maintenance of normal fluid balance and immune surveillance but they also provide a pathway for metastasis in many types of cancers. VEGFR-3 was identified as a receptor expressed in the lymphatic endothelium and its ligands, VEGF-C and VEGF-D were reported to induce lymphangiogenesis.

The work presented here demonstrated that VEGFR-3 mediates signals for the survival, migration and proliferation of lymphatic endothelial cells, and these signals are essential for the normal development of lymphatic vessels during embryonic development. Inhibition of VEGFR-3 signaling by using a soluble VEGFR-3 protein resulted in a specific and complete inhibition of lymphangiogenesis in a mouse model. Also in tumor models the soluble VEGFR-3 has been shown to specifically inhibit VEGF-C-induced lymphangiogenesis and tumor spread via lymphatic vessels (Karpanen *et al*., 2001, He *et al*., 2002). In cancers which show high lymphogenous metastasis, lymphatic eradication by inhibiting VEGFR-3 signaling may thus provide a novel strategy to inhibit metastasis.

Until now, only few cell lines of lymphatic endothelial cells have been available for molecular biological studies, and these cells were mainly derived from lymphatic tumours. We showed the isolation and maintenance of primary cultures of dermal blood vascular and lymphatic endothelial cells (BECs and LECs, respectively), which allows now further characterisation of the molecular properties of these cells. Using gene expression profiling, we identified genes which distinguish these two cell lineages, and showed that the homeobox transcription factor Prox-1 is involved in establishing the molecular identity of LECs. Molecular discrimination of the vessels is essential in studies of diseases involving the blood and/or lymphatic vessels and in the targeted treatment of such diseases. Thus the BEC and LEC specific molecules may provide new targets for the treatment of these diseases.

In the future, the next challenges in lymphatic research include the identification of new molecular regulators of lymphangiogenesis. The ability to isolate and to culture LECs facilitate the cloning of novel LEC specific genes the characterisation of which should give us a better understanding of the mechanisms of lymphangiogenesis. The functional analysis of the new lymphangiogenic regulators may also give us novel insights into the pathogenesis of diseases of the lymphatic system.

ACKNOWLEDGEMENTS

These studies were carried out at the Molecular/Cancer Biology Laboratory, Haartman Institute and Biomedicum Helsinki, University of Helsinki during the years 1997-2002.

I wish to thank my supervisor Professor Kari Alitalo for giving me the possibility to join his group and work at the world's leading edge of lymphatic research. It has been a privilege to work in a laboratory with so many opportunities, excellent working facilities and international connections.

I warmly thank all my co-authors for fruitful collaboration. I want to express my special thanks to Birgitta Olofsson and Tanya Petrova for inspiring teamwork and for their delightful enthusiasm for science. It has been a real pleasure to know you and to work with you!

I thank all the former and present members of M/CBL and also all the other people on the 5th floor for their collaboration and for creating a stimulating working atmosphere. I especially want to thank Kristiina Iljin and Eola Valdre for support and friendship both in and outside the lab. Our discussions during the lunch and coffee breaks have been an important source of joy and, when needed, comfort for me. I also thank Terhi Kärpänen, Michael Jeltsch, Yulong He and Marja Lohela for their friendly attitude, discussions and opportunities to exchange ideas. I owe my special thanks to Jussi Taipale, whose extensive knowledge of molecular biology and willingness to teach helped me immensely during my first days in lab, and later on his advice through e-mail were very educative and important when I continued a project he had started.

I am grateful to all the skillfull technicians in our laboratory for their kind help. Special thanks to Mari Helanterä for her excellent work (and refreshing chats!), Tapio Tainola for all his help in numerous practical matters, and Heikki Vuokko for enthusiastic attitude.

I kindly thank Vijay Kumar for revising my English language, and Miia Putkinen and Marja-Leena Saastamoinen for secretarial help.

I thank Heikki Rauvala for all the opportunities and benefits I had as a member of the Helsinki Graduate School of Biotechnology and Molecular Biology. I warmly thank Irma Thesleff and Juha Partanen for their interest towards my work and for their valuable comments and advice when reviewing and improving the manuscript for this thesis as well as when giving me thesis follow-up.

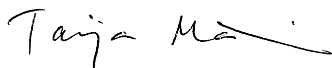
I warmly think back of the time I started my studies of biology. I thank all the people with whom I studied and had carefree and joyful years filled with all sorts of happenings, and all those people who have taught me and made me feel that I had made the right choice. Although I moved to the medical campus, due to my background I'll always believe that the beauty of biology is seen in the light of evolution.

I am thankful to my friends, Marika, Marko, Tiina, Kati and Toni, for many enjoyable moments and good memories. I thank Anne for relaxing riding sessions.

Very special thanks to my parents, Anneli and Risto, my sister, Tytti, and her family for their support and encouragement throughout my life. The memorable times in Ostrobothnia are due to Juha's family, especially Pirjo, Heli and Tapani, for which I thank them.

My dearest thanks belong to Juha for his unselfish support and love. I truly admire his genuine and broad interest in science.

I have been financially supported by the Helsinki Graduate School of Biotechnology and Molecular Biology and by the Finnish Cancer Organisations, Ida Montin Foundation, Emil Aaltonen Foundation and the Finnish Cultural Foundation.



Helsinki, June 2002

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