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HEM- AND LYMPH-ANGIOGENESIS IN CANCER METASTASIS

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To my dearest family

ABSTRACT

Angiogenesis, the process of sprouting new microvessels from the pre-existing vasculature, is known to promote tumor growth. However, the role of tumor angiogenic vessels in facilitating metastasis remains poorly understood. In addition to hemangiogenesis, various types of tumors often contain lymphatic vessels, which may facilitate lymphatic metastasis. Cancer metastasis employs complex processes that are collectively termed as the metastatic cascade, which involves multiple-step defined mechanisms. A clinical detectable metastatic mass represents the ultimate consequence of the complex metastatic cascade that includes dissemination of tumor cell from the primary site; intravasion of tumor cells into the circulation or lymphatic system; transport of tumor cells along blood circulation or lymphatic system to distal tissues or organs; extravasation of tumor cells from the circulation of lymphatic system; formation of the primary metastatic niche in distal tissues; manipulation of metastatic microenvironment; and regrowth of metastatic nodules to a visible metastatic mