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LYMPHANGIOGENESIS AND LYMPHATIC METASTASIS

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Cover Image: Intratumoral networks of blood- and lymphatic vessels.

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ABSTRACT

The major cause of cancer mortality is metastasis, which relies on the growth of blood vessels (haemangiogenesis) and lymphatic vessels (lymphangiogenesis). Whereas the field of haemangiogenesis has been relatively thoroughly studied, little is known about the mechanisms regulating lymphangiogenesis. Recent research efforts in studying lymphangiogenesis have been focused on two members of the VEGF-family, VEGF-C and VEGF-D. However, it seems unlikely that these would be the sole factors regulating the formation and maintenance of the lymphatic system. In this thesis work, we have identified several novel lymphangiogenic factors, including members of the PDGF-, IGF-, and VEGF families, and investigated the role of bone marrow-derived circulating endothelial precursor cells (CEPCs) in promoting lymphangiogenesis.

It has previously been demonstrated that members of the PDGF family are potent haemangiogenic factors. In this thesis, we provide compelling evidence that PDGFs display direct lymphangiogenic activity. We have localized expression of the PDGF receptors on lymphatic endothelium and demonstrated direct stimulatory effects of PDGFs on primary lymphatic endothelial cells *in vitro*, as well as lymphangiogenic activities *in vivo*. Overexpression of PDGF-BB in murine fibrosarcomas stimulated tumoral lymphangiogenesis and promoted lymphatic metastasis. VEGF-A is another key angiogenic factor frequently utilized by tumors and other tissues to switch on their angiogenic phenotypes. This factor was previously thought to act as a specific haemangiogenic factor. However, we and others have identified VEGF-A as a novel lymphangiogenic factor *in vivo*. We found that overexpression of VEGF-A in murine fibrosarcomas induced the accumulation of peritumoral lymphatic vessels and promoted metastasis to the regional lymph nodes. The mechanism by which VEGF-A induces lymphangiogenesis might involve both direct effects, through activation of VEGFR-2 expressed on lymphatic endothelium, and indirect effects, by recruiting inflammatory cells that secrete lymphangiogenic cytokines and growth factors. Members of the IGF family are widely expressed in various types of solid tumors. IGF-1R, the major receptor of the IGF family, may indirectly stimulate lymphangiogenesis by upregulating the expression of several known lymphangiogenic factors. However, we have demonstrated that IGF-1 and IGF-2 also can directly induce lymphangiogenesis by activating the cognate receptor expressed in lymphatic endothelium. Our findings suggest that these factors may contribute to lymphatic metastasis. Bone marrow constitutes a rich reservoir of organ-specific pluripotent and committed stem cells. CEPCs have recently been shown to contribute to postnatal vasculogenesis. Although sprouting of new lymphatics from the pre-existing lymphatic network is a critical mechanism for postnatal lymphangiogenesis, it is possible that lymphvasculogenesis also occurs. In this thesis work we show that CEPCs were incorporated into newly formed lymphatic vessels at both physiological and pathological conditions, making CEPCs an interesting target in the development and evaluation of new therapeutic drugs and strategies for the treatment of lymphatic metastasis.

Increased molecular understanding of regulators contributing to lymphangiogenesis will increase the possibility to prevent lymphatic, in addition to haematogenous, spread of tumors.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Renhai Cao*, **Meit A. Björndahl***, Stina Garvin, Dagmar Galter, Björn Meister, Fumitaka Ikomi, Steen Dissing, David Jackson, Toshio Ohhashi, and Yihai Cao. PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell*. 2004 Oct;6(4):333-45.

II. **Meit A. Björndahl***, Renhai Cao*, Jeremy B. Burton, Ebba Brakenhielm, Piotr Religa, Dagmar Galter, Lily Wu, and Yihai Cao. Vascular endothelial growth factor-a promotes peritumoral lymphangiogenesis and lymphatic metastasis. *Cancer Res*. 2005 Oct 15;65(20):9261-8.

III. **Meit A. Björndahl**, Renhai Cao, L. Johan Nissen, Steve Clasper, Louise Johnson, Yuang Xie, Zhongjun Zhou, David Jackson, Anker J. Hansen, and Yihai Cao[1]. Insulin-like growth factors 1 and 2 induce lymphangiogenesis in vivo. *Proc Natl Acad Sci* U S A. 2005 Oct 25;102(43):15593-8.

IV. Piotr Religa, Renhai Cao, **Meit Bjorndahl**, Zhongjun Zhou, Zhenping Zhu, and Yihai Cao. Presence of bone marrow-derived circulating progenitor endothelial cells in the newly formed lymphatic vessels. *Blood*. 2005 Sep 1.

* These two authors contributed equally.

Other related publications and manuscripts by the same author:

- Ekstrand AJ, Cao R, **Bjorndahl M**, Nystrom S, Jonsson-Rylander AC, Hassani H, Hallberg B, Nordlander M, Cao Y. Deletion of neuropeptide Y (NPY) 2 receptor in mice results in blockage of NPY-induced angiogenesis and delayed wound healing. *Proc Natl Acad Sci U S A*. 2003 13(10):6033-8.

- **Meit A. Björndahl**, Renhai Cao, Anna Eriksson, and Yihai Cao. Blockage of VEGF-induced angiogenesis by preventing VEGF secretion. *Circ Res*. 2004 94(11):1443-50.

- Renhai Cao, **Meit A. Björndahl**, Marta I. Gallego, Shaohua Chen, Anker J. Hansen, and Yihai Cao. Hepatocyte growth factor acts as a novel lymphangiogenic factor. *Submitted Manuscript*

- Levent M. Akyürek, Xiaowei Zheng, Xianghua Zhou, **Meit A. Björndahl**, Hidetaka Uramoto, Teresa Pereira, Lakshmanan Ganesh, Yihai Cao, Lorenz Poellinger, and Jan Borén. Filamin-A deficiency impairs nuclear translocation of HIF-1α and reduces VEGF activity. *Submitted Manuscript*

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ABBREVIATIONS

Ang	angiopoietins
BM	bone marrow
BEC	blood endothelial cells
CEPC	circulating endothelial precursor cell
c-Met	hepatocyte growth factor receptor
DNA	deoxyribonucleic acid
EC	endothelial cells
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ERK	extracellular regulated kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
HGF	hepatocyte growth factor
HIF-1α	hypoxia-inducible factor
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
i.v.	intra venous
LEC	lymphatic endothelial cells
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
PAE	porcine aortic endothelial
PCR	polymerase chain reaction
PDGF	platelet growth factor
PDGFR	platelet growth factor receptor
PKB/Akt	protein kinase B
RT-PCR	reverse transcriptase-Polymerase chain reaction
s.c.	subcutaneous
SMC	smooth muscle cell
TGF-β	transforming growth factor-beta
TIMP	tissue inhibitors of metalloproteases
TNF	tumor necrosis factor
TUNEL	terminal nick-end labeling
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
uPA	urokinase plasminogen activator
wt	wild-type

1 INTRODUCTION

1.1 INTRODUCTION OF ANGIOGENESIS

The formation of the mammalian circulatory system consists of two main processes, vasculogenesis and angiogenesis, and is essential for a variety of physiological processes including organ formation, embryonic development, female reproduction, wound repair, and tissue regeneration[1]. Vasculogenesis is initiated during early embryogenesis and represents the *de novo* formation of blood vessels from differentiating endothelial cell precursors, angioblasts[2]. After development of a primary vascular plexus, additional blood vessels are generated through a process termed angiogenesis; the sprouting of new capillaries from pre-existing blood vessels[3]. In the adult mammals, the vasculature generally remains quiescent with the exception of transient phases of neovascularisation occurring during the female menstrual cycles and pregnancy, or at sites of wound healing[1,3,4]. If upregulated, abnormal neovascularisation can contribute to development and progression of several pathological conditions[5,6].

1.1.1 Vasculogenesis: Establishment of a primary vascular network

During the earliest stages of embryogenesis, the embryo develops in the absence of vascularisation, receiving oxygen and nutrition by simple diffusion. Formation of the mammalian cardiovascular system is a complex process requiring precisely regulated differentiation and assembly of multiple cell lineages to form capillaries, arteries, and veins of the mature circulatory system. The earliest vascular structures consist of clusters of mesodermally-derived precursor cells (hemangioblasts) that aggregate in the developing yolk sac to form extraembryonic blood islands at the primitive streak

stage. Differentiation of pluripotent embryonic precursor cells into hemangioblastic cells involves fibroblast growth factor (FGF)[7]. Within the blood islands, the hemangioblasts undergo their first critical steps of differentiation. The peripheral cells of the blood islands flatten and give rise to angioblasts, while those at the centre become haematopoietic stem cells (HSCs)[8,9]. The close association of angioblasts and HSCs in the blood islands has led to the assumption that a common precursor cell might exist, *i.e.* the hemangioblast[10]. Another reason for assuming a common origin is that angioblasts and HSCs share a number of markers, such as PECAM-1 (CD31), CD34[11], VE-cadherin[12], Tie-2[13], and VEGFR-2[14]. Further, target disruption of the genes encoding vascular endothelial growth factor-A (VEGF-A) and its angiogenic receptor (VEGFR-2) disrupts both haematopoietic and endothelial cell (EC) function during development, in support of the hypothesis of a bipotent precursor cell[10,14,15]. Following differentiation from angioblasts, ECs migrate to form endothelial tubes, which interconnect with each other in order to organize a primitive blood vessel network[16]. As soon as the first primordial vessels are formed, vascular stabilizing cells, pericytes and mesenchymal cells, are recruited by growth factors such as platelet-derived growth factor B (PDGF-B) and epidermal growth factor (EGF) produced by proliferating and migrating ECs. The mesenchymal cells become vascular smooth muscle cells (SMCs) of the media or fibroblasts of the adventitia depending on their locations in the vessel. The association of pericytes and SMCs with the newly formed endothelial network, as well as the production of extracellular matrix (ECM), stabilise nascent vessels by regulating EC proliferation, differentiation, migration, survival, vascular branching, blood flow, and vascular permeability, leading to the formation of stable and mature blood vessels[17,18].

1.1.2 Angiogenesis: Formation of the mature circulatory system

After development of a primary vascular network, the developing embryo requires the formation of additional blood vessels. This process, called angiogenesis, is largely elicited by tissue hypoxia, which induces the expression of several angiogenic growth factors such as VEGF-A, PDGF, angiopoietin-2 (Ang2), and others[19,20]. In response to these angiogenic factors, ECs of the primitive vasculature proliferate and sprout to form a branching network of capillaries (Figure 1). The basement membrane surrounding the ECs is locally degraded by proteases produced by the ECs themselves, allowing the chemotactic migration of ECs towards an angiogenic stimuli. Proliferating ECs invade and degrade the ECM to form the advancing front of the vessel sprout[1,3,21,22]. Degradation of the extracellular matrix not only dissolves the physical barrier for the migrating ECs but also results in the liberation of matrix-bound angiogenic factors such as FGF-2, VEGF-A, Insulin-like growth factor-1 (IGF-1), and transforming growth factor-β (TGF-β). Today, over 20 different matrix metalloproteinases (MMPs) have been identified and implicated in cell proliferation and angiogenesis. The liberated ECs change morphology, proliferate, and adhere tightly to each other in order to establish the tunica intima membrane of a new vessel wall. Newly formed capillary sprouts are fragile and highly susceptible to remodelling unless endothelial support cells are recruited to solidify and stabilise the endothelial tubules[1,18]. The supporting cells include pericytes for small capillaries, SMCs for larger vessels and myocardiocytes in the heart[18,22]. Like the ECs, pericytes

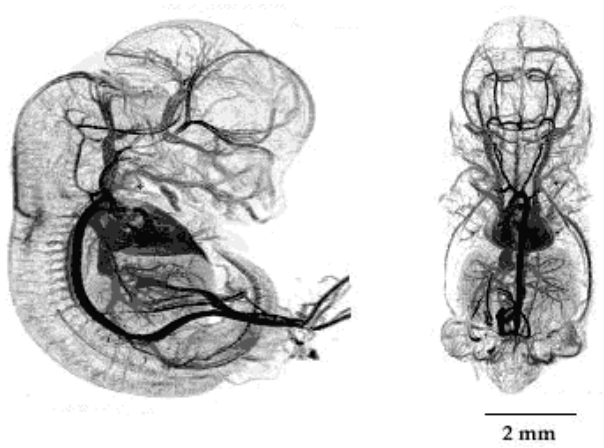

Figure 1. *Gadolinium-injected vasculature of a 12.5 day mouse embryo.*

and SMCs proliferate and migrate in parallel to the growth of the vascular sprout. Once the vessels are stabilised, the ECs become quiescent with a remarkable resistance to exogenous factors, and a survival time of many years. Insufficient recruitment of stabilizing cells results in continuous growth of EC, vascular permeability and fragility, leading to tissue edema[23]. Formation of new capillaries can also occur through a non-sprouting mechanism called intussusception. Non-sprouting angiogenesis is based on splitting of pre-existing vessels, which can occur either by proliferation of ECs inside of a vessel, producing a wide lumen that can be split through the formation of transcapillary pillars, or simply by fusion and subsequent splitting of capillaries[24]. Through this process, larger blood vessels can be divided into smaller capillaries, which then grow separately[22].

1.1.3 Morphological features of blood vessels

The wall of large blood vessels such as veins and arteries consists of three layers: (1) the innermost layer, the *tunica intima*, consisting of a single layer of flattened ECs surrounded by a basement membrane; (2) the intermediate muscle layer, the *tunica media*, comprising SMCs and fibrocytes intermingled with sheets of elastin and collagen; and (3) the outermost connective tissue layer called the *tunica externa* or *tunica adventitia*, containing loose connective tissue, smaller blood vessels, and nerves. Although the walls of veins have the same three layers as the arteries, there is less smooth muscle and connective tissues present making the walls thinner and less rigid than arteries, and capable of holding more blood. Large and medium-seized veins have valves that prevent the backflow of blood and keep the blood flowing toward the heart. A single layer of microvascular ECs, surrounded by a continuous basement membrane, lines small blood vessels such as arterioles, capillaries, and

venules. Pericytes instead of SMCs surround the endothelial layer, which in turn is encircled by a thin layer of connective tissue, the *adventitia*. The distinct functional and molecular characteristics of arteries and veins suggest that the EC cell population is highly heterogenous. ECs differ considerably in the arterial, capillary, and venous compartments, and there is further heterogeneity in vascular beds of different organs[5,167,168].

1.1.4 Regulation of angiogenesis

Angiogenesis is a complex process precisely regulated by a local change in the balance between angiogenic factors and inhibitors[6,21]. The quiescent nature of the adult vasculature is maintained by high levels of local or circulating angiogenic inhibitors that counteract the angiogenic stimulus produced by metabolically active neighbouring cells. The activation of an angiogenic response requires both up-regulation of angiogenic factors and/or decreased expression of endogenous inhibitors[1,6,25]. Both angiogenic and anti-angiogenic factors may arise from various sources such as cancer cells, ECs, stromal cells, inflammatory cells, as well as the extracellular matrix. Cells suffering from hypoxia start to release angiogenic factors to establish a better contact with the circulating blood providing oxygen. Additional signals that can trigger an angiogenic switch include metabolic stress, including low pH and hypoglycaemia, mechanical stress, as well as genetic mutations of oncogenes or tumor-suppressor genes that control the production of angiogenic regulators. It has for instance been shown that malignant cells with mutations in the p53 tumor suppressor gene can survive toxic and hypoxic conditions that kill less malignant cells, resulting in clonal selection of more malignant tumor cells[26].

In contrast to angiogenesis in the embryo, adult angiogenesis is often associated with inflammation, attracting monocytes/macrophages, platelets, mast cells, and other leukocytes. All of these infiltrating inflammatory cells are rich sources of angiogenic factors such as VEGF-A, VEGF-C, VEGF-D, angiopoietins, FGF-2, PDGF, TGF-β (transforming growth factor-β), HGF (Hepatocyte growth factor), IGF-1 (Insulin-like growth factor-1), monocyte chemotactic protein 1 (MCP-1), and several proteolytic enzymes [27,28]. The list of angiogenic stimulators is constantly growing, but among the known regulators of angiogenesis, members of the VEGF family are best characterized. Three major growth factor families with potant angiogenic activities are illustrated in Figure 2. Similarly, a number of potent endogenous inhibitors of angiogenesis have been identified including endostatin, angiostatin, thrombospondin-1, and kringle 1-5 of plasminogen[25,29-32].

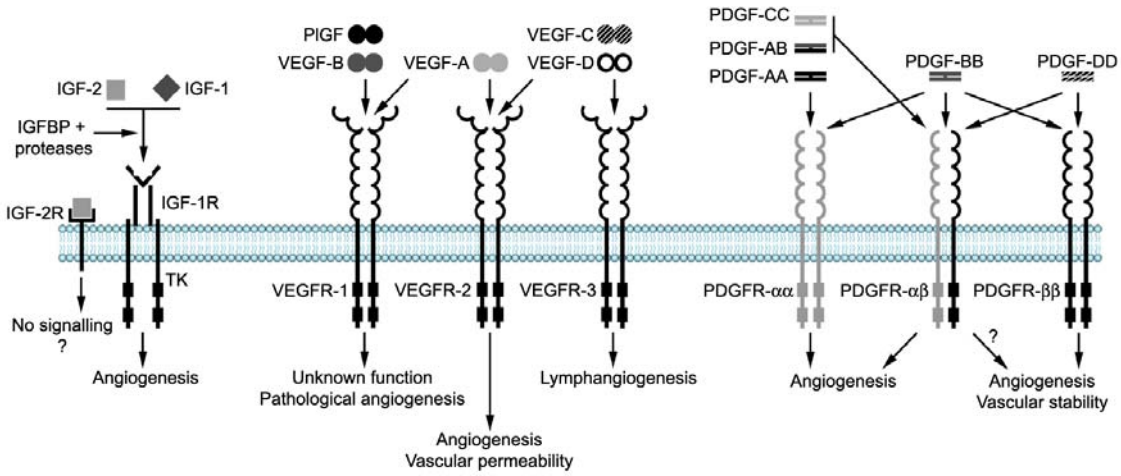

Figure 2. *Schematic illustration of the vascular events induced by members of the IGF-, VEGF-, and PDGF families.*

1.1.4.1 Stimulators

1.1.4.1.1 Vascular endothelial growth factor family

VEGF-A, the best-characterized positive regulator of blood vessel development[21], is the prototype for a number of structurally related growth factors, including VEGF-B, -C, -D, -E, and placental growth factors (PlGF)[33]. The factors of the VEGF family form homo- and heterodimers with distinct biological activities[34]. The angiogenic activities of members of the VEGF-family are mediated through two structurally related tyrosine kinase receptors, VEGFR-1and VEGFR-2, both of which are mainly expressed on vascular ECs[19]. VEGFR-1 is also expressed on monocytes/macrophages[35] and VEGFR-2 was recently reported to be expressed occasionally on lymphatic endothelium[36-38]. In addition to VEGFR-1 and VEGFR-2, a lymphatic EC specific tyrosinase kinase receptors, VEGFR-3, has been identified[19,39]. VEGF-C and VEGF-D can activate both VEGFR-3 and VEGFR-2 to induce lymphangiogenesis and angiogenesis, respectively[40,41].

VEGF-A is an abundant endothelium-specific growth factor that stimulates proliferation, migration, sprouting, and tube formation of ECs, as well as vascular integrity[42]. Moreover, VEGF-A acts as a chemoattractant for SMC, implicating a role for VEGF-A in vessel stabilization[43]. VEGF-A was initially described as a permeability factor, as it increases vascular permeability through the formation of intercellular gaps, vesicular organelles, vacuoles, and fenestrations[44]. VEGF-A is expressed in a wide variety of cell types including activated macrophages, keratinocytes, pancreatic β-cells, hepatocytes, SMCs, and embryonic fibroblasts[45]. The expression of VEGF-A is markedly up regulated in hypoxic conditions *via* hypoxia inducible factor (HIF) regulated elements of the VEGF-A gene. Stabilization of VEGF-A mRNA also contributes to increased VEGF-A levels under oxygen

deprivation[46]. VEGF-A stimulates angiogenesis in a strict dose-dependent manner. Among the known angiogenic factors, VEGF-A is considered the most essential for development of the vascular system as loss of a single VEGF-A allele results in defects in early vascular development and embryonic lethality in mice at embryonic day 11-12 (E11-E12). Homozygous VEGF-A knockout mice die at an even earlier time point, around E9, from severe defects in the formation of the blood islands, lack of development of ECs, and reduced angiogenic sprouting[47,48].

VEGFR-1 is the only known signalling receptor for PlGF. Expression of PlGF is mainly restricted to the placenta, heart, lung, and some tumors[33]. The direct role of PlGF still remains unknown, but the factor has been suggested to indirectly stimulate angiogenesis by occupying VEGFR-1 and thereby increasing the fraction of VEGF-A molecules available to activate VEGFR-2. Under pathological conditions, PlGF has been shown to enhance VEGF-A-induced angiogenesis lest in part through a unique cross-talk between VEGFR-1 and VEGFR-2. Deletion of PlGF in mice does not affect embryonic angiogenesis, and p*lgf*-deficient mice are born at a Mendelian frequency and are healthy and fertile. However, these mice show impaired pathological angiogenesis in response to ischemia, inflammation, wound healing, and cancer, possibly due to a critical role for PlGF in mediating recruitment of bone marrow-derived cells[49].

VEGF-B is expressed in most tissues, but most abundantly in the developing heart, as well as in skeletal muscles, and the spinal cord[50]. VEGF-B binds exclusive to VEGFR-1 and stimulates EC growth and the regulation of plasminogen activator activity in ECs, suggesting a role for VEGF-B in the regulation of ECM degradation, cell adhesion, and migration[51]. VEGF-B deficient mice are fertile and develop normally, with a normal life span[52]. Today, the precise physiological function of VEGF-B *in vivo* is not known.

VEGF-C and VEGF-D are the only known activating ligands for the lymphatic receptor VEGFR-3. In addition to activating VEGFR-3, VEGF-C and VEGF-D also activates VEGFR-2[40,41]. Both growth factors are produced as precursor proteins, whose receptor binding specificity is regulated by proteolytic cleavage. Partially processed and mature forms of VEGF-C and VEGF-D bind VEGFR-3 with high affinity, whereas only the fully processed forms bind VEGFR-2 and induce angiogenesis[53,54]. VEGF-C and VEGF-D will be discussed in further detail in the lymphangiogenic section.

Targeted disruption of the receptors of the VEGF-family results in severe defects in vascular development and early embryonic lethally[14,15,47,48,55]. Each VEGFR knockout produces a distinctive phenotype, indicating that each of these tyrosine kinases controls a specific but complementary function in ECs. VEGFR-1-deficient mice die *in uterus* between E8.5-9.5 as a result of defective assembly of vascular tubes and overgrowth of ECs. Thus, this receptor seems to be critical for blood vessel organisation and assembly, whereas differentiation of ECs and their continued proliferation does not seem to be affected by the absence of receptor signalling[55]. VEGFR-2 knockout mice also die *in uterus* at E8.5-E9.5 due to lack of development

of the embryonic blood islands, and subsequent differentiation of hemangioblasts into mature ECs and HSCs.[14,15] Targeted inactivation of VEGFR-3 in mice is embryonic lethal at E9.5 due to defective remodelling of the primary vascular plexus and disturbed haematopoiesis. Vasculogenesis and angiogenesis occur in these embryos, but large vessels are abnormally organized leading to fluid accumulation in the pericardial cavity followed by cardiovascular failure[56].

1.1.4.1.2 Fibroblast growth factor family

The FGF family, with its prototype members acidic FGF (aFGF, also called FGF-1) and basic FGF (bFGF or FGF-2), encompasses at present at least twenty factors, which are 30-70% identical in their primary sequences[57]. FGF-1 and FGF-2 lack signal sequences for export out of the producer cell, whereas most other members of the FGF-family are secreted[58]. FGFs are pleiotropic factors displaying their biological effects by binding to four structurally related tyrosine kinase receptors, the fibroblast growth factor receptors (FGFR-1, -2, -3, -4)[59], present on a wide variety of different cell types, including ECs[60,61]. Upon ligand binding the receptor undergo dimerization, which is a prerequisite for activation of the tyrosine kinase[62]. Heparan sulphate proteoglycans (HSPs) are cell membrane attached or extracellular matrix proteins required for FGF receptor function[63]. In their absence, FGFs fail to bind and activate FGF receptors. During embryonic development, the expression patterns of the different FGF receptors are distinct yet overlapping[64-66]. Members of the FGF-family, mainly FGF-1 and FGF-2, have been shown to promote EC proliferation[67], migration[68], differentiation[61], protease production[69], integrin and cadherin receptor expression[70], and intercellular gap-junctions *in vitro*, and to stimulate blood vessel formation *in vivo*[71]. The FGFR-1 is expressed on ECs *in vivo*, however FGFR-1-mutant mouse embryos are developmentally retarded and die during gastrulation prior

to a stage in which the role of the FGFR-1 in blood vessel development can be evaluated[72]. However, adenovirus-mediated expression of a dominant-negative FGFR-1 significantly impairs blood vessel development in mouse embryos cultured *in vitro*[73], suggesting that FGFs/FGFR-1 plays a role in the development and maintenance of a mature vascular network in the embryo[74]. Moreover, an intimate cross-talk exists between FGF-2 and members of the VEGF-family during vasculogenesis, angiogenesis, and lymphangiogenesis. FGF-2 appears to partly induce neovascularisation indirectly through induction of the VEGF/VEGFR system[75,76].

1.1.4.1.3 Family of angiopoietins

The angiopoietins work in concert with VEGF-A to regulate blood vessel formation. These factors are ligands for the Tie receptors, a family of receptor tyrosine kinases that are selectively expressed within the vascular endothelium[13,77]. Both the Tie-1 and Tie-2 receptors play a critical role in embryonic development, which has been demonstrated through establishment of knockout mice. Tie-$1^{-/-}$ embryos fail to establish structural integrity of vascular ECs and die *in uterus* between day E14.5 and birth from edema and localised haemorrhage. In Tie-2-deficient mouse embryos, slightly reduced numbers of ECs are present and assembled into tubes, but the blood vessels are immature, lacking branching networks and proper organisation into both small and large vessels[77,78]. The finding of immature vessels lacking intimate encapsulation by stabilizing support cells suggests an important role of Tie-2 in controlling the capacity of ECs to recruit stromal cells. Tie2-deficiency is embryonic lethal at E9.5-10.5 due to insufficient branching of the cardinal vein and capillaries in the pericardium, lack of vessel remodelling in the yolk sac, and insufficient heart development[79].

Angiopoietin-1 (Ang1) is a Tie-2 agonist widely expressed in both embryonic and adult tissues[79]. During early vascular development, VEGF-A and Ang1 appear to work in complementary fashion, with VEGF-A initiating vascular formation[47,48] and Ang1 promoting subsequent vascular remodelling, maturation, and stabilization, presumably by stimulating tight interactions between ECs and their surrounding support cells and extra cellular matrix[77-80]. Mice with a targeted deletion of the *Ang1* gene develop vascular defects highly reminiscent of those previously described in Tie-2-deficient mice, demonstrating that Ang1 is the primary physiological ligand for Tie2[79].

Ang2 is expressed around large vessels in the embryo and in adults at sites of angiogenic sprouting and vascular remodelling[81]. The actions of Ang2 are complex, context-dependent, and far from fully understood. On some cells, Ang2 seems to activate Tie-2, while it blocks the receptor on other cells. Ang2 appears to be a key regulator of vascular remodelling that plays a critical role in both vessel sprouting and vessel regression. During post-natal vascular remodelling events, Ang2 is presumed to destabilise blood vessels by acting as a Tie-2 antagonist and interfering with interactions between ECs and stabilizing cells. In presence of VEGF-A, the destabilised vessels undergo angiogenic sprouting, whereas they regress by EC apoptosis in the absence of VEGF-A[81,82]. Unlike Ang1, Ang2 is dispensable for embryonic vascular development but is specifically required for subsequent postnatal vascular remodelling. Ang2-deficient mice generally die within two weeks of birth from severe chylous ascites and lymphatic dysfunction shortly after feeding[83].

1.1.4.1.4 Insulin-like growth factor family

The IGF family consists of two ligands (IGF-1 and IGF-2) that are structurally homologous to pro-insulin[84,85]. They bind to two transmembrane receptors (IGF-1R and IGF-2R), and are regulated by multiple IGF-binding proteins (IGFBPs), and IGFBP cleaving proteases. The IGFs are produced in many tissues and are thought to function both in a local autocrine or paracrine fashion, as well as like classical hormones circulating in the plasma in association with IGFBPs. In the body, less than 1% of the IGFs circulate in a free form[86]. The IGFBPs play an essential role in coordinating and regulating the availability and biological activities of the IGFs[87].

IGF-1 has a long-term impact on cell proliferation and differentiation, and functions as an anti-apoptotic survival factor by up-regulating the expression of anti-apoptotic proteins such as Bcl-xL[88]. In contrast to IGF-1, most physiological actions of IGF-2 appear to be restricted to embryonic and fetal growth[89]. The tyrosine kinase receptor IGF-1R has been identified as the major receptor for both IGF-1 and IGF-2[90], whereas the IGF-2R is only considered a scavenger receptor lacking intrinsic tyrosine kinase activity albeit its high affinity for IGF-2[91]. IGF-1 exerts all its known physiological effects by binding to the IGF-1R[90,92]. Although the. Association of IGF-2 with the IGF-2R results in internalization, processing, and degradation of the ligand rather than induction of cellular signaling cascades[93].

The IGF-1R is expressed on EC of both macrovessels and microvessels[94,95]. Signalling through this receptor has been correlated to angiogenesis in several systems[96]. *In vitro*, IGF-1 directly stimulates proliferation, migration, and tubule formation of ECs[97], all of which are key-steps in the process of sprouting angiogenesis. In addition, IGF-1 acts as a potent mitogen and anti-apoptotic factor for

vascular SMC, and also stimulates their migration[98]. IGF-1-signalling through the IGF-1R has further been shown to modulate angiogenesis by stimulating the production of VEGF-A, at both mRNA and protein level, in neoplastic[99-102] and non-neoplastic tissues[103,104]. IGF-2 directly induces angiogenesis by stimulating EC migration and tube formation on *in vitro*. In addition, IGF-2 promotes migration of human umbilical vein endothelial cells (HUVECs) by up-regulating the expression of the proteolytic enzyme MMP-2[105]. IGF-2 was previously found to be a potential regulator of hemangioma growth, which strongly suggests a role of IGF-2 in promoting tumor-associated angiogenesis[106]. Activation of IGF-1R may contribute to tumor angiogenesis by stimulating cancer cells to produce angiogenic factors including VEGF-A, VEGF-C, angiopoietins, FGFs, and proteolytic enzymes such as urokinase-type-plasminogen activator (uPA), MMP-2, and MMP-9[99,101,107-109]. Interestingly, members of the IGF family have been reported to be frequently over-expressed in a variety of neoplasms and activation of the IGF-1R has been implicated in the malignant progression of several types of human cancers[110-113].

The IGF-system is necessary for embryonic development and normal postnatal growth, which has been elegantly demonstrated by selectively disrupting the genes coding for the IGF-1 and IGF-2 ligands, and the IGF-1R separately and in combination[114-118]. Noteworthy, no vascular phenotype has yet been identified in mice deficient in any of the members of the IGF-family.

1.1.4.1.5 Platelet-derived growth factor family

PDGF was originally described as the major mitogenic compound in serum[119]. The PDGF family consists of four ligands, PDGF-A to –D, and two receptor subtypes, PDGFR-α and PDGFR-β. All ligands are secreted as full-length, disulfide-linked homodimers, but when expressed in the same cell, PDGF-A and PDGF-B monomers can dimerize with each other to form functional heterodimers[120]. Based on proteolytic processing, the ligands are segregated into two subfamilies. PDGF-A and PDGF-B, which comprise one subfamily, are both synthesized as longer precursor molecules that are extensively processed intracellularly before secreted in their active form. Cleavage of amino terminal pro-domains and dimerization occurs shortly after synthesis of both PDGF-A and PDGF-B monomers, whereas PDGF-B undergoes additional cleavage of the carboxy termini extracellularly. The other family of ligands consists of PDGF-CC and PDGF-DD, which possess a novel amino terminal domain referred to as a CUB domain. This domain constitutes repeat regions that are found in many proteins, but their function remains unknown. Proteolytic removal of the CUB domain is a prerequisite for generation of biologically active ligands that can bind to PDGF receptors[121,122].

The two PDGFR genes may be expressed individually or together in cells, and the gene products can assemble into both homo- and heterodimers that differ in their affinities for the various ligands. The PDGFR-αα demonstrate affinity for PDGF-AA, PDGF-BB, PDGF-AB, and PDGF-CC, the PDGFR-αβ binds to PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD, whereas PDGF-ββ is selective for PDGF-BB and PDGF-DD[122-124]. Upon ligand binding, the receptors dimerize, which triggers an intracellular signalling cascade that ultimately leads to cellular responses such as proliferation and migration[125].

PDGFs have been extensively characterized *in vitro*, where activation of PDGF receptors have been shown to drive multiple cellular processes including proliferation, differentiation[126], migration[127], actin reorganization, survival[128], and deposition of ECM components and tissue remodelling factors[129]. Over-expression or expression of abnormal members of the PDGF family can result in progression of proliferative diseases such as atherosclerosis[130], inflammatory joint disease[131], fibrosis[132], and cancer[133]. Through their action on mesenchymal cells members of the PDGF family regulate important functions during development, which has been demonstrated using gene-targeting approaches[134-136]. In the mouse embryo, expression of PDGF-BB is mostly restricted to vascular endothelium and megakaryocytes, whereas PDGFR-β is expressed in pericytes, SMCs, and mesenchyme surrounding blood vessels[137,138]. Targeted deletion of either *pdgf-b* or *pdgfr-β* genes generates phenotypically identical mice that die during late gestation (E16-19) from a sudden onset of edema, dilation of the heart and large blood vessels, capillary haemorrhage, and subsequent cardiovascular failure[135,139-141]. A widespread microvascular bleeding occurs in these mice due to failure of recruitment of pericytes and SMCs to newly formed blood vessels[23]. Histological examination of these mice has revealed additional pathological phenotypes, including abnormal kidney glomeruli[135], cardiac muscle hypotrophy[140], defective development of the labyrinthine layer of the placenta[142], and haematopoietic deficiencies[138]. PDGF-A- and PDGFR-α-deficiency are both lethal in mice but results in substantial phenotypic differences. The most severe phenotype is observed in PDGFR- α-deficient mice, which die at E8-16 displaying a number of severe developmental abnormalities, including defective plate closure and nasal septum formation, subepidermal blistering, reduced thymus size, malformed intestinal villi, and cardiac, vascular, and skeletal

defects[136,143,144]. PDGF-A-deficient mice develop a wide range of phenotypes and die at different time-points depending on genetic background. Animal surviving E10, which is when the earliest lethality occurs, die shortly after birth or survive for up to six weeks postnatally[134]. These mice display various defects such as a lung emphysema-like phenotype due to complete failure of alveolar septum formation, interstitial villus dysmorphogenesis, progressive loss of dermal mesenchyme and disrupted hair cycles, spermatogenic arrests, and tremor due to severe hypomyelination of neuronal projections in the CNS[134,144-146]. The expression pattern for PDGF-AA and PDGF-CC are strikingly similar at E9.5-12.5. Thus, it is very likely that these factors have overlapping functions and that PDGF-CC represent the missing link between PDGF-AA and PDGFR-α null mice. Indeed, PDGF-A/-C double mutant mice recapitulate most if not all of the phenotypes seen in PFGFR-α null mice[147].

Members of the PDGF family do not appear to be critical for the initial formation of the vascular system since no apparent vascular or lymphatic phenotypes were observed during embryogenesis in mice with targeted deletion of PDGF ligands or receptors. However, PDGFs may play an important role in regulating angiogenesis in specific organs[148]. PDGF receptors are expressed on capillary ECs[149,150], whereas the ligands of this family are secreted from aggregated platelets, ECs, SMCs, and macrophages in vascular tissues[151]. Co-expression of both receptors and ligands strongly suggests a possible autocrine or paracrine activation of ECs, which might contribute to angiogenesis. Indeed, PDGFs have been shown to activate ECs *in vitro*[152,153], and to stimulate angiogenesis in the chick choriallantoic membrane and the avascular cornea after growth factor implantation[154,155]. In regard to pathology, the PDGF family is involved in multiple tumor-associated processes such as autocrine

growth stimulation, recruitment of fibroblasts to the tumor stroma, and regulation of tumor interstitial fluid pressure. Recent studies have further provided experimental evidence for a role of PDGFs in tumor angiogenesis and metastasis[133,156].

1.1.4.2 Inhibitors

Angiogenesis is thought to depend on a delicate balance between endogenous stimulators and inhibitors. Components of the vascular basement membrane can modulate EC behaviour in addition to providing structural and functional support. A number of endogenous angiogenesis inhibitors have been described that are cryptic fragments of naturally occurring proteins of the ECM or the basement membrane.

1.1.4.2.1 Endostatin

Endostatin is a specific inhibitor of EC proliferation[32], migration[157], and angiogenesis. It corresponds to a 20-kDa fragment derived from the COOH-terminal domain of type XVIII collagen[32], and has been localized to vessel walls and basement membranes[158]. The anti-angiogenic activity of endostatin seems to depend on the interactions with α_5 and α_v integrins[159], E-selectin[160], and HSPs. Endostatin does not affect proliferation of tumor cells or other non-endothelial cell lines, including fibroblasts and SMCs, *in vitro* and thus appears to be specific for ECs[32]. Treatment of ECs with recombinant endostatin induces a 15- to 30-fold increase in apoptosis. This increase in cell death rate has been associated with a reduced expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L[161], as well as induced activation of Caspase-3. Endostatin efficiently blocks tumor-associated angiogenesis and thereby suppresses primary tumor growth and dissemination in experimental animal models without any apparent side effects,

toxicity, or development of drug resistance[32,162]. Interestingly, tumor dormancy can be induced following repeated cycles of endostatin treatment[162].

1.1.4.2.2 Angiostatin

Angiostatin is a 38- to 45-kDa proteolytic fragment containing the first four (K1-4) disulfide linked kringle modules of plasminogen[25]. Prior to the discovery of angiostatin, scientists had observed that in some clinical malignancies, such as breast- and colon cancer, the primary tumor inhibited the growth of metastases, and that the resection of the primary tumor was often followed by a rapid growth of distant metastases[163]. In an animal metastatic model, angiostatin was purified from both serum and urine of mice bearing a transplantable murine Lewis lung carcinoma. Angiostatin was identified as a circulating angiogenesis inhibitor that accumulates in the circulation in the presence of a growing primary tumor but disappears from the circulation shortly after removal of the primary tumor. The anti-angiogenic activities possessed by angiostatin are not shared by the parent molecule plasminogen. Angiostatin specifically inhibits EC proliferation *in vitro*, but not proliferation of other non-endothelial cell types such as tumor cells[25]. In addition, angiostatin inhibits migration of Ecs and induce apoptosis[164]. *In vivo*, angiostatin suppresses neovascularisation in the chick chorioallantoic membrane assay and in the mouse corneal assay, and it also impairs neovascularisation and growth of primary tumors and metastases without toxicity[25,163]. The production of angiostatin is regulated enzymatically whereby several members of the MMP-family hydrolyze plasminogen to generate active angiostatin fragments[165,166]. Smaller fragments of angiostatin have been shown to display differential inhibitory effects on EC proliferation, and the fragments can be ranked as follows, starting with the most potent inhibitor; K1-5>K5>K1-3>K1-4>K1>K3>K2>K4[25,29-31].

1.1.5 Arterial or venous cell fate

The vascular system is a highly heterogeneous and non-uniform organ consisting of an arterial and a venous system with distinct functional and molecular characteristics. Recent gene profiling data has demonstrated that arterial and venous ECs express entire sets of distinct and specific genes[169], suggesting that establishment of the identities of the arterial and venous vasculatures are under the control of related, yet distinct, genetic programs[170]. Both arteries and veins acquire a molecular definition before they become functional and deliver blood. EphrinB2 and EphB4 have been implicated in determining arterial and venous cell fates, presumably by mediating a repulsive signal separating arterial and venous endothelium[171]. In the blood vascular system, ephrinB2 is expressed in arterial ECs, pericytes, SMCs, and mesenchyme at sites of vascular remodelling[170-172]. In contrast, EphB4 expression is mainly restricted to venous and lymphatic ECs[171]. Additional studies have revealed that members of the Notch signalling family mediate the choice of fate between arterial and venous ECs, through a molecular cascade by which arterial identity is induced at the expense of the venous fate[173]. Notch signalling promotes arterial cell fate, at least partly, *via* the activity of gridlock, a transcriptional repressor that negatively regulates venous cell fate[174]. Sonic hedgehog- (shh) and VEGF-A-signalling pathways act upstream of Notch/gridlock to determine arterial cell identity[175].

1.2 INTRODUCTION TO LYMPHANGIOGENESIS

Whereas the development of blood vessels, angiogenesis, has been studied extensively relatively little is known about the development of lymphatic vessels, lymphangiogenesis. From a historical point of view, Hippocrates gave the initial description of putative lymphatic structures, which he described as "white blood nodes". Later, Aristotle made the first observation of lymphatic vessels, which he described as fibres with colourless fluid arranged between blood vessels and nerves. After these early observations, Gasparo Asellius did the first true characterisation of the lymphatic vascular system in 1627[176].

" De Lacteibus sive lacteis venis Quarto Vasorum Mesaroicum genere novo invente Gasp. Asellii Cremonensis Antomici Ticiensis Qua Sententiae Anatomicae multae, nel perperam receptae illustrantur ". Milan: Mediolani, 1627

A few years later, Louis Petit detected breast cancer metastases in axillary lymph nodes and was thereby the first to describe tumor spread *via* the lymphatic system[177-179]. Until the last few years, research on lymphatic vessels and lymphangiogenesis has been very limited due to the lack of histological, ultrastructural, and immunohistochemical markers to accurately discriminate between blood ECs and lymphatic endothelial cells (LECs)[180]. This changed recently with the identification of novel markers specifically expressed on the surface of LECs, such as VEGFR-3[181], prospero related homebox gene-1 (Prox-1)[182], podoplanin[183], and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)[184].

1.2.1 Specific markers of the lymphatic endothelium

Although the lymphatic system was morphologically described almost 400 years ago it was not until recently that a range of markers with lymphatic specificity was identified.

1.2.1.1 Vascular endothelial growth factor receptor-3

VEGFR-3 was the first lymphatic endothelium-specific cell surface molecule to be characterized. However, further studies revealed that VEGFR-3 is also expressed by ECs of the cardinal veins and by angioblasts of the head mesenchyme before mid gestation. In adults, expression of VEGFR-3 becomes mainly confined to the lymphatic system where the receptor is expressed predominantly in the LECs that line the inner surface of lymphatic vessels[39]. In addition, VEGFR-3 is still expressed in splenic and hepatic sinusoids, pancreatic duct epithelium, and in capillaries of kidney glomeruli and endocrine glands, as well as on monocytes, macrophages, and certain dendritic cells (DCs)[28,181,185]. Further, VEGFR-3 expression is up regulated in blood vessels during pathological conditions characterized by neoangiogenesis, including inflammation, wound healing, and tumor growth[186,187]. VEGFR-3 was previously employed as a marker for lymphatic vessels in both normal and pathological tissues[188]. Although VEGFR-3 is expressed almost exclusively on lymphatic endothelium in normal adult tissues, the fact that it is widely expressed in embryonic blood vascular endothelium and re-expressed in tumor blood vessels has complicated the use of VEGFR-3 as a selective marker for lymphatic vessels, especially in studies of tumor lymphangiogenesis.

1.2.1.2 Prospero related homebox gene-1

Prox-1 is a transcription factor involved in the budding and elongation of lymphatic vessels sprouts during development[189,190], and the expression of Prox-1 persists in adult lymphatic endothelium. Prox-1 was also found expressed in non-endothelial cells of the heart, lens, liver, nervous system, and pancreas[191,192]. Targeted deletion of the *Prox-1* gene in mice results in neonates completely devoid of a lymphatic vascular system[189].

1.2.1.3 Lymphatic vessel endothelial hyaluronan receptor-1

A third selective marker of the lymphatic endothelium is the CD44-related hyaluronan receptor LYVE-1. The expression of LYVE-1 is largely restricted to lymphatic endothelium, with the exception of normal hepatic blood sinusoidal ECs[193] and placental syncytiotrophoblasts[194]. The LYVE-1 receptor is distributed equally among the luminal and abluminal surfaces of lymphatic endothelium and has been identified as a novel endocytotic receptor for the ECM glycosaminoglycan hyaluronan (HA)[184,195]. HA is a key mediator of cell migration during embryonic morphogenesis and also in adult processes such as wound healing and tumor metastasis[196]. LYVE-1 is a type I integral membrane glycoprotein sharing 41% homology with the metastatic-related CD44 receptor for HA[184]. Nevertheless, there are distinct differences between LYVE-1 and CD44 suggesting that the two homologues differ either in the mode or regulation of HA-binding. While the expression of LYVE-1 is almost exclusively restricted to lymphatic endothelium[195], CD44 is expressed abundantly in blood vessels and largely absent in lymphatic vessels[197]. LYVE-1-deficient mice are healthy and fertile, and display no pathological phenotype consistent with a defect in lymphatic function (personal communication with Professor David Jackson, The John Radcliff Hospital in Oxford, United

Kingdom). However, further studies are required to explore the role of this receptor in HA-transport, leukocyte migration, and tumor metastasis.

1.2.1.4 Podoplanin

Another promising marker for differentiating between lymphatic and blood vascular endothelium is podoplanin[183], a glomerular podocyte membrane mucoprotein required for lymphatic development. Podoplanin knockout mice have defects in lymphatic vessel, but not blood vessel, patterning, demonstrate a phenotype of lymphatic edema, and die at birth due to respiratory failure[198]. Podoplanin is expressed in lymphatic endothelium, but not in the blood vasculature[183,199]. In the mouse embryo, podoplanin is expressed between E10.5-E11 in ECs of the cardinal vein and in budding Prox-1-expressing progenitor cells committed to the lymphatic phenotype[198]. Within the lymphatic system, podoplanin is preferentially expressed in small lymphatic capillaries lined by a single layer of LECs, such as lymphatic capillaries of the skin. The marker is, however, not expressed in large lymphatics invested by pericytes or SMCs or in endothelial venules of the lymph nodes[183]. Further, podoplanin is co-expressed with VEGFR-3 in lymphatic endothelium of the skin and kidney, and in ECs of benign vascular tumors and angiosarcomas[183,199-201]. Although podoplanin is expressed in some non-endothelial cell types, including lung alveolar type I epithelial cells[202], choroid plexus epithelial cells, and osteoblasts[203], it constitutes a useful marker for detection of lymphatic capillaries.

1.2.2 Formation of the lymphatic system

Our present knowledge about the genesis of the lymphatic system is based on studies performed at the beginning of the 20th century. At that time, two diverse opinions about the origin of LECs were discussed. In 1902, Florence Sabin proposed that the lymphatic system develops by budding of ECs from the venous system, resulting in the formation of the initial lymph sacs from which LECs then sprout towards the periphery and into surrounding organs to form the mature lymphatic system. According to his hypothesis, all LECs are derived from venous endothelium[204-206]. Competing with Sabins' "centrifugal" theory, Huntington, McClure, and Kampmeier proposed that the first lymphatic vessels arise independently in the mesenchyme and only later establish connections with the centripetally located veins, suggesting that all precursors of LECs develop from mesenchymal cells close to the veins, but independently form their endothelial lining[207,208]. Recent studies in *prox-1*-null mice and expression studies of the specific lymphatic marker VEGFR-3 have provided strong evidence supporting Sabins' original theory[39,189,190,209]. In mice, prox-1 expression is initiated at E9.5 in a polarized manner in a subset of uncommitted ECs of the cardinal vein[189]. At this stage, the venous endothelium also expresses the lymphatic markers LYVE-1, secondary lymphoid tissue chemokine (SLC), and VEGFR-3[181,195,210]. Prox-1$^+$ lymphatic progenitor cells subsequently bud and migrate from veins, giving rise to the embryonic lymph sacs. In mammalian embryos, eight primary lymph sacs have been identified; the unpaired retroperitoneal lymph sac, the paired jugular, posterior, and subcla*via*n lymph sacs, and the cisterna chyli. These lymphatic sacs later give rise to primary lymph nodes in mammals (Figure 3)[206,211]. Within the embryonic lymph sacs, the prox-1$^+$ lymphatic progenitor cells progressively down-regulate the expression of blood vascular genes such as CD34 and laminin, while increasing the expression of markers specific for the lymphatic

endothelium such as VEGFR-3[190]. The association of VEGF-C with the VEGFR-3 appears to be critical for proliferation and migration of prox-1^{+} lymphatic progenitor cells from the cardinal veins[212,213]. During further development, the lymphatic- and blood vascular networks become separated, leaving only the thoracic duct and the right lymphatic trunk connected to the venous system. Recent studies have revealed that local recruitment of lymphangioblasts might contribute to the formation of the lymphatic vascular system in the early wing buds, limb buds, and the chorioallantoic membrane of birds[214,215]. However, it still remains unclear if lymphangioblasts contribute to lymphangiogenesis in mammals.

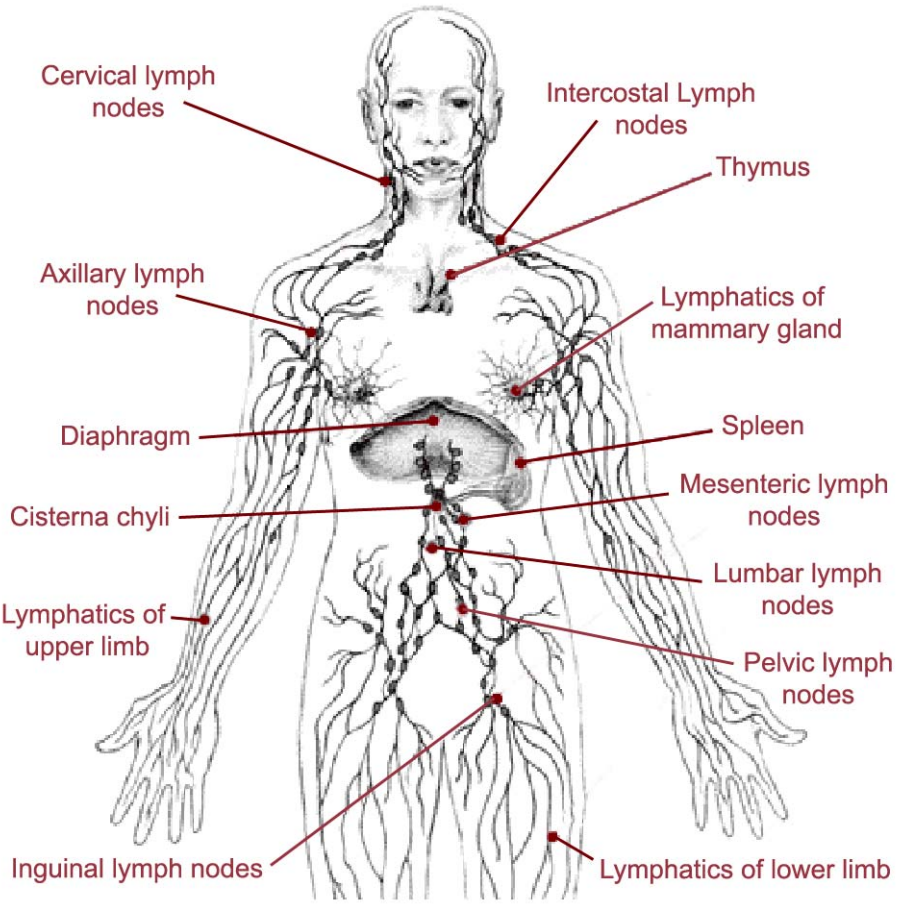

Figure 3. *The human lymphatic system.*

Deletion of the *prox-1* gene in mice leads to multiple phenotypic abnormalities, including the complete absence of lymphatic vessels, arrested migration of hepatocytes during liver development, and embryonic lethality[189-191]. Today, these mutant mice represent the only known model completely devoid of a lymphatic vascular system. In *prox-1* null mice, budding and sprouting of lymphatic endothelial progenitor cells from the veins is arrested at E11.5[189]. Notably, these progenitor cells fail to up regulate expression of the lymphatic endothelial-specific markers LYVE-1, SLC, and VEGFR-3, instead they continue to express blood vascular markers such as CD34 and laminin. Thus, Prox-1 appears to function as a master regulator of LEC phenotype, by providing essential signals required for the commitment of venous ECs to a lymphatic phenotype and subsequent development of the lymphatic vasculature[189,190]. Transcriptional experiments have demonstrated that adenoviral expression of Prox-1 in blood endothelial cells (BECs) is sufficient to re-program the gene expression profile toward a LEC phenotype, with up-regulation of LEC-specific genes and concomitant down-regulation of BEC-specific genes[216,217]. Interestingly, vasculogenesis and angiogenesis of the circulatory system is unaffected by functional inactivation of the *Prox-1* gene, demonstrating the *Prox-1* activity is critical only for normal development of the lymphatic system and that the vascular and lymphatic system thus develop independently[189].

In the mouse, VEGF-C is expressed in the mesenchyme surrounding the region of the cardinal veins, in which the embryonic lymph sacs develop[209]. In the absence of VEGF-C, lymphatic development is arrested whereas the blood vascular system develops normally. In VEGF-C-deficient mice, prox-1^{+} cells appear in the cardinal veins, but they fail to migrate and form embryonic lymph sacs and later disappear, possibly by apoptosis. This indicates that LEC specification and subsequent cell

migration are two separate events presumably regulated by distinct signalling pathways. Interestingly, application of VEGF-C and VEGF-D, but not VEGF-A, rescued the sprouting defect of the committed lymphatic endothelial progenitor cells, demonstrating the necessity of VEGFR-3-mediated signalling for lymphangiogenesis during early embryogenesis[212]. Together, these data demonstrate that Prox-1 is required for differentiation of venous ECs into LECs[189,190], whereas VEGF-C-signalling through the VEGFR-3 is essential for sprouting of prox-1^+ LECs from the cardinal veins[212].

1.2.3 Lymphatic vascular factors and receptors

Since the discovery of the first specific markers for lymphatic endothelium less than a decade ago, tremendous efforts have been made in order to understand the molecular mechanisms of lymphangiogenesis. VEGF-C and VEGF-D were the first growth factors found to activate LECs and stimulate the growth of lymphatic vessels. Recent studies have, however, identified several new lymphangiogenic factors, and many of these appear to be functionally important for the development of the lymphatic system.

1.2.3.1 Vascular endothelial growth factor family

During early embryogenesis, VEGF-C is expressed along with its receptor VEGFR-3 predominantly in regions where the initial lymphatic vessels sprout and develop, strongly suggesting that it plays a role in the development of the lymphatic system[209]. The expression then decreases in most tissues, but remains high in the lymph nodes. Like VEGF-A, VEGF-C stimulates the migration and proliferation of ECs *in vitro*, increases vascular permeability, and stimulate angiogenesis at both physiological and

pathological conditions *in vivo*, albeit at higher concentrations than VEGF-A[41,53,218]. Further, VEGF-C directly activates LECs and contributes to formation and maintenance of the venous and lymphatic vascular systems[39]. VEGF-C has been shown to induce lymphatic vessel growth in various experimental models[219,220]. In contrast to VEGF-A, the expression of VEGF-C is not regulated by hypoxia[221] but rather in response to pro-inflammatory cytokines[222]. Both VEGF-C and VEGFR-3 are prominently expressed by activated macrophages[28,223]. Thus, VEGF-C appears to play a role in inflammatory responses. Mice with targeted deletion in both VEGF-C alleles fail to develop a lymphatic system and die at E15.5-17.5 due to tissue edema. Surprisingly, the blood vascular system develops normally in these mice, demonstrating that VEGF-C is dispensable for blood vessel development[212].

VEGF-D shares 61% sequence identity with VEGF-C and binds to the same receptors, VEGFR-2 and VEGFR-3[54]. Interestingly, VEGF-D only binds to VEGFR-3 in mice, while it binds to both VEGFR-2 and VEGFR-3 in human, suggesting the VEGF-D might have a somewhat different function in these species[224]. During embryogenesis, VEGF-D expression is most abundant in the developing lung and skin. In adults, VEGF-D is expressed in numerous tissues, but particularly in the lung, heart, skeletal muscle, colon, and small intestine[225]. VEGF-D is mitogenic for ECs and is involved in growth regulation of lymphatic and blood vessel endothelium[40,54,226]. However, VEGF-D deficient mice are *via*ble and lack profound blood- and lymphatic vascular phenotypes, suggesting that VEGF-D is not essential for development of either the vascular- or the lymphatic system[227]. In experimental tumors, VEGF-D induces growth of intratumoral lymphatics and promotes lymphatic metastasis[228,229].

During early development, VEGFR-3 is expressed on venous endothelium at sites of lymphatic vessel growth. Only later, VEGFR-3 expression is progressively down-regulated by venous ECs and becomes mainly restricted to lymphatic endothelium[39,188]. The expression of VEGFR-3 on lymphatic endothelium suggests a role for the receptor and its two known ligands, VEGF-C and VEGF-D, in regulating lymphatic vessel growth[40,41]. However, the role of VEGFR-3 in development of the lymphatic system during embryogenesis has remained impossible to evaluate as VEGFR-3 knockout mice die at E9.5 when the lymphatic system is just about to develop[56]. In adults, the expression of VEGFR-3 is mainly restricted to lymphatic endothelium[39], but it is also detected in haematopoietic cells of monocytic lineage[230] and certain fenestrated blood capillaries, although it is absent in endothelia of large blood vessels[181]. In addition, the expression of VEGFR-3 is up-regulated in vascular endothelium in certain pathological conditions such as inflammation- and tumor-associated angiogenesis[38,223].

VEGF-A has been identified as a major angiogenic factor over-expressed in most of human cancers and murine experimental tumor models[3]. The angiogenic effects of VEGF-A are mediated principally *via* VEGFR-2, which was previously considered to be expressed exclusively on vascular endothelium[19]. However, it has recently been shown that, like vascular endothelium, lymphatic endothelium also expresses VEGFR-2 *in situ* and *in vitro* and that VEGF-A promotes survival, proliferation, and migration of LECs[231,232]. Moreover, VEGF-A has also been shown to induce lymphangiogenesis *in vivo*. During wound healing of full-thickness skin wounds in transgenic mice over-expressing VEGF-A specifically in the epidermis, VEGF-A promotes lymphangiogenesis in the granulation tissue *via* activation of VEGFR-2[37]. In another experiment, adenoviral over-expression of VEGF-A in the rabbit ear

resulted in formation of giant, hyperplastic lymphatics that once formed persisted indefinitely, independent of VEGF-A[233]. Very recently, Hirakawa et al demonstrated that chronic transgenic delivery of VEGF-A specifically to the skin not only promotes skin carcinogenesis, but also induces formation of tumoral VEGFR-2-expressing lymphatics[36].

1.2.3.2 Fibroblast growth factor family

FGF-2 is a pleiotropic factor that in addition to its angiogenic properties also can induce lymphangiogenesis. *In vitro*, FGF-2 promotes LEC proliferation, migration, and assembly into capillary-like tube structures[234]. In the cornea assay, FGF-2 stimulates lymphatic vessel growth indirectly *via* up-regulation of VEGF-C expression in vascular endothelial and perivascular cells. Blockage of VEGFR-3-signalling suppresses FGF-2-induced lymphangiogenesis, demonstrating that FGF-2 acts as an indirect stimulator of lymphangiogenesis[186,235]. In this model, FGF-2 appear to induce a dose-dependent stimulation of lymphangiogenesis, with robust lymph vessel growth and minimal or no angiogenesis at low concentrations, demonstrating that lymphangiogenesis can occur in the absence of angiogenesis[235].

1.2.3.3 Angiopoietins

In addition to destabilizing blood vessels during sprouting of new vessels[82], Ang2 was recently suggested to play a role in the development of functional lymphatic vessels. Ang2 knockout mice display defects in the patterning and function of the lymphatic vasculature, and develop highly disorganized and hypoplastic intestinal and dermal lymphatic capillaries, as well as larger collecting lymphatic vessels poorly invested by SMCs. The mice develop subcutaneous edema and generally die by two weeks of age from severe chylous ascites, a condition that is characteristic of defective lymphatic function. Chylous ascites is the result of poor uptake and

transport of chyle, a milky fluid produced by the intestine after feeding that fills the peritoneal cavity[83].

1.2.3.4 EphrinB2

As described previously, ephrinB2 and EphB4 interaction appears to be critical for the specification of arteries and veins, presumably by mediating a repulsive signal separating arterial and venous endothelium[171]. Molecularly, ephrinB2–EphB4 interactions results in bidirectional signal transduction into both receptor- and ligand-expressing cells[236]. Phosphorylation of the cytoplasmic tail of ephrinB2 provides docking sites for intracellular signaling molecules[237]. In addition, the cytoplasmic tail also contains motifs required for binding of proteins containing PDZ-domains[238]. A very recent study demonstrated that ephrinB2 is required not only for the development of blood vasculatures, but also for lymphatic vasculatures[239]. In mice expressing ephrinB2 with a deficient PDZ target site, major defects in the morphogenesis and function of the lymphatic vasculature were detected. This finding suggests that interactions with PDZ-domain proteins are critical for the reverse signalling of ephrinB2 *in vivo*, and further suggests a requirement for ephrinB2 reverse signalling in lymphatic endothelium.

1.2.3.5 Insulin-like growth factor family

The IGF-1R is expressed in most tissues, including vascular ECs[94,95]. Both IGF-1 and IGF-2 act *via* IGF-1R to stimulate EC proliferation, migration, and tube formation, all critical steps in the process of angiogenesis[96,97]. Due to lack of specific markers to reliably distinguish between blood- and lymphatic endothelium, as well as established LEC lines, it has been difficult to evaluate any direct effect of the IGF-family in the process of lymphangiogenesis. Many solid tumors such as cancers of the breast, prostate, and colon utilizes lymphatic vessels as the main route for metastatic spread[240]. Recent studies have suggested that intratumoral lymphatic vessels are critical structures for lymphatic metastasis[36,223,229,241-243], and that expression of lymphangiogenic factor within the primary tumor may govern the growth of intratumoral lymphatics. Signalling through the IGF-1R has been shown to induce the expression of VEGF-A, VEGF-C, angiopoietins, and FGF, all of which are potent lymphangiogenic factors[99,100,108,109]. Thus, IGF-1R-activation might indirectly induce intratumoral lymphatic vessel growth and thereby promote lymphatic metastasis.

1.2.4 Morphological Features of lymphatic vessels

The lymphatic system comprises a tree-like hierarchy of capillaries, collecting vessels, and ducts that are present in most tissues[244]. Lymphatic capillaries are blind-ended vessels lined by a single layer of non-fenestrated LECs[245]. Unlike blood capillary endothelium, LECs have poorly developed junctions with frequent large inter-endothelial gaps. Lymphatic capillaries harbour a discontinuous or completely absent basement membrane, and are not invested by pericytes or SMCs. The abluminal surfaces of LECs are anchored to the perivascular ECM through fine strands of elastic fibers[246]. These fibers keep the vessels from collapsing and also

promote their dilation during changes of interstitial pressure, allowing the flow of interstitial fluid to the lymphatics[178,179]. Overlapping inter-cellular junctions serves as valves in the lymphatic capillaries. As the interstitial pressure increases a pulling force is exerted through the anchoring filaments, causing the junctions to open and permitting the uptake of fluid and particles. When the pressure equalizes, the valves close, preventing the fluid to flow back into the interstitium. From the capillaries the lymph is transported to the collecting lymphatics, and ultimately into the venous circulation *via* the thoracic duct[247]. The larger collecting lymphatics contain perivascular SMCs with an intrinsic contractile function that increases the efficiency of fluid transport. Moreover, these vessels have valves, composed of cytoplasmic protrusions from the lymphatic endothelium, to avoid retrograde flow of lymph[178,179,194].

1.2.5 Function of the lymphatic vascular network

The lymphatic vascular system plays a critical role for tissue fluid homeostasis. Extravasated fluid and macromolecules are collected by initial lymphatics, small lymphatic capillaries, and transported unidirectionally from tissues and organs back to the blood vascular system for recirculation[248]. The lymphatic system is also essential for maintaining a constant immune defence by filtering lymph and any foreign material present in it through a chain of lymph nodes before entering the venous circulation. Lymphocytes and antigen-presenting cells (APCs) are transported from the periphery through afferent lymphatic vessels into lymph nodes and lymphoid organs. In collaboration with APCs, lymphocytes recognize non-self antigens and mount an immune response upon activation[249]. In the small intestine, lymphatic vessels serve as a major conduit for lipid adsorption[247].

Lymphangiogenesis occurs during both normal development and during pathological conditions such as inflammation, lymphedema, and metastatic dissemination of tumors. Defects in lymphatic development, maintenance, and function can lead to lymphedema, a pathological condition characterized by disabling and disfiguring swelling of the affected area[250]. When the uptake of lymph from the interstitium is prevented, protein-rich interstitial fluid accumulates within the tissues. Lymphedema can occur as a manifestation of heritable malformations of the lymphatic vasculature[251], or as a consequence of a previous infection[252,253], tissue trauma[254], or post-surgical disruption of the lymphatics[255]. In addition, the lymphatic vasculature serves as a major route for metastatic spread of tumor cells to regional lymph nodes, and possibly also distant organs[179,180,229,256].

1.3 CIRCULATING ENDOTHELIAL PROGENITOR CELLS

As early as 1934, non-haematological cells were reported in the blood of certain cancer patients[257], and in the 1960s circulating ECs were detected in several animal graft models[258,259]. Lately, mature circulating ECs have been detected in peripheral blood under physiological conditions and during vascular trauma, and more recently also in patients with sickle cell anaemia[260,261]. Circulating ECs can be derived from two sources, either from the wall of blood vessels shedding damaged or dying cells into the circulation or from the adult bone marrow (BM), which constitutes a rich reservoir of organ-specific pluripotent stem- and progenitor cells, including immature endothelial precursor cells. The existence of a BM-derived EC precursor was strongly supported by the results obtained in a graft model of BM-transplanted animals. In this experiment, Darcon grafts implanted 6-8 months after BM-transplantation became colonized by ECs derived from the donor-BM[262]. Today, we know that at least a fraction of all circulating non-haematological cells present in peripheral blood constitute mature circulating ECs and circulating endothelial progenitor cells (CEPCs)[260,263]. Ever since their identification, both mature circulating ECs and CEPCs have been described in several pathological conditions involving vascular injury[261,264-266].

1.3.1 Postnatal vasculogenesis

Vasculogenesis is essential for the establishment of the initial cardiovascular system during early embryonic development and has been considered restricted to embryogenesis, whereas angiogenesis occurs in both the developing embryo and during postnatal life. However, the distinction between vasculogenesis and angiogenesis is far from absolute[267]. Recent observations indicate that vasculogenesis

may not only be restricted to early embryogenesis, but may also be implicated in neovascularisation processes in the adult[268,269]. The existence of postnatal vasculogenesis is supported by the presence of both mature ECs and CEPCs in the circulation. Moreover, CEPCs have been reported to specifically "home" to sites of vascular trauma and tumor growth, where rapid neovascularisation is required[262,270-275]. Revascularisation of injured tissues and neovascularisation of tumors was previously considered to be mediated solely through the recruitment of ECs from adjacent pre-existing blood vessels[1,3]. Today, accumulating evidence strongly suggests that recruitment of BM-derived CEPCs play an essential role in promoting tissue vascularisation after injury as well as in pathological stastes[262,270,274,276].

1.3.2 Selective markers of circulating endothelial precursor cells

It has proven difficult to isolate and characterize CEPCs from the peripheral circulation, mostly due to the lack of specific markers and functional assays that can distinguish CEPCs from mature ECs sloughed from vascular wall structures. CEPCs share many endothelial-specific markers with mature ECs, including VEGFR-2[277], Tie-1[13], Tie-2[278], VE-cadherin[279,280], CD34, and E-selectin. The discrimination between CEPCs and mature EC is further complicated by the fact that subsets of haematopoietic cells also express endothelial markers such as CD34[281], CD31, von Willebrand factor (vWF), and tyrosine kinase receptors such as VEGFR-1[282], VEGFR-2[283], Tie-1, Tie-2[284], and Eph[285].

A novel haematopoietic stem cell marker prominin (CD113) was recently identified. This marker, with unknown function, was found to be expressed on CEPCs but not on mature ECs[286]. Apparently, the expression of CD133 is rapidly down-regulated as haematopoietic progenitors and CEPCs differentiate into more mature postmitotic cells[274,286]. In agreement with this finding, a small population of CD34+ cells co-expressing both VEGFR-2 and CD133 was identified and isolated. Interestingly, isolated populations of CD34+/VEGFR-2/+CD133+ cells proliferate in an anchorage-independent manner *in vitro*, but differentiate into mature adherent CD34+/VEGFR-2+/CD133- ECs when cultured in the presence of VEGF-A and FGF-2 or VEGF-A and stem cell growth factor[272,274]. As mature ECs do not express CD133, it appears that CD34+/VEGFR-2+/CD133+ CEPCs constitute a population phenotypically and functionally distinguishable from circulating mature ECs[272-275,286,287]. At physiological conditions, CD34+/VEGFR-2+/CD133+ CEPCs represent less than 0.01% of circulating mononuclear cells in the peripheral circulation.

Although several lines of evidence clearly indicate that postnatal neovascularisation does not solely rely on angiogenesis, and that CEPCs recruited from the BM contribute to postnatal vasculogenesis[262,271,288,289], it is still a matter of debate to which extent CEPCs contributes to neovascularisation. Several studies have demonstrated that BM-derived CEPCs can integrate into tumor blood vessel endothelium[268,287]. The tumor vasculature is generally characterized by a persistence of immature, rather than maturely differentiated blood vessels. Therefore, postnatal vasculogenesis may actively contribute to the process of neovascularisation through the recruitment of CEPCs, which migrate and differentiate in the tumor stroma[271,288]. Tumor-derived VEGF-A has been shown to be the most potent inducer of mobilization of CEPCs from the BM, and depletion of BM-derived CEPCs retards

tumor growth[287]. In contrast to these reports, others could not define a critical role for BM-derived CEPCs in tumor vascularisation[290,291]. These contradictory findings suggests that the extent to which BM-derived CEPCs contribute to tumor neovascularisation might depend on a plethora of factors, such as tumor type, size, and position, as well as time-point of analysis and choice of markers used for their detection. It is possible that for certain types of cancers, inhibition of the recruitment of circulating CEPCs to the tumor vasculature may constitute an important approach for cancer therapy.

BM-derived CEPCs have the ability to differentiate into mature ECs *in vivo* when cultured in the presence of VEGF-A together with FGF-2 or stem cell growth factor[272,274]. When isolated from the circulation, this cell population co-expresses CD34 and VEGFR-2, two cell surface markers shared by embryonic endothelial progenitor cells[292]. Recently, a population of $CD133^{+}/CD34^{+}$ cells co-expressing the lymphatic endothelial marker VEGFR-3 was identified. These cells appear to define a population of lymphatic endothelial precursor cells. However, it is not known if these cells are able to incorporate into the newly formed lymphatic vessels *in vivo*.

1.4 INTRODUCTION TO CANCER

Cancer is a genetic disease resulting from accumulating gene mutations. Most mutations in a single non-reproductive cell have no effect upon the overall function of an organism, even if they lead to the death of that particular cell. If the mutations, however, result in the failure of the systems controlling replication, recombination, and repair of DNA, a cell with the capacity for uncontrolled growth may be formed[293]. This cell in turn will give rise to a relentlessly growing mass of abnormal cells, what we refer to as a tumor or a neoplasm. As long as the neoplastic cells remain clustered together in a single mass and do not penetrate adjacent tissue borders or spread -*metastasise*- to distant sites, the tumor is defined as benign and will remain as a local overgrowth in the area in which it arose. At this stage, a complete cure can usually be achieved by surgical removal of the neoplasm. In contrast to benign cells, malignant cells have acquired the ability to break loose and invade neighboring tissues. By entering the blood stream or lymphatic vessels malignant cells can metastasise to distant sites where subpopulations of malignant cells take up residence[294]. A tumor that spread to distant sites in the body is almost impossible to eradicate surgically or by localized irradiation, and therefore often becomes deadly.

For a single abnormal cell to give rise to a neoplasm, it must be able to pass on its abnormality to its progeny. Heritable aberrations can be due either to genetic chances, alterations in the DNA sequence, or to *epigenetic* changes, changes in the pattern of gene expression without a change in the DNA sequence. Several lines of evidence indicate that tumorigenesis is a multi-step process[295], in which a succession of genetic and/ or epigenetic alterations, each conferring one or another type of survival and growth advantage, contributes to the progressive conversion of normal cells into

highly malignant cells[296]. For a neoplasm to be successful, the cells within the tumor must acquire a whole range of aberrant properties, and different cancers appear to require different combinations of properties. It was recently suggested that the following six alterations in cell physiology are required to promote the transformation of normal cells into cancer cells; (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) evasion of apoptosis, (4) limitless proliferative potential, (5) capacity to induce angiogenesis, and (6) invasive growth properties and metastatic ability[297]. Thus, many different regulatory systems have to be disrupted before a cell can behave defiantly as a malignant cancer cell. In addition, tumor cells constantly meet new barriers to further expansion. For example, tumors deprived of oxygen and nutrients must promote the formation of new blood vessels before they can grow.

1.4.1 Tumor angiogenesis

Nearly a century ago, the observation was made that tumor growth is accompanied by increased neovascularisation. Mammalian cells require adequate oxygen and nutrition supply, as well as removal of toxic molecules, for metabolism and growth. In 1971 Dr. Folkman proposed that both tumor growth and spread are dependent on angiogenesis[298]. Today, it is widely accepted that in the absence of blood vessels tumors cannot grow beyond the size of a few mm^3 or metastasise to distant organs[5,6,25,32,299]. At the pre-vascular stage, equilibrium is reached between cell proliferation and apoptosis[300], but once vascularised the growth rate of the tumor increases exponentially[6]. Whereas in normal tissues the vasculature maintains quiescent by the dominant influence of endogenous angiogenic inhibitors over pro-angiogenic stimuli, tumors often over-express several angiogenic factors to induce

neovascularisation. As a tumor grows it becomes avascular and hypoxic, resulting in marked production of VEGF-A[20,46]. Tumor-derived angiogenic factors acts in a paracrine manner to recruit blood vessels from the surrounding stroma into the tumor for nutritional support, allowing survival and further growth of the tumor[22]. In addition to stimulating angiogenic sprouting, small avascular masses of tumor cells can co-opt pre-existing host vessels and thereby start off as well vascularised small tumors[82,301]. In response to this inappropriate co-opting, the host vessels usually regress as a defence mechanism trying to shake off the tumor. As the host vessels regress a secondarily avascular and hypoxic tumor is formed, resulting in a marked expression of tumor-derived VEGF-A followed by a new robust angiogenic response. Many naturally occurring tumors initially arise in this manner[1]. If the tumor, however, obtains sufficient blood supply from the pre-existing vasculature in the neighbouring tissue the induction of an angiogenic switch might not be necessary for continuous tumor growth.

1.4.2 Morphological features of tumor blood vessels

Tumor vessels are structurally and functionally abnormal. The vasculature is highly disorganized, consisting of tortuous and dilated vessels, with excessive branching and uneven diameter. Consequently, intratumoral blood flow is chaotic and highly variable[302], resulting in acidic and hypoxic tumor regions[303]. ECs within tumor vessels are irregularly shaped and disorganized, sometimes overlapping each other. In addition, tumor vessels have numerous openings in their walls, widened inter-endothelial junctions, and a discontinuous or even totally absent basement membrane[304]. Cancer cells have also been reported to be located within the walls of tumor vessels, intermingled with neoformed ECs. This event, in which the tumor cells

seem to have gained the ability to build-up blood channels within the tumors, is referred to as "vascular mimicry"[305]. It is worth pointing out that the neovascularisation of a tumor further provides cancer cells with conduits for entry into the circulation, and thus allows metastatic dissemination of invasion-competent cells. Tumor cells can easily penetrate actively growing or newly formed capillaries, providing a ready opportunity for cancer cells to enter the circulatory system and begin the metastatic process. It has previously been reported that nearly one million cells per gram of tumor mass are in the process of shedding into the blood stream daily[306,307].

1.4.3 Control of metastatic tumor growth

As previously mentioned, the switch to an angiogenic phenotype requires a change in the local balance between angiogenic stimulators and inhibitors[1,6,21]. One of the most mysterious aspects of angiogenesis is that a primary tumor will often secrete a substance that inhibits angiogenesis and thereby growth of its metastases. Several inhibitors such as angiostatin, endostatin, and serpin antithrombin have been discovered in association with tumor growth[25,32]. One explanation is given by the following: as a result of their longer half-life, the systemic concentration of angiogenic inhibitors may exceed that of angiogenic stimulators and thereby inhibit growth of distant metastases without affecting the growth of the primary tumor itself, where angiogenic stimulators are constantly maintained at high levels. However, surgical removal of the primary tumor depletes circulating levels of endogenous angiogenesis inhibitors, and in turn promotes the growth of secondary tumors.

1.4.4 Intratumoral lymphatic vessels

In contrast to the extensive studies on tumor-associated angiogenesis, little is known about the mechanisms by which tumor cells gain entry to the lymphatic system. Analysis of the presence and potential function of lymphatic vessels in tumors was previously hampered by a lack of molecular markers to reliably distinguish lymphatics from blood vessels. The lymphatic marker VEGFR-3 was originally thought to be expressed specifically on the lymphatic endothelium, but further studies demonstrated that in the neovasculatures of tumors VEGFR-3 is up-regulated[308]. The recent discovery of improved lymphatic-specific markers such as prox-1, podoplanin, and LYVE-1, along with the establishment of techniques for isolation of LEC has made it possible to study the formation of tumor-associated lymphatic vessels, and to investigate their contribution to lymphatic metastasis. Although it is known that induction of tumor angiogenesis is a complex process involving the interplay of a dozen tumor-derived growth factors, it is poorly understood how tumors induce lymphangiogenesis and what growth factors are critical for the process.

The two ligands for VEGFR-3, VEGF-C and VEGF-D, are by far the best characterized lymphangiogenic growth factors[40,41]. Both VEGF-C and VEGF-D induce proliferation and migration of LECs *in vitro* and lymphangiogenesis *in vivo*[213,232]. Recent work using experimental xenotransplanted tumor models in animals have provided fundamental evidence that increased levels of VEGF-C and VEGF-D induce tumor lymphangiogenesis and promote the formation of lymph node metastasis[223,228,229,309,310]. Both tumor and stromal cells can contribute to the expression of VEGF-C and VEGF-D, suggesting the existence of close tumor cell-host interactions[228]. Inhibitors of the VEGF-C/-D/VEGFR-3 signalling pathway may have the potential to block lymphatic tumor spread[311].

An increasing number of clinopathological studies have recently described a direct correlation between intratumoral VEGF-C expression, tumor lymphangiogenesis, and the formation of lymph node metastases in a variety of human tumors, including thyroid[312], head and neck[241], oesophageal[313], breast[223], lung[314], prostate[315], endometrial[316], cervical[317], gastric[318], and colorectal carcinomas[319]. Less is known about the expression patterns of VEGF-D in human tumors. VEGF-D expression correlates with metastasis in some tumors[229,320], but demonstrates an inverse correlation with metastasis in others[314]. The diverse clinical outcome associated with VEGF-D expression could be due, at least in part, to differences in the degree of proteolytic processing of the growth factor within different tumors, or simply due to expression of other unknown lymphangiogenic stimulators within the same tumor. In addition to VEGF-C and VEGF-D, other growth factors have also been shown to participate in the molecular mechanism of lymphangiogenesis, including FGF-2[235], VEGF-A[200,233], Ang-1[321], Ang-2[322], HGF[323], ephrinB2[239], and members of the PDGF-[242] and IGF-families[324]. In this thesis, the role of three of these factors/families will be presented in more detail, namely VEGF-A, PDGFs, and IGFs.

1.4.5 Metastatic spread

1.4.5.1 Invasion

The ability to escape from the parent tissue and to survive and grow in a foreign tissue at a distant site in the body are key properties that cancer cells must acquire in order to become metastatic. The invasion of tumor cells into the surrounding tissue and subsequent formation of metastasis defines malignancy and is the principal cause of death in cancer. Malignant cells can spread through direct invasion of neighboring tissues, but the two major routes of metastatic spread are *via* blood- and lymphatic

vessels[6,179,180]. All cells of an intact tissue are surrounded by an ECM consisting of a complex meshwork of connective tissue molecules, including fibrillar collagens, basement membrane molecules, connective glycoproteins, and proteoglycans. Basement membranes constitute highly organized structures of the ECM that form molecular barriers preventing movement of cells from one compartment to another. A growing tumor induces alterations in the surrounding stromal ECM, often resulting in a fibroproliferative response, characterized by the expression of type I and type III pro-collagens[325]. Formation of a tumor stroma can be viewed as a non-specific host attempt to isolate the tumor, and is thought to have a negative influence on tumor progression and invasion. During the process of metastasis, malignant cells of a typical solid tumor must detach from the primary tumor mass, penetrate the basement membrane underlying the tumor, enter a blood- or lymphatic vessel, survive and arrest within the circulating blood or lymph, extravasate, and survive and grow as a metastatic leison in the new tissue (Figure 4). The entire metastatic process from the initial escape of tumor cells from the tissue of origin to the growth in a distant site requires a number of different properties of the malignant cells.

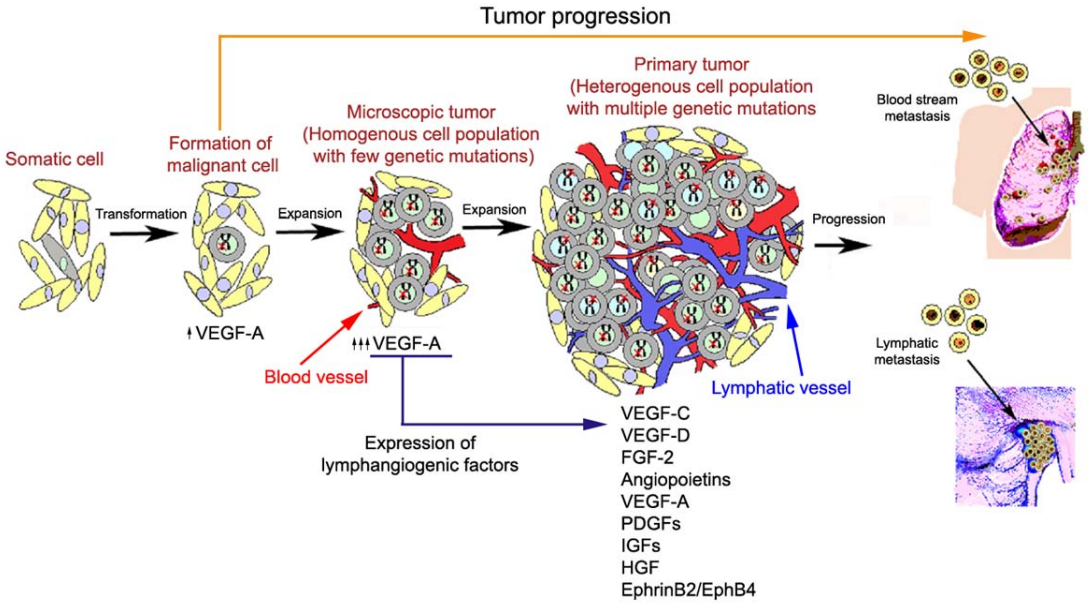

Figure 4. *The multi-step process of tumorogenesis, strating with a single genetic event and ending with the spread of invasive tumor cells via the vascular- or lymphatic system.*

1.4.5.2 Detachment from the primary tumor

For a cancer cell to metastasise, it must first detach from the parent tumor and subsequently invade neighboring tissues. Although invasiveness is not thoroughly understood, it almost certainly requires a disruption of the adhesive mechanisms that normally keep cells tethered to their neighboring host cells as well as to molecules of the ECM. Several classes of surface molecules, including integrins and cadherins, and their ligands mediate these interactions. Integrins are transmembrane adhesion receptors that mediate attachment of cells to a variety of adhesive molecules in the ECM[326], whereas cadherins are intercellular adhesion receptors required for maintenance of tissue architecture[327]. Further, the restriction of a normal cell type to a given organ or tissue is maintained by cell-to-cell recognition and by physical barriers such as the basement membrane, which underlies layers of epithelial cells as well as surrounds the ECs of blood vessels. Cancers cells are usually less stringently

regulated by cell-to-cell- and cell-to-matrix interactions than normal cells and can also sometimes manage to overcome constraints on cell movement provided by the basement membrane and other barriers. Most cancer cells down-regulate the expression of cell surface adhesion molecules, making the cells less adhesive than normal cells[328,329]. In addition, tumor cells often produce elevated levels of cell-surface receptors specific for components of the basement membrane, such as collagens, proteoglycans, and glycosaminoglycans[330]. A great deal of research remains to be done regarding the signaling pathways that regulate cell invasion *in vivo*. The Rho family GTPases Rac, Rho, and Cdc42 appears to function as molecular switches that transduce intracellular signals regulating critical cell functions during an invasion of cells, including gene expression, adhesion, migration, and invasion[331].

1.4.5.3 Proteolytic enzymes and tumor progression

Cell invasion requires controlled proteolytic activity and degradation of ECM components. Malignant cells generally secrete a variety of proteases, including serine-, cysteine-, threonine-, aspartic-, and metalloproteinases that together cooperate to digest matrix proteins. Some metalloproteases, such as the *collagenases*, appears to be an important determinant of the ability of cancer cells to digest and penetrate through basement membrane to invade underlying connective tissue[332]. Many proteases are secreted as inactive precursors that can be activated locally when needed. For example, plasminogen is an inactive circulating protein that is locally cleaved by tumor secreted plasminogen activator (PA), converting it to the active protease plasmin[333]. Cell surface receptors that bind proteases have been identified at the leading edge of some migrating cancer cells. Expression of these receptors helps confining the enzyme to the sites where they are needed to clear a pathway for the migrating cancer cell. The proteolysis of matrix proteins contributes to cell

detachment and promotes cell invasion by simply clearing a path through the ECM. Digestion of the ECM can also expose cryptic sites on the cleaved proteins that promote cell binding, cell migration, or both, as well as a release of sequestered growth factors that stimulate cell migration. The action of proteases is further confined to specific areas by various secreted protease inhibitors, including the tissue inhibitors of metalloproteases (TIMPs) and the serine protease inhibitors known as serpins[334,335]. Protease inhibitors have the potential to inhibit angiogenesis through their mechanisms of action[336].

1.4.5.4 Establishment of a secondary tumor.

A molecular understanding of the metastatic spread of tumors is of greatest importance for the development of successful strategies to treat cancer. Although the mechanisms of tumor cell dissemination are not fully understood, metastatic spread is most likely not a random process[337]. To metastasise successfully, a cancer cell must be able to cross the basal lamina and the endothelial lining of a blood- or lymphatic vessel to enter the circulation. The site of initial detachment will most likely influence whether the route of spread is *via* the lymphatic- or vascular system. The importance of lymphatic vessels in tumor spread is beyond doubt. For many tumors the regional lymph nodes are often the first sites to develop metastases. Intra-lymphatic tumor cells can also pass directly into the blood vascular system *via* lymphatic-venous shunts or by drainage through the thoracic duct[178,180]. Thus it is not always easy based on primary tumor site to predict the localization of its potential metastases.

Tumor cells can enter the circulation by penetrating through proliferating capillaries that have fragmented basement membranes and are leaky. During the process of intravasation tumor cells secrete angiogenic factors that induce proliferation of ECs. Upon their activation, ECs increase their production of proteolytic enzymes, such as PA and collagenases, which further contribute to the degradation of the basement membrane and thus facilitates the entry of tumor cells into the circulation[338]. When leaving the vascular system the cancer cells extravasate as single cells by adhesion to and spreading along the vessel wall[339], often using pseudopodial projections to migrate across an EC lining of a capillary, and then move into the underlying tissue without disrupting the microcirculation. To survive in the new environment in which it finds itself, a tumor cell must be able to multiply in the absence of a surrounding mass of identical cells and establish cell-to-cell interaction with host tissue stromal cells and cell-to-matrix interactions with components of the local ECM. Interestingly, many cancer cells can persist for a long time as dormant cells, neither dividing nor undergoing apoptosis. Actually, only a small fraction of extravasated tumor cells ever begin to divide to form micrometastases, and only a very small fraction of these micrometastases will continue to grow to form tumors[340].

Tumor metastasis is the most difficult aspect of cancer to tackle from a therapeutic standpoint. A thorough understanding of the mechanism of metastatic spread is therefore critical if any progress towards its control is to be made. The complex multi-step nature of the process may explain why metastasis is a highly inefficient phenomenon, with less than 0.1% tumors cells estimated to be able to overcome all the obstacles and survive to form new colonies[341]. In recent years, a large body of research has helped to elucidate many critical molecular changes that endow tumor cells with metastatic properties. The mechanism determining whether regional lymph

nodes or other sites will develop metastases remains poorly understood. However, most disseminated tumor cells have a limited life span and only a few cells manage to develop into clinically detectable micrometastases. Nevertheless, identification of those occult tumor cells, and prevention of their growth and spread would be of great clinical significance.

2 AIMS

The general aim of the studies presented in this thesis work was to increase our understanding of the molecular mechanisms of lymphangiogenesis. In particular, we aimed to investigate the role of tumoral lymphangiogenesis in promoting lymphatic metastasis.

The specific aims were:

I. To identify novel lymphangiogenic factors (Paper I-III) and study their potential role in inducing lymphatic tumor spread (Paper I and II).

II. To investigated the role of bone marrow-derived circulating endothelial precursor cells in promoting lymphangiogenesis (Paper IV).

3 METHODS

Angiogenesis is a complex multi-step process that involve critical events such as proteolytic degradation of the ECM, directed migration of ECs, proliferation of ECs, deposition of a new ECM, and formation of vascular tubules[3,22]. The principal cells involved are ECs whose migration, proliferation, differentiation, and structural rearrangement into tubules are central for the angiogenic process. It is important, however, to remember that these are far from the only cells involved in angiogenesis. Supporting cells, circulating inflammatory cells, and components of the ECM all play important roles. One of the major problems in the research field of angiogenesis is to select appropriate methods for assessing the angiogenic process. Today, no *in vitro* assay exist that can be readily translated into *in vivo* effects on angiogenesis due to its complex nature. Although angiogenesis has been axtensively studied, lymphangiogenesis is a relatively new field of vascular biology. The mechanisms och lymphangiogenesis are not well-characterized, but the methods used for studying angiogenesis can be adopted for studies of the lymphangiogenic process.

In vitro assays are practical for the study of specific cellular functions, such as migration, proliferation, and tubule formation, or for screening of large numbers of potentially inhibitory or stimulatory compounds. Although these assays are rapid, reproducible, and easy to quantify, they do not permit study of the complex physiological interactions occurring *in vivo*. When evaluating the effect of different compounds and factors on EC proliferation, migration, and structural rearrangements one must remember that the response may vary with the source of ECs. In addition, primary ECs are difficult to maintain in culture, thus agents inhibiting EC properties *in vitro* may do so for reasons that have no relevance to angiogenesis *in vivo*. There are also a number of cytotoxic compounds whose effects are not specific to ECs, and thus are unlikely to selectively block angiogenesis. Further, *in vitro* systems do not take the marked diversity among ECs of different origin into account and fail to identify indirect angiogenic effects of a compound. Thus, *in vitro* assays should be performed using ECs from several sources, and must always be followed by *in vivo* studies.

3.1 IN VITRO ASSAYS

3.1.1 Cell proliferation assay

ECs are normally quiescent in adult blood vessels whereas established EC lines in culture have gained the ability of cell division. Proliferating ECs undergo significant changes after prolonged culture, including alterations in activation state, karyotype, growth properties, and expression patterns of cell surface antigens. It is generally not feasible to use only primary ECs that have not been expanded *in vitro* although that would be the optimal. Today, there are a number of highly reproducible and quantitative cell proliferation assays available. Net cell number can easily be measured by using an electronic counter such as the Coulter counter. In Paper I-III, we have used this assay to investigate the role of several growth factors, including

VEGF-C, PDGF-BB, IGF-1, and IGF-2, on proliferation of primary human and mouse LECs. Further, we have assayed the *in vitro* growth properties of a fibrosarcoma cell line transfected with different growth factors, such as VEGF-A, VEGF-C, and PDGF-BB (Paper I and II). Another method is to quantify DNA synthesis as a measure of proliferation, which can easily be done by the thymidine incorporation assay. Alternatively, proliferation can be assayed using DNA-binding molecules coupled with flow cytometric analysis. This method has the advantage of measuring both proliferating and apoptotic cells[342].

3.1.2 Morphological changes

During angiogenesis the phenotype of activated ECs changes in a reversible manner from a resting to a sprouting state. Following activation, ECs adopt an elongated migratory phenotype through a process involving actin rearrangements. *In vitro*, these morphological changes can be seen following stimulation of ECs with various growth factors. Porcine aortic endothelial cells expressing VEGFR-3 (PAE-R3) are strongly activated by recombinant VEGF-C treatment. In paper I and II, we evaluated the ability of two antagonists of the VEGF-C/-D/VEGFR-3 pathway, an anti-VEGFR-3 antibody and a soluble VEGFR-3-Fc, to block morphological changes induced by VEGF-C. Actin filaments of PAE cells treated with VEGF-C alone or in combination with one of the two antagonists were stained with TRITC-phalloidin and elongated ECs were visualised using a light/fluorescence microscope.

3.1.3 Chemotaxis assay

Angiogenesis and lymphangiogenesis involve a co-ordinated migration of several cell types, including ECs, pericytes, and stromal fibroblasts. Both processes are regulated by interactions between ECs, soluble factors, and components of the ECM. During vessel sprouting ECs move towards a gradient of angiogenic stimulus. Cell migration is most frequently assayed using a modified Boyden chamber[343]. In this method, ECs are plated on top of a membrane coated with a matrix protein and permitted to migrate across the membrane in response to an angiogenic factor placed in the lower chamber. A major advantage with this assay is that it allows a distinction to be made between *chemotaxis*, directed cell movement towards a concentration gradient of a soluble factor, and *chemokinesis*, random cell movement in response to an isotropic distribution of a soluble growth factor. The system is very sensitive to small differences in concentration gradients, and thus may well reflectconditions *in vivo*. We have used the modified Boyden chamber in Paper I-III to study migration of primary human and mouse LECs in response to factors such as VEGF-C, PDGF-BB, IGF-1, and IGF-2. We have also evaluated the ability of an anti-VEGFR-3 antibody and a soluble VEGFR-3-Fc to block VEGF-C-induced migration of PAE-R3 cells. In another migratory assay, ECs are cultured in monolayer to confluence. By using a scraping tool, a part of the monolayer is "wounded", cleared of ECs. This creates a margin from which ECs migrate back to "heal", re-form, the monolayer. This assay does not distinct between chemotaxis and chemokinesis, but is considered to represent one aspect of wound healing[344]. In paper I, this assay was used to study the migratory potential of PDGF-BB on primary rat LECs.

3.1.4 Intracellular signalling

Cell growth, differentiation, and survival are triggered by the recognition of extracellular signals at the cell surface. The specific way that a cell reacts to it environment depends both on the set of receptor proteins that the cell expresses as well as its intracellular machinery by which the cell integrates and interprets the information that it receives. Several signalling molecules work in concert to regulate the behaviour of the cell, and many cells require multiple signals only to survive and additional signals to proliferate or carry on some more specialized function. If deprived of the appropriate signals, a cell will undergo programmed cell death. Upon ligand binding, a conformational alteration in the extracellular domain results in receptor dimerization and trans-phosphorylation. In turn, this results in the activation of linked cytoplasmic and nuclear signalling cascades activating gene transcription. In many cases the transcriptional response is divided into two steps where the first is the direct induction of the transcription of a small number of specific genes, known as the primary response. Some of these early gene products activate expression of the secondary response genes, whereas others turn off the primary response genes.

All eukaryotic cells possess multiple Mitogen-activated protein kinase (MAPK) pathways, which co-ordinately regulate several diverse cellular activities such as gene expression, mitosis, motility, survival and apoptosis, and differentiation. Convincing evidence has linked cell proliferation to activation of Extracellular regulated kinase-1 and -2 (ERKs), which are members of the MAPK family[345]. Once activated, ERK causes activation of specific mitogenic transcription factors such as c-fos and c-myc. The Src kinase family consists of nine structurally related cytoplasmic tyrosine kinases containing SH2, SH3, and catalytic domains. The Src family kinases were initially implicated in mitogenic signalling through receptor tyrosine kinases such as those for PDGF[346]. More recently, Src activation has been linked to additional processes such as cell adhesion, motility, carcinogenesis, and immune cell function. PKB/Akt is activated in response to a wide variety of growth factors, including PDGFs, insulin, IGF-1, IGF-2, EGF, and FGFs[347,348]. Several research groups have independently identified PKB/Akt as a critical regulator of cell survival[349]. We have studied intracellular signalling cascades in primary LECs in response to several growth factors, including PDGF-BB, PDGF-AA, VEGF-C (paper I), IGF-1, and IGF-2 (paper II).

3.1.5 Polymerase chain reaction

The polymerase chain reaction technique (PCR) was invented by Mullis in the mid-1980s[350], and is based on enzymatic amplification of a specific target DNA sequence. The genetic material used for a PCR is often total genomic DNA extracted from cells or tissues. A PCR can, however, be used to study gene expression. For this purpose, mRNA is converted by the enzyme reverse transcriptase into cDNA, which serves as the template for the PCR. By using reverse transcriptase (RT)-PCR, mRNA from a single cell can be quantified, making this system far more sensitive than other commonly used methods for mRNA quantification, such as Northern blot and RNase protection assay. In paper I and III, RT-PCR was performed to amplify PDGFR-α and PDGFR-β, as well as IGF-1R and IGF-2R using cDNA prepared from human and mouse LECs.

A new method, Real-Time PCR, have been developed that reflects the initial amount of target cDNA. Real-Time PCR is also based on the principles of reverse transcriptase and is today the most sensitive and reproducible technique available for detection and quantification of mRNA. The concept of Real-Time PCR is very similar to that of standard PCR-reactions, with the major difference being the incorporation of a fluorescent probe for detection of the PCR-product. The system is based on quantification of a fluorescent signal emitted from the molecular probe when bound to dsDNA. The emission increases in a direct proportion to the amount of PCR-product formed. In a typical reaction, the PCR-product is amplified exponentially, and can be quantified by generating a standard curve from RNA of known concentration or simply relative to an internal control gene, such as actin and GAPDH mRNA, or preferentially 18S rRNA. Real-Time PCR was used in paper I to analyse if PDGF-BB up-regulates the expression levels of Prox-1, a transcription factor involved in the development and differentiation of LECs, in primary mouse LECs. Moreover. the method was also used to investigate whether PDGF-BB induces lymphangiogenesis *via* upregulation of Ang-2, a Tie-2 receptor ligand that has recently been shown to induce lymphagiogenesis. Expression of Ang-2 in murine melanoma B16 cells, which are known to express PDGFRs, was analysed post treatment with PDGF-BB. Specific primers and FAM-labelled probes for Real-Time PCR were designed and the reactions were performed in multiplex using the 7700 Sequence detection system by Perkin Elmer. As an internal loading control 18S ribosomal RNA was used.

3.1.6 Microarray

Microarray analysis has become a widely used technique for the study of gene-expression patterns on a genomic scale. Traditional methods in molecular biology generally focus on one gene at a time, thus giving very limited information about gene function. The microarray technology, however, has the potential of monitoring the whole genome on a single chip and can therefore provide us with a more comprehensive picture of the interactions occurring among thousands of genes simultaneously. The underlining principle of DNA microarray is base-pairing (A-T and GC for DNA; A-U and G-C for RNA) or hybridization. The two major application fields for the microarray technology are identification of genomic sequences and determination of gene expression levels. In paper III, we used the Affymetrix GeneChip microarray analysis to detect IGFBP- and IGFR transcripts in primary human and mouse LECs.

3.1.7 Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) is a flow sorting technique used to isolate individual cells or cellular subpopulations from a heterogeneous cell mixture. Flow cytometers analyses the physical and chemical properties of cells or particles as they travel one by one in a moving fluid stream past a sensing point, a fixed argon-laser beam. As a single cell interrupts the laser beam, it produces a scattering of light from the beam. The scattered and fluorescent light generated is collected by photo-detectors, which convert the photon pulses into electronic signals relayed to a computer for interpretation of the result. The system is designed to identify, calculate

and sort cells possessing pre-selected properties. When analysing a heterogenous suspension the cytometer will ignore the cells or particles whose light scatter characteristics do not meet the previously defined parameters. Up to six parameters can be measured simultaneously using a flow cytometry system, including forward scatter (size), side scatter (granularity), and four fluorescence parameters (using a four-colour immunofluorescence system). Other measurable properties are volume, contents of DNA, RNA and enzymes, and also surface antigens[351,352]. To study the role of specific BM-derived subpopulations of CEPCs in postnatal lymphangiogenesis *in vivo*, we established chimeric mice reconstituted with $EGFP^+$ sorted BM cells (Paper IV). In Brief, femoral BM cells were collected by flushing femur and tibiae from $EGFP^+$ donor mice. Cells were washed carefully and suspended in FACS-buffer to obtain a single-cell suspension before staining with antibodies directed against CD34, VEGFR-2, and VEGFR-3. Sorting of different subpopulations of CEPCs was performed using a FACS sorter.

3.2 IN VIVO ASSAYS

3.2.1 Mouse corneal neovascularisation assay

Gimbrone et al first described the cornea micropocket assay in 1974 for use in rabbits[353]. Further, the assay has been performed in both rat-[354] and mouse cornea[355]. The assay is based on the implantation of an angiogenic inducer, for example a growth factor or a piece of tumor tissue, into a corneal micropocket in order to elicit a vascular response. As the cornea is an avascular tissue, any vessels penetrating from the peripheral limbal vasculature into the corneal stroma can be identified as newly formed vessels[353-355]. The assay allows repeated non-invasive measurement of corneal vascularisation, which is useful when evaluating the stimulatory or inhibitor potential of a compound. Compared to the chicken chorioallantoic membrane assay, the mouse corneal assay is more expensive and far more technically demanding, making it unsuitable for large-scale screening. Although the usage of mice instead of rabbits and rats make the assay less expensive and increases the number of tests that can be performed, the surgery becomes far more complicated as the size of the eye decreases. The mouse corneal assay, however, offers several advantages, such as the availability of a large number of well-defined mouse strains, including transgenic- and knockout mice, and the wide variety of commercial reagents established for mice. Lastly, systemic treatment of mice requires only a small amount of drugs[355].

The Mouse corneal neovascularisation assay presented in paper I-IV was performed according to procedures previously described[356]. Briefly, slow-release polymers containing the angiogenic substances were implanted into corneal micropockets. The vascular response, measured as vessel length, vessel area, and clock hours of circumferential neovascularisation, was scored at day 5, 14, and 25 after implantation by using a slit-lamp biomicroscope. By staining corneal whole-mount preparations with antibodies directed against CD31 and LYVE-1 we could visualise the angiogenic- and lymphangiogenic responses induced by various growth factors, including members of the VEGF-, PDGF-, IGF- and FGF families. Further, we have used the mouse corneal tumor model, described by Muthukkaruppan in 1982[357], to study the growth of intratumoral blood- and lymphatic vessels (Paper I and II). In this

assay, a piece of freshly dissected microscopic tumor tissue was surgically implanted into the micropocket. The eyes were examined with a slit-lamp biomicroscope on day 14 after tumor implantation, photographed, and enucleated for histological examination.

3.2.2 Mouse tumor and metastasis model

Many different *in vivo* tumor models have been established in mice to investigate the effect of potential anti-cancer therapies. Tumors can be grown syngeneically or orthotopically in syngenic mice, or as xenografts in immodeficient mice, with tumor size and animal survival reflecting the effect of the test substance. In the absence of angiogenesis tumors will not grow beyond the size of a few cubic millimeters. Therefore, tumor models can also be used to examine the uptake and distribution of an anti-angiogenic drug, as well as its efficacy. A number of histological techniques can be used to investigate the effect of a drug candidate on tumor angiogenesis and growth, such as H&E staining for general morphology, CD31- or CD34 staining for microvessel density, and proliferating cell nuclear antigen (PCNA)- or TUNEL staining for tumor cells proliferation or apoptosis. When evaluating an anti-angiogenic drug as a potential anti-cancer treatment, it is important to determine whether the drug is *anti-angiogenic*, will only inhibit the growth of new vessels to the tumor, or *anti-vascular*, will damage pre-existing blood vessels and therefore potentially cause side effects, in its mode of action. In paper I and II, tumors were grown syngeneically to study the role of VEGF-A, VEGF-C, or PDGF-BB on tumor growth, as well as tumor-associated angiogenesis and lymphangiogenesis. A T241 fibrosarcoma cell line was transfected with VEGF-A, VEGF-C, or PDGF-BB cDNA according to a stepwise procedure previously described[358]. Green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria*[359] was used as a reporter protein and positive transfectants were sorted using a FACStar+ (Becton Dickinson, San Jose, CA). Tumor cells were implanted s.c. in the middle dorsum of mice, and tumor volumes were measured as previously reported[358]. Tumors grown close to the ethical size limit (1.5 cm^3) were surgically removed for histological analysis. Approximately 4-6 weeks post surgery metastasised GFP-positive tumor cells were detected in the brachial lymph nodes of VEGF-A, VEGF-C, and PDGF-BB tumor-bearing mice. Conditions of low oxygen, which often exists in both experimental and clinical tumors, affect GFP gene expression. Thus, the GFP marker is optimal for the study of tumor cell spread but less suited as a reporter gene under hypoxic tumor conditions. To further support our results we transduced VEGF-A-T241 tumor cells with a lentivirus expressing the *renilla luciferase* (RL) reporter gene[360]. Transduced cells were transplanted s.c. in the upper right back of mice. Post implantation, mice were imaged weekly using an optical imaging system. To detect secondary tumors in the regional lymph nodes, brachial lymph nodes were dissected at various time-points and imaged ex vivo for presence of RL signals (Paper II).

3.2.3 Radiation and BM-transplantation

To investigate whether BM-derived CEPCs contributes to postnatal lymphangiogenesis, depleted BM of irradiated mice was reconstituted with $EGFP^+$ whole BM from donor mice. Prior to transplantation, recipient mice were irradiated by a sub lethal dose of 900 rad. Femoral BM cells were injected intravenously immediately after irradiation. To further specify what subpopulations of CEPCs that incorporate into the newly formed lymphatic vessel wall, sorted populations of $EGFP^+/CD34^+/VEGFR\text{-}2^+$ or $EGFP^+/CD34^+/VEGFR\text{-}3^+$ cells were transplanted to irradiated recipient mice (Paper IV).

3.3 HISTOLOGICAL TECHNIQUES

3.3.1 In situ hybridization

Today, pathological diagnosis of human tumors is in general based on conventional morphological and histopathological features. Human cancers are pathological diverse and vary in their responsiveness to clinical treatment, which is at least in part due to variations in cellular gene expression patterns. Cellular mRNA levels are determined by the rate at which mRNA precursors are synthesised and processed in the nucleus, and by the rates of nuclear export and cytoplasmic degradation. Gene expression profiling does not only complement conventional histopathology, but also helps to identify potential drug targets and predict clinical outcome and response to therapy. Genomic instability of tumor cells leads to expression of multiple angiogenic- and lymphangiogenic factors[361]. *In situ* hybridization is a method used for mRNA expression analysis in cryopreserved histological samples. In paper I, *in situ* hybridization was used in combination with conventional immunohistochemistry in order to analyse the expression of PDGFR-α and –β on lymphatic vessels present in corneal tissue. The hybridization was performed by using two independent oligo-probes complementary to PDGFR-α (nucleotides 423-470 and 3083-3130) and PDGFR-β (946–996 and 2610-2657) according to a procedure recently published[356]. For immunohistochemistry, the same sections were stained with a rabbit anti-mouse LYVE-1 antibody, and positive signals were developed with a streptavidin peroxidase kit combined with a DAB peroxidase substrate. According to the same procedure we also analysed the expression of VEGF-C mRNA in tumor tissues (Paper II) by using an oligo-probe complementary to VEGF-C (nucleotides 536-585).

3.3.2 Immunohistochemistry

Immunohistochemistry provides an alternative method to *in situ* hybridization for studying gene expression by direct microscopic observations of tissue sections *in situ*. The main distinction is that, whereas with hybridization probing the target gene itself is directly identified, an immunological method instead detects the protein coded by the gene of interest. The principle of immunohistochemistry is to use a primary antibody produced against a specific cellular constituent, an antigen, as a tool for detection of this specific antigen in a tissue. A primary antibody is isolated and

purified from the blood of an animal inoculated with a specific antigen of interest. This antibody can in turn be used as an antigen in another animal for production of a secondary antibody. The primary antibody or, more common, the secondary antibody can be directly conjugated with a marker molecule, such as a fluorescent or chemiluminescent label, to allow detection of the target protein. The method is quick and more than one antigen can be revealed on the same section.

In the field of angiogenesis, immunohistochemistry offers a plethora of applications, such as identification of ECs, grading of vessel density, quantification of EC proliferation or apoptosis in correlation with treatment with potent angiogenic inducers or inhibitors, as well as analysis of vessel maturity and structure. Since the endothelium is a highly heterogeneous tissue[5,167,168], the choice of primary antibody can profoundly influence the number of vessels available for detection. Many vascular endothelial markers, such as those directed against vimentin, lectin, and type IV collagen, suffer from low specificity and are further expressed on many non-endothelial structures as well. More specific markers have been developed over the years, including CD31, CD34, VEGFR-1, VEGFR-2, factor VIII related antigen, E-selectin, and von Willebrand Factor (vWF). The most specific and sensitive vascular endothelial marker currently available is CD31, which is constitutively expressed on the surface of most vascular ECs. The marker is also to a lesser extent expressed on LECs, platelets, granulocytes, and a sub-set of CD8+ lymphocytes. CD34 is a good alternative antigen to CD31, although some stromal cells also express this vascular endothelial marker. Until recently, the process of lymphangiogenesis has been difficult to investigate simply due to a lack of markers that discriminate between lymphatic- and blood vessels. This, however, came to a change with the discovery of improved markers such as prox-1[182], podoplanin[183], LYVE-1[184], and VEGFR-3[181].

In this thesis work we have mainly used a combination of CD31 and LYVE-1 antibodies to detect blood- and lymphatic vessels in mouse corneas (paper 1-IV) and tumor tissues (paper I, II and IV). To further confirm their identity as blood- and lymphatic vessels we have co-stained the vascular structures with other specific markers such as CD34 for blood vessels and prox-1, podoplanin, and VEGFR-3 for lymphatic vessels. We have further studied expression patterns of PDGFRs in cryosections of PDGF-BB implanted corneas (Paper I), as well as infiltration of macrophages in VEGF-A- and VEGF-C-over-expressing fibrosarcomas (Paper II).

3.3.3 Confocal microscopy of whole-mount specimens

Laser Scanning Confocal Microscopy (LSCM) is an advanced microscopic technique routinely used for generating high resolution images and three-dimensional reconstructions of a variety of biological specimens. In this microscopic technique, which is based on point scanning principles, a laser beam is expanded through reflection off a dichroic mirror in order to optimise the use of the optics in the objective. Subsequently, the laser beam hits two scanning mirrors, which directs the laser across the sample and the point of illumination in the specimen is brought to focus by the objective lens. A mixture of reflected light and emitted fluorescent light is captured by the same objective and passed on by the two scanning mirrors in the direction of the photo detector. Only emitted fluorescent light passes through the dichroic mirror and is focused onto the pinhole, an aperture placed in front of the photo detector. The confocal pinhole obstructs fluorescent light from points of the

specimen outside of the focal plane, which results in efficient rejection of out-of-focus information. Stacking many thin 2-D optical sections collected in series generates a very clean three-dimensional reconstruction of a specimen. We have used the Zeiss LSM 510 system to generate high-resolution images of blood- and lymphatic vessels in corneal (paper I-IV) and tumor (paper I, II and IV) whole-mount samples.

4 RESULTS

4.1 THE ROLE OF PDGF-BB IN PROMOTING TUMOR LYMPHANGIOGENESIS AND LYMPHATIC METASTASIS (PAPER I)

Members of the PDGF family have been reported at high expression levels in tumors, especially in carcinomas of the breast, and have been suggested to contribute to their progression and spread[362,363]. We alongside with other groups have previously shown that members of the PDGF family are potent angiogenic factors[152-155]. Together, these findings raised the questions whether PDGFs are lymphangiogenic factors and whether PDGFs may contribute to pathological lymphangiogenesis.

To address the question whether members of the PDGF family might be involved in lymphangiogenesis, we established a corneal lymphangiogenesis assay. In Brief, PDGF-AA, PDGF-AB, or PDGF-BB was implanted into the mouse cornea as previously described[356]. At day 5, 14, and 25 post implantation the corneas were dissected from enuclated eyes and stained with a mixture of CD31- and LYVE-1 antibodies. Interestingly, all three factors evaluated in this experiment induced lymphangiogenesis in the cornea, with PDGF-BB being be most potent inducer. Due to its high lymphangiogenic activity, we decided to mainly focus on PDGF-BB during the rest of our study. PDGF-BB stimulated the growth of LYVE-1 positive lymphatic vessels of larger diameter at an almost equal efficiency as FGF-2, which was used as a positive control. In contrast, implantation of the negative control, the slow-release polymer soaked in PBS, did not induce angiogenesis or lymphangiogenesis, and the only vessels detected in these corneas were the pre-existing lymphatics of the limbus. Lymphatic vessels induced by PDGF-BB were

readily detectable at day 5 after implantation, and a maximal lymphangiogenic response was visualised at day 14 after implantation. At this time-point, the primitive newly formed lymphatic capillaries detected at day 5 after implantation had undergone remarkable remodeling into large vascular tree-like structures. These findings demonstrated that PDGF-BB is indeed a potent lymphangiogenic factor *in vivo*.

The LYVE-1 positive corneal lymphatics detected in this assay were also positive for other lymphatic markers such as VEGFR-3 and podoplanin, but generally lacked or exhibited only a weak expression of the vascular marker CD31. Moreover, double staining with LYVE-1 and the highly specific vascular endothelial marker CD34 revealed that LYVE-1 was not detected on CD34-expressing blood vessels, which further confirmed their identity as lymphatic vessels.

Knowing that PDGF-BB induces lymphangiogenesis, the next step in our study was to investigate the molecular mechanisms of PDGF-BB-induced lymphangiogenesis. We hypothesized that PDGF-BB might induce lymphangiogenesis indirectly, similar to FGF-2, *via* activation of VEGFR-3. To investigate this possibility, we evaluated the ability of a neutralizing VEGFR-3 antibody and a soluble VEGFR-3-Fc, both of which efficiently block VEGF-C-induced EC activities *in vitro*, to block PDGF-BB-induced lymphangiogenesis in the corneal lymphangiogenesis model. Corneas were co-implanted with PDGF-BB or VEGF-C alone or in combination with either of these neutralizing compounds. As expected, both neutralizing agents efficiently blocked VEGF-C-induced lymphangiogenesis. In contrast, neither of these blocking compounds had any effects on either PDGF-BB induced angiogenesis nor

lymphangiogenesis, suggesting that PDGF-BB induces lymphangiogenesis independently of the VEGF-C/-D/VEGFR-3 pathway, at least in this model system.

The fact that PDGF-BB-induced lymphangiogenesis is not mediated *via* the VEGF-C/-D/VEGFR-3 pathway suggested to us that PDGF-BB might act as a direct lymphangiogenic factor. Indeed, PDGF-BB directly induced migration of primary human and mouse LECs and activated various intracellular signaling pathways, such as the MAP kinases *Erk*1/2 and *Akt* in the very same cells. Further, we managed to localize the expression of PDGFR-α and PDGFR-β, both mRNA and proteins, in primary LECs *in vitro* by performing RT-PCR and Affimetrix gene array analysis, as well as on newly formed lymphatic vessels *in vivo* by performing in situ hybridization and conventional immunohistochemistry. These findings strongly suggest that members of the PDGF family act as direct lymphangiogenic factors.

PDGFs have been reported over-expressed in various tumors, we studied the effect of PDGF-BB in promoting tumor growth, as well as tumor lymphangiogenesis and lymphatic tumor spread. For this purpose, we established a fibrosarcoma cell line over-expressing PDGF-BB (T241-PDGF-BB), as well as a positive control over-expressing VEGF-C (T241-VEGF-C). Implantation of either of these two cell lines resulted in accelerated tumor growth as compared to control tumors. Interestingly, histological examination of the primary tumors revealed a high density of LYVE-1 positive intratumoral lymphatics in the PDGF-BB-over-expressing tumors. A similar but more robust response was detected in the T241-VEGF-C tumors, whereas only pre-existing lymphatic vessels located at the tumor margins were found in the control tumors. We next investigated whether this increased degree of tumor lymphangiogenesis observed in T241-PDGF-BB tumors resulted in lymphatic tumor

spread by establishing a mouse lymphatic metastatic model, in which subcutaneously grown primary tumors located at the middle dorsum were surgically removed at the size of 1.5 cm^3. The majority of animals implanted with T241-PDGF-BB cells developed metastatic lesions in the regional lymph nodes. All T241-VEGF-C tumor-bearing mice developed lymphatic metastasis, both regional and distal, whereas no animals implanted with control tumors developed lymphatic metastasis. To summarize, our results clearly shows that PDGF-BB is a potent lymphangiogenic factor that, when expressed in tumors, can promote tumoral lymphangiogenesis and lymphatic metastasis.

4.2 LYMPHANGIOGENIC ACTIVITIES OF VEGF-A (PAPER II)

VEGF-A was previously though to act solely as an angiogenic factor by activating VEGFR-2 expressed exclusively in vascular ECs[19]. However, recent work has demonstrated that, like vascular endothelium, lymphatic endothelium also expresses VEGFR-2[36-38] and that VEGF-A activates LECs *in vitro*[232] as well as induces lymphangiogenesis *in vivo*[36,37,233]. VEGF-A is over-expressed in most solid tumors and contributes to tumor angiogenesis and presumably to tumor growth and haematogenous spread. Considering its lymphangiogenic potential we speculated that this factor, due to its high expressions levels in majority of solid tumors, might contribute to lymphatic metastasis.

To evaluate the lymphangiogenic activity of VEGF-A, we performed the corneal lymphangiogenesis model previously described. Immunofluorescence analysis of VEGF-A-implanted corneas revealed a robust lymphangiogenic response from day 14 after implantation and onward. Compared to the positive control FGF-2, there was a

clear delay in the lymphangiogenic response induced by VEGF-A during early stages. At day 5, distinct and well-organized lymphatic vessels were readily detectable in FGF-2-implanted corneas, whereas in the corneas implanted with VEGF-A only diffuse LYVE-1 positive structures were found in close connection to the pre-existing lymphatic vessels in the limbus, and these lymphatic structures completely lacked a vessel-like phenotype. However, between day 5 and day 14 after implantation these primitive LYVE-1 positive structures underwent dramatic remodeling into distinct sprouting lymphatic vessels growing in the direction of the pellet. Quantification analysis demonstrated that VEGF-A and FGF-2 were equally potent in inducing lymphangiogenesis two weeks or more after growth factor implantation. These findings show that VEGF-A is a potent lymphangiogenic factor *in vivo*, although the vascular event seems to be somewhat delayed.

Angiogenesis was previously considered a prerequisite for lymphatic vessel growth. Surprisingly, we found that on the backside of the circumferential eye globe, meaning the side opposite to the implantation site, VEGF-A stimulated lymphangiogenesis in the absence of blood vessel growth. This very interesting finding demonstrates that VEGF-induced lymphangiogenesis can exist independent of an angiogenic event.

VEGF-A has been suggested to induce lymphangiogenesis through both direct and indirect mechanisms of action. To identify potential indirect mechanisms by which VEGF-A can induce lymphangiogenesis *in vivo*, we co-implanted VEGF-A with a neutralizing VEGFR-3 antibody in the corneal lymphangiogenesis model, as described above. Importantly, we observed no blocking effect of this neutralizing compound on either VEGF-A-induced angiogenesis or lymphangiogenesis. In contrast, VEGF-C-induced lymphangiogenesis was completely blocked by this agent,

demonstrating that in this *in vivo* setting, the VEGF-C/-D/VEGFR-3 pathway is not required for the lymphangiogenic activity of VEGF-A.

Since VEGF-A is over-expressed in most solid tumors, we wanted determine whether VEGF-A can induce tumoral lymphangiogenesis. Implantation of a VEGF-A-over-expressing fibrosarcoma cell line (T241-VEGF-A) in the middle dorsum of mice resulted in robust peritumoral infiltration of lymphatic vessels as well as dilation and enlargement of surrounding pre-existing lymphatic vessels. Implantation of T241-VEGF-C tumor cells resulted in a marked lymphangiogenic response spanning the entire tumor tissue, whereas in T241-VEGF-A tumors lymphatic vessels were rarely detected in the more central areas. VEGF-A tumors are very fast growing tumor with an invasive phenotype, making it possible that the lymphatic vessels detected in the peritumoral area did not constitute newly formed lymphatic vessels but rather pre-existing lymphatics engulfed by the primary tumor during its expansion. To further evaluate the origin of the peritumoral lymphatics, we implanted a small piece of T241-VEGF-A tumor tissue in the cornea according to procedures previously described[357]. Because the cornea is an avascular tissue, tumor-associated angiogenesis and lymphangiogenesis exclude the involvement of pre-existing blood- or lymphatic vessels. Implantation of control or VEGF-A-transduced tumor tissues in the cornea resulted in growth of tumors, and neovascularisation of the tumors became visible by direct gross examination 2 weeks after implantation. Control tumors induced the growth of distinct intratumoral blood vessels with fine tree-like structures, whereas lymphatic vessels only grew in the surrounding tumor stroma. In contrast to control tumors, T241-VEGF-A tumors were highly vascularised and contained giant lymphatic vessels growing throughout the entire tumor tissue. These results shows that VEGF-A stimulates growth of tumoral lymphatics, and that the peritumoral

lymphatics most likely constitute newly formed lymphatic capillaries rather then co-opted pre-existing lymphatics.

As VEGF-A stimulates lymphangiogenesis in the peritumoral area and enlargement of pre-existing lymphatic vessels at the tumor margin, we investigated whether VEGF-A promotes lymphatic metastasis when over-expressed in tumors using our established lymphatic metastatic model. At 4-6 weeks after removal of T241-VEGF-A tumors, 55-60% of animals developed metastatic lesions in the brachial lymph nodes. Lymphatic tumor spread was detected by gross examination and histological analysis for detection of GFP positive T241-VEGF-A tumor cells. To further validate our results, *renilla* luciferase-transduced T241-VEGF-A tumor cells were implanted in the right upper back of mice. Two weeks after tumor implantation, bioluminescence signals were readily detectable in *ex vivo* dissected ipsilatheral brachial lymph nodes by using an optical imaging system. These signals were intensified more than 10-fold at 4 weeks post implantation, and at this time point signals were also observed in contralateral lymph nodes. This clearly demonstrated the growth of T241-VEGF-A secondary tumors in the lymph nodes, and further confirmed that VEGF-A promotes lymphatic metastasis.

VEGF-A induces migration of macrophages *via* activation of VEGFR-1. Activated macrophages were recently shown to contribute to VEGF-A-induced lymphangiogenesis in an *in vivo* model of inflammatory neovascularisation[27]. To investigate the ability of VEGF-A to recruit inflammatory cells in tumors, we stained T241-VEGF-A tumor sections with an antibody directed against macrophages. Immunohistochemical staining revealed a high infiltration of macrophages in the peritumoral areas, where a high lymphatic vessel density was previously observed.

T241-VEGF-C tumors were also infiltrated by macrophages, but not to the same extent as T241-VEGF-A tumors, whereas no inflammatory cells were detected in control tumors. Thus, macrophage recruitment appears to contribute to VEGF-A-induced lymphangiogenesis *in vivo*. We next performed an *in situ* hybridization analysis to analyse whether VEGF-A can up-regulate the expression of VEGF-C in tumors. No increased expression levels of VEGF-C mRNA were detected in T241-VEGF-A tumors as compared to control tumors. This further confirms our previous finding in the cornea assay that VEGF-A induces lymphangiogenesis independent of the VEGF-C/-D/-VEGFR-3 pathway.

4.3 A DIRECT ROLE OF THE IGF FAMILY IN INDUCING LYMPHANGIOGENESIS (PAPER III)

The IGF family has been demonstrated to induce angiogenesis in several systems, but a potential role of this growth factor family in the mechanism of lymphangiogenesis has not been reported. Interestingly, IGF-1R-signalling was recently shown to positively regulate the expression of VEGF-A, VEGF-C, and FGF-2, three potent lymphangiogenic factors[99,100,108,109]. This suggested to us that the IGF family might induce lymphangiogenesis, at least through an indirect mechanism.

Implantation of IGF-1 or IGF-2 induced a robust lymphangiogenic response in the cornea assay. Both factors stimulated the sprouting of large lymphatic vessels, consisting of well-organized tree-like structures, from the pre-existing lymphatics of the limbus. Although not as potent as FGF-2, these results demonstrates that both ligands of the IGF family induce lymphangiogenesis *in vivo*.

To investigate whether IGF-1 promotes lymphangiogenesis directly *via* activation of its cognate receptor or indirectly *via* activation of the VEGF-C/-D/VEGFR-3 pathway, we co-implanted IGF-1 with a soluble VEGFR-3-Fc, known to efficiently block VEGF-C-induced lymphangiogenesis *in vivo*, in the cornea of mice. Interestingly, this soluble receptor did not display any inhibitory activities on either IGF-1-induced angiogenesis or lymphangiogenesis, indicating that IGF-1 induces lymphatic vessel growth *via* activation of a signaling pathway functioning independent of the VEGF-C/-D/VEGFR-3 system.

We next wanted to investigate whether the lymphatic endothelium expresses IGFRs, which would implicate a direct effect for IGFs in inducing lymphangiogenesis. Immunohistochemical staining demonstrated a strong expression of IGFR-1 protein in cultured primary human LECs. Further, RT-PCR and DNA microarray analysis revealed presence of IGFR-1 and IGFR-2 mRNA in both human and murine primary LECs. This strongly suggests that IGFs act, at least to some extent, as direct lymphangiogenic factors. To further validate these results, we wanted to evaluate any potential direct effects of IGFs of primary LECs. Importantly, both IGF-1 and IGF-2 stimulated proliferation and migration of cultured LECs, and activated phosphorylation cascades of intracellular signaling components, such as *Akt*, *Erk*1/2, and *Src*, in the same cells. These results show that IGFs directly activate LECs *in vitro*, further demonstrating a potential direct role of IGFs in promoting lymphangiogenesis. We can however not exclude any indirect mechanisms of actions since activation of the IGF-1R has been associated with increased expression of three potent lymphangiogenic factors, namely VEGF-A, VEGF-D, and FGF-2.

Further experiments are required to investigate whether members of the IGF family can also induce growth of intratumoral lymphatic vessels and thereby promote lymphatic metastasis.

4.4 CONTRIBUTION OF CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN LYMPHANGIOGENESIS (PAPER IV)

Vasculogenesis was previously considered to be restricted to embryogenesis. However, recent studies have suggested that this vascular event may not only be restricted to early development, but also be implicated in postnatal neovascularisation. CEPCs have been identified in the blood of healthy adults, and have been shown recruited to sites of vascular trauma or tumor growth, where extensive neovascularisation is required. Although, CEPCs have been demonstrated to contribute to angiogenesis, their possible role in postnatal lymphatic vessel formation is not clear.

To study the role of BM-derived cells in lymphangiogenesis, we established a BM-reconstituted mouse model, in which BM form $EGFP^+$ donor mice were transplanted into normal radiated C57/Bl/6 recipient mice. In BM-depleted mice, FGF-2-induced corneal angiogenesis and lymphangiogenesis were significantly suppressed, but reconstitution of the BM with $EGFP^+$ donor cells almost completely restored both vascular processes, demonstrating that the BM is an important organ for both angiogenesis and lymphangiogenesis.

Implantation of FGF-2 in corneas of $EGFP^+$ BM-reconstituted mice resulted in robust angiogenesis and lymphangiogenesis. By using confocal analysis, more precisely an orthogonal sectioning tool of the Zeiss LSM software, we studied the association between $EGFP^+$ cells and LYVE-1 positive lymphatic vessels in three-dimensional settings. Interestingly, $EGFP^+$ cells were indeed incorporated into the lymphatic wall. To further validate this finding, high-resolution microscopy of thin sections of FGF-2-implanted corneas was performed. The sections were stained with LYVE-1 antibodies and subsequently with PI, which stains the cell nuclei and thereby reveal cellular identity. As expected, overlapping signals between EGFP and LYVE-1 were found in association with cell nuclei, demonstrating that these structures constituted $EGFP^+$ LECs. Further, $EGFP^+$ and VEGFR-3^+ overlapping signals were also found in the lymphatic vessel wall. In addition, $EGFP^+$ cells were also found incorporated into blood vessels. This clearly shows that $EGFP^+$ BM cells were incorporated into lymphatic vessels, and further confirms previous findings that CEPCs contribute to angiogenesis. We further detected incorporation of $EGFP^+$ cells in peritumoral lymphatics of *wt* T241 tumors grown s.c. on the back of these $EGFP^+$ BM-transplanted mice.

To identify what subpopulations of $EGFP^+$ BM cells that incorporated into the lymphatic vessel wall, we established two new mice models, by transplanting sorted populations of $EGFP^+/CD34^+/VEGFR\text{-}3^+$ cells or $EGFP^+/CD34^+/VEGFR\text{-}2^+$ cells into radiated recipient mice. Transplantation of isolated cell populations resulted in a dramatical increase in the incorporation of $EGFP^+/CD34^+/VEGFR\text{-}3^+$ cells in FGF-2-induced corneal lymphatic vessels. Interestingly, $EGFP^+/CD34^+/VEGFR\text{-}2^+$ cells were also found incorporated into lymphatic vessels. These findings identify BM-derived CEPCs as the primary circulating cells contributing to lymphangiogenesis.

Again, we performed high-resolution microscopy of corneal sections to validate that the $EGFP^+$ cells incorporated into the lymphatic vessel wall were of endothelial origin. Importantly, we found that $EGFP^+/CD34^+/VEGFR\text{-}3^+$ and $EGFP^+/CD34^+/VEGFR\text{-}2^+$ cells were detected in both VEGFR-3 and VEGFR-2 positive structures. In addition, $EGFP^+/CD34^+/VEGFR\text{-}2^+$ cells were readily detectable in CD31 and CD34 positive structures, just as expected. Surprisingly, $EGFP^+/CD34^+/VEGFR\text{-}3^+$ cells were also found incorporated into blood vessels. These findings confirm that CEPCs contribute to lymphangiogenesis. It also suggests to us that CEPCs might constitute a mixture of subpopulations that can incorporate into both blood- and lymphatic vessels, perhaps depending on presence of different growth factors that promote their differentiation in either direction. However, these speculations need to be further investigated.

5 DISCUSSION

Lymphatic vessels provide one of the main routes for tumor cell dissemination especially for tumors of the breast, lung, and gastrointestinal tract, which frequently metastasise to regional lymph nodes. The prognostic value of lymph node metastasis was recognized long before the concept of peritumoral and intratumoral lymphangiogenesis was described[364]. Until recently, the presence and potential function of lymphatic vessels inside the tumor tissue was a rather disputable issue. However, several studies have elegantly demonstrated that infiltrating tumoral lymphatics do exist, at least in the periphery of many experimental and human tumors[179,180,223,229,243,256,309,365]. The question of whether intratumoral lymphatics are functional or not still remains controversial[365,366]. Some authors argue that tumors metastasise solely by invasion of enlarged pre-existing lymphatics present at the tumor border[366], while others state that tumors metastasise by promoting *de novo* formation of intratumoral lymphatics[241].

Tumor dissemination to regional lymph nodes represents the first step of lymphatic metastasis and serves as an important prognostic factor in many cancers. Compared to the blood vascular system, the lymphatic system appears to be an excellent conduit for tumor cell dissemination. First of all, lymphatic vessels are much larger in size than blood capillaries and a continuous basal membrane does not invest them making the transit easier for the tumor cells. In addition, the composition of lymph and interstitial fluid is very similar, readily allowing cell survival inside of lymphatic vessels. Lastly, flow velocities inside the lymphatic vessels are much lower than those of blood vessels[178-180,194]. Conversely, the blood stream constitutes a highly aggressive environment for tumor cells due to serum toxicity, host immunity, and

high fluid velocities and shear stress resulting in mechanical deformation of cells[178,179,367,368]. Compared with the well-characterized tumor blood vasculature, little is known about the biology of intratumoral lymphatics, what factors are involved in inducing tumor lymphangiogenesis, or what role the intratumoral lymphatics play in metastasis.

5.1 IDENTIFICATION OF NOVEL LYMPHANGIOGENIC FACTORS

VEGF-C has been identified as an essential factor during the development of the lymphatic system[212]. Both VEGF-C and VEGF-D have direct effects on LECs in vitro[213,232], and have been shown to induce tumoral lymphangiogenesis and promote lymphatic metastasis when expressed at high levels in various human cancers[223,228,229,243,309,310]. There is no doubt that VEGF-C and VEGF-D are critical lymphangiogenic factors contributing to both physiological and pathological lymphangiogenesis. However, when considering the complex nature of angiogenesis, it seems unlikely that these would be the sole factors regulating lymphangiogenesis. Indeed, several other factors have recently been demonstrated to participate in the molecular mechanism of lymphangiogenesis, such as FGF-2[235], VEGF-A[200,233], Ang-1[321], Ang-2[322], and members of the PDGF-242 and IGF-families[324]. In this thesis work we provide compelling evidence that PDGFs and IGFs display direct lymphangiogenic activities via activation of their cognent receptors, PDGFRs or IGFRs, present on lymphatic endothelium. Ligands of both growth factor families activate primary LECs in vitro and potently stimulate lymphangiogenesis in the cornea assay. In addition, we have shown that VEGF-A-implantation in the cornea also induces a robust, yet somewhat delayed, lymphangiogenic response. Our finding that, IGF-1-, PDGF-BB-, and VEGF-A-induced corneal lymphangiogenesis could not be blocked by a neutralizing VEGFR-3 antibody or by a soluble VEGFR-3, suggest

that activation of the VEGFR-3 is not critical for lymphangiogenesis induced by these growth factors. Although our results identify PDGFs and IGFs as direct lymphangiogenic factors, the mechanism of VEGF-A-induced lymphangiogenesis is somewhat more complex. VEGF-A probably induces lymphangiogenesis through both direct (activation of VEGFR-2 expressed on proliferating lymphatic endothelium[36-38]) and indirect (recruitment of inflammatory cells[27]) mechanisms of action.

Interestingly, members of the PDGF- and IGF-families have been reported to be expressed at high levels in several tumors with lymphatic metastatic ability, raising the possibility that growth factors other than VEGF-C and VEGF-D may contribute to tumor lymphangiogenesis and lymphatic metastasis clinically[110-113,133,369]. In this thesis work, we revealed the occurrence of pronounced lymphangiogenesis within experimental xenotransplanted tumors in mice, using an antibody directed against LYVE-1 as a specific lymphatic marker[184]. We observed that over-expression of PDGF-BB or VEGF-A in a fibrosarcoma cell line resulted in both enlargement of pre-existing lymphatic vessels at the tumor implantation border as well as increased infiltration of peritumoral lymphatics into the tumor. As a positive control, we used a VEGF-C over-expressing fibrosarcoma. Post-implantation of VEGF-C over-expressing fibrosarcoma cells, infiltrating lymphatic vessels were detected throughout the entire tumor tissue at much higher densities than in tumors over-expressing PDGF-BB or VEGF-A. This indicates that VEGF-C is more potent than both PDGF-BB and VEGF-A in inducing intratumoral migration of LECs. However, the tumor microenvironment appears to plays a critical role in either promoting or preventing infiltration of tumor lymphatics. Interestingly, over-expression of VEGF-C in the fibrosarcoma used in our experiments results in formation of extremely soft and

liquidous tumors. Considering the high density of lymphatic vessels spanning the entire tumor tissue, it is rather surprising that the interstitial fluid remains within the tumor instead of being transported away. This strongly suggests that these VEGF-C-induced intratumoral lymphatics are non-functional in regard of fluid drainage or that VEGF-C causes blood vessel leakage. Numerous studies have failed to identify functional intratumoral lymphatics[370], suggesting that lymphangiogenesis may not represent an essential event in tumor growth and progression. Noteworthy, the formation of an intratumoral lymphatic network, functional or not in transport of fluids and macromolecules, might still promote tumor dissemination simply by increasing the surface area between the hyperplastic lymphatic endothelium and the invading tumor cells and thereby creating increased opportunities for invasive tumor cells to leave the primary tumor site. In contrast to VEGF-C over-expressing tumors, implantation of fibrosarcoma cells over-expressing VEGF-A results in formation of solid tumors with a high interstitial pressure, presumably due to the blood-filled necrotic area detected at the center of all tumors. In VEGF-A over-expressing tumors, enlarged and dilated lymphatics are frequently present in the surrounding tumoral stroma whereas only fine and irregularly shaped LYVE-1 positive vessel structures penetrate into the tumor periphery. One possible explanation for the abnormal phenotype of these peritumoral lymphatics, and the absence of lymphatic vessels at the more central areas of VEGF-A-tumors, could be that the high interstitial pressure within the tumors generates mechanical stress or compresses the newly formed lymphatic channels thus preventing lymphatic sprouting.

5.2 ON THE ROLE OF PDGFs IN TUMOR LYMPHATICS (PAPER I)

We have detected both mRNA and protein expression of PDGFRs in primary LECs in vitro and in lymphatic endothelium in vivo. In isolated LECs, mRNA expression levels of PDGFRs are rather moderate, whereas the expression levels of PDGFR proteins are high in vivo. FGF-2 has been reported to enhance the expression of PDGFR-β in mural cells[373]. Perhaps, FGF-2, or some other growth factor, similarly induces the up-regulation of PDGFRs in proliferating lymphatic endothelium and thereby increases the lymphangiogenic response. In PDGF-BB over-expressing tumors, considerable regional heterogeneity in lymphatic vessel density was found within individual tumors. This may reflect local variations in the tumor microenvironment such as differences in the composition of the ECM, blood vessel supply, proteolytic activity, mechanical forces, or the presence of an inflammatory response.

5.3 ON THE ROLE OF INTRA- AND PERITUMORAL LYMPHATICS IN THE FORMATION OF LYMPH NODE METASTASIS

Tumor cells can gain access to the lymphatic system either by inducing intratumoral lymphangiogenesis or by co-opting neighboring pre-existing lymphatic vessels during tumor expansion[180,241]. In our studies, the lymphangiogenic responses induced by VEGF-C-, PDGF-BB-, or VEGF-A-over-expressing fibrosarcomas were dramatically different. However, all three factors promoted lymphatic metastasis, albeit at different time-points and with varying efficiency. All animals implanted with VEGF-C over-expressing tumors developed lymphatic metastasis in both regional and distant lymph nodes, although the main spread of tumor cells was to the brachial lymph nodes, which were the lymph nodes located at the shortest distance from the primary tumor. The majority of animals implanted with PDGF-BB over-expressing tumors developed

lymphatic metastasis in the brachial lymph nodes, and in rare cases in distant lymph nodes. About 55-60% of animals implanted with VEGF-A-over-expressing tumors, also developed metastases in the brachial lymph nodes. Consistent with our finding that VEGF-A-over-expressing fibrosarcomas induce a peritumoral lymphangiogenic response and contributes to lymphatic metastasis, Hirakawa et al recently showed that VEGF-A induces active proliferation of VEGFR-2-expressing tumoral lymphatic vessels and promotes tumor spread to both regional and distant lymph nodes using a murine transgenic skin tumor model[36].

5.4 ON THE ROLE OF VEGF-A IN LYMPHATIC METASTASIS (PAPER II)

VEGF-A is a key angiogenic factor expressed at high levels by most tumors. The fact that VEGF-A induces lymphangiogenesis suggests that this factor may not only have a major impact on tumor progression and angiogenesis, but also on lymphatic tumor spread. In animals implanted with the VEGF-A-over-expressing fibrosarcoma cell line, tumor cell dissemination to regional lymph nodes appeared to be strictly dependent on the growth rate of the primary tumor as well as the size of the primary tumor at the time-point of surgical removal, with a slower growth rate and a larger tumor size being favorable for the establishment of metastasis. VEGF-A-over-expressing tumors generally have a very fast growth rate due to the robust angiogenic response induced. Histological examination of primary tumors at various time-points demonstrated a strong correlation between lymphatic vessel density in the peritumoral area and enlarged tumor volume as well as a slower growth rate. Larger- as well as slow growing tumors exhibited a higher density of peritumoral lymphatics compared to smaller tumors or fast growing ones. In support of this finding, there appears to be a delay in the lymphangiogenic response induced by VEGF-A, as elegantly demonstrated in the cornea assay. Many lymphangiogenic factors, such as FGF-2,

VEGF-C, PDGF-BB, IGF-1, and IGF-2, induce a robust lymphangiogenic response with well-organized vessel structures detectable directly at day 5 after implantation. At this time-point, the LYVE-1+ structures detected in VEGF-A-implanted corneas completely lacked a vascular tree-like structure and remained in close contact to the pre-existing limbal lymphatics. At day 14, however, these primitive lymphatic structures had undergone remarkable remodeling into distinct lymphatic vessels growing towards the pellet, but not until day 25 after implantation was the lymphangiogenic response induced by VEGF-A comparable to that of FGF-2 for example. This finding suggested to us that indirect mechanisms of action might be critical for VEGF-A-induced lymphangiogenesis.

5.5 ON THE CONSEQUENCE OF INFLAMMATION

There is increasing evidence that inflammatory cells contribute to tumor progression, tumor-associated angiogenesis and lymphangiogenesis, and metastasis[28,374,375]. The expression of VEGF-A is readily induced by pro-inflammatory cytokines. VEGF-A recruits monocytes and macrophages, which express VEGFR-1[35], and these cells constitute a rich source of many angiogenic and lymphangiogenic factors, including FGF-2, VEGF-A, VEGF-C, VEGF-D, PDGFs, HGF, EGF, angiopoietins, tumor necrosis factor-α (TNF-α), TGF-β, as well as proteolytic enzymes with pro-angiogenic activity, such as MMP-2, MMP-9, and urokinase plasminogen activator (uPA)[27,28]. In agreement, during our experiments, we observed a high infiltrate of macrophages in the lymphatic-rich peritumoral areas of VEGF-A-over-expressing tumors. VEGF-C-over-expressing tumors were also infiltrated by macrophages, but not to the same extent as VEGF-A-over-expressing tumors. Consistent with this finding, others have demonstrated that macrophages promote tumor progression and metastasis[28]. VEGF-A also directly up-regulates adhesion molecules on the vascular

endothelium resulting in increased transmigration of leukocytes, which secrete several cytokines and other regulatory factors[376]. Lastly, VEGF-A, as well as VEGF-C, could indirectly support inflammatory lymphangiogenesis by inducing vascular leakage thus increasing the ease of infiltration of inflammatory cells. Furthermore, on the potential significance of increased vascular permeability, it remains an intriguing possibility that the mechanical stress induced by increased interstitial pressure may stimulate the production of lymphangiogenic growth factors in an effort to reduce the pressure. Taken together, the indirect effects of members of the VEGF family in stimulation of angiogenesis and lymphangiogenesis remain plausible. In relation, at the time being, the potential role of inflammatory cells in lymphangiogenesis induced by members of the PDGF- or IGF families has not been investigated.

5.6 ON THE SIGNIFICANCE OF TUMOR INTERSTITIAL PRESSURE

Although it seems evident that VEGF-C, PDGF-BB, as well as VEGF-A can induce, at least to some extent, the growth of new tumoral lymphatic vessels many key questions remain unanswered. For example, it is not known whether the enlarged and dilated pre-existing lymphatic vessels present in the peritumoral stroma alone are sufficient for lymphatic metastasis[366] or whether the *de novo* formation of intratumoral lymphatic capillaries may increase the propensity of a tumor to metastasise[241]. Although dilated pre-existing peritumoral lymphatics with large lumens and low flow velocities provide a readily accessible conduit for tumor cell extravasation and dissemination[179], the formation of an intratumoral lymphatic network theoretically provides the most direct route for tumor cell spread by making a larger surface area for the tumor cells to leave the primary tumor. However, intratumoral lymphatics are usually collapsed due to the high interstitial pressures generated by rapidly growing solid tumors[303], impairing the lymphatic transport

capacity. Conversely, tumor cell spread *via* the lymphatic vascular bed may be facilitated by a high interstitial pressure, especially since recent findings show that intratumoral lymphatics are often filled with tumor cells that occlude the lumen[366]. If present inside of the lymphatic lumen, tumor cells may directly enter the lymphatic system simply by shedding. Tumor-cell-secreted factors might also facilitate metastasis by increasing vascular permeability or by changing the adhesive properties of the lymphatic endothelium. For instance, VEGF-A and VEGF-C can increase vascular permeability contributing to an increase in interstitial fluid pressure[44,53]. Perhaps these factors, or other tumor-produced growth factors such as members of the PDGF-, IGF-, or FGF families, can further increase the permeability of the lymphatic endothelium as well.

5.7 ON THE POTENTIAL FUNCTION OF LYMPHATIC ENDOTHELIUM IN TUMOR CELL SPREAD

Morphologically, newly formed intratumoral lymphatics appear to be highly immature with small lumens that comprise only a small number of ECs. Due to this immature phenotype intratumoral lymphatics may be more prone to tumor cell invasion. Furthermore, tumor cells may secrete factors that activate LECs and promote an interaction between tumor cells and the lymphatic endothelium that may ease tumor cell entry into the lymphatics. Tumoral lymphatics may be involved in chemotactic recruitment and intralymphatic transport of certain tumor cells. For example, lymphatic endothelium secretes the chemokine secondary lymphoid chemokine (CCL21), which binds to its receptor CC chemokine receptor 7 (CCR7). This receptor has been detected on some breast cancer cells as well as some malignant melanoma cell lines[371]. Injection of B16 malignant melanoma cells over-expressing CCR7 into the footpad of mice resulted in a dramatic increase in the

incidence of metastatic spread to regional lymph nodes[372], in support of a chemotactic role of lymphatic-derived chamokines. Further, LYVE-1, the lymphatic receptor for HA, has been implicated in the trafficking of cells within lymphatic vessels and lymph nodes. This receptor may, however, also be directly involved in the transmigration process of tumor cells into the lymphatics. This possibility remains to be investigated in LYVE-1-deficient mice.

5.8 ON THE ROLE OF CIRCULATING ENDOTHELIAL PRECURSOR CELLS IN THE ADULT (PAPER IV)

It is now clear that vasculogenesis also plays an important role in neovascularisation and tissue healing in response to ischemia276. The finding that CEPCs are rapidly mobilized from the BM to colonize areas of vascular trauma, where they differentiate into mature ECs has supported an important role of CEPCs in physiological and pathological angiogenesis270,273. The origin of LECs is not known, but sprouting of new lymphatics from the pre-existing lymphatic network appears to be a critical mechanism for postnatal lymphangiogenesis377. However, it is not clear if BM-derived CEPCs may also actively contribute to lymphangiogenesis. Recently, a population of CD34+/CD133+ endothelial progenitor cells co-expressing the lymphatic marker VEGFR-3 was identified. We and others have found that these cells are functionally non-adherent endothelial precursor cells that can differentiate into mature adherent vascular- or lymphatic ECs in the presence of vascular growth factors272,274. Although, the number of circulating CD34+/CD133-/VEGFR-3+ cells is very low in healthy adults, these cells might be mobilized from the BM to the peripheral circulation during postnatal lymphangiogenesis.

In this thesis work we show that BM-derived circulating cells are incorporated into newly formed lymphatic vessels in both physiological and pathological conditions. Depletion of whole BM in mice by irradiation results in remarkable suppression of FGF-2-induced angiogenesis and lymphangiogenesis in the cornea assay, whereas BM-reconstitution with donor cells largely restores the vascular responses. In order to identify what BM-derived cell populations contribute to postnatal lymphangiogenesis, we implanted FGF-2 into corneas of mice transplanted with isolated donor $EGFP^+/CD34^+/VEGFR\text{-}2^+$ cells or with $EGFP^+/CD34^+/VEGFR\text{-}3^+$ cells immediately after total body irradiation. Interestingly, both cell populations were found incorporated into newly formed lymphatic vessels. VEGFR-2 was previously thought to be expressed exclusively in blood ECs[19]. However, recent findings have demonstrated that this receptor is also expressed in proliferating lymphatic endothelium[36-38]. Thus, the circulating population of $EGFP^+/CD34^+/VEGFR\text{-}2^+$ cells may actually constitute subpopulations of vascular- or lymphatic ECs, just like the population of $EGFP^+/CD34^+/VEGFR\text{-}3^+$ cells does. Another explanation could be that $EGFP^+/CD34^+/VEGFR\text{-}2^+$ cells further differentiate into $EGFP^+/CD34^+/VEGFR\text{-}3^+$ cells prior to incorporation. Several angiogenic factors, such as VEGF-A, VEGF-C, and VEGF-D, could potentially contribute to this differentiation. In contrast to our finding, a recent study showed that newly formed lymphatic vessels mainly sprout from the pre-existing lymphatic network, and that only little, if any, incorporation of BM-derived lymphatic endothelial progenitor cells occurs[377]. The discrepancy between our and this finding may, however, be less evident. We agree that sprouting from pre-existing lymphatic vessels is critical for postnatal lymphangiogenesis, but nevertheless we uphold that lymphatic endothelial precursor cells contribute to postnatal lymphangiogenesis. In our study, we further suggest that BM-derived CEPCs cells might also promote tumor lymphangiogenesis. This makes CEPCs an

interesting target for the development and evaluation of new therapeutic drugs and strategies for the treatment of lymphatic metastasis.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

A PubMed search for the term “lymphangiogenesis” identifies 389 citations, all but 24 of which postdate 2000, whereas the term “angiogenesis” identifies as many as 24,600 publications. This clearly shows that in contrast to its sister field, angiogenesis, lymphangiogenesis is a relatively new and unexplored vascular research field that has gained an enormous interest recently after many decades of dormancy. Since the discovery of central regulators of lymphatic development, such as prox-1, VEGF-C, and VEGFR-3, and the development of techniques for the isolation and culture of primary LECs great advances have been made at the molecular understanding of lymphangiogenesis. VEGF-C and VEGF-D were the first lymphangiogenic factors identified. Today, additional regulators of lymphangiogenesis have been identified, such as FGF-2, VEGF-A, HGF, angiopoietins, PDGFs, and IGFs, and the list of lymphangiogenic stimulators will undoubtedly continue to grow. In addition, the search of specific lymphangiogenesis inhibitors presents an exciting new area of research.

Many regulatory similarities of the angiogenic- and lymphangiogenic processes have been reported, and these two distinct vascular systems seem to work in a tightly co-ordinated manner. Angiogenesis was previously considered a prerequisite for lymphangiogenesis, with one theory being that blood vessels themselves stimulate the growth of lymphatic vessels in order to secure removal of fluid and macromolecules leaking out from the newly formed, immature blood capillaries. However, others and we have experimentally demonstrated that lymphangiogenesis can occur in the absence of blood vessels[235,324].

Understanding the biology of lymphangiogenesis and how it is connected to intratumoral vessel growth and lymphatic spread is fundamentally important in understanding the concept of lymphatic metastasis. For some carcinomas, intratumoral lymphangiogenesis has been associated with nodal and distal metastasis. However, there is no solid evidence to support a significant correlation between intratumoral lymphatic vessel growth and lymphatic metastasis in certain human cancers. Thus, many critical questions regarding the mechanism of lymphatic tumor spread remain unanswered. For example, do tumor-secreted lymphangiogenic factors promote intratumoral lymphangiogenesis and lymphatic vessel dilation? If so, does this increase the probability of lymphatic tumor spread beyond that which would occur solely through pre-existing lymphatic vessels in the tumor stroma? Moreover, what is the reason for the great heterogeneity observed between different tumors in regard to expression and secretion of lymphangiogenic factors? What role does the regional lymph node metastasis play in the development of tumor immune responses or in the development of distal non-lymphatic metastasis? Further work is required to clarify if there is a phenotypic difference between proliferating- and established lymphatics that could make newly formed lymphatic vessels more prone to tumor cell invasion and spread. Regarding tumor cell intravasation into lymphatic vessels, it is not clear whether this process is similar or completely different from that which occurs during intravasation of blood vessels. Do lymphatic vessels simply constitute passive conduits for tumor cell spread or can they activate tumor cells through the production of trophic, mitogenic, or chemotactic factors, as well as change their adhesive- and transport capacities? Extensive studies of adhesive interactions between tumor cells and blood vessels are required to increase our understandning of the malignant process.

In the field of angiogenesis, an extensive effort has been made to identify potent anti-angiogenic agents that can be used in anti-cancer therapy, and many potentially useful compounds has already entered late stages of clinical trials. The enormous interest in lymphangiogenesis will for certain direct us towards similar objectives. At the time being, we do not know whether inhibition of lymphangiogenesis represent a realistic strategy for prevention of lymphatic metastasis. Characterisation of the molecular effect of these lymphangiogenic factors in lymphatic endothelium will increase the possibility to block lymphatic metastasis. The identification of further selective markers that reliably separate lymphatic endothelium form blood vascular endothelium will facilitate these studies. As the molecular mechanisms regulating growth and function of lymphatic vessels become better understood, it might be possible to monitor for markers of tumoral lymphangiogenesis in vivo, and thus potentially prevent lymphatic metastasis by modulating the primary lymphangiogenic response.

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☺
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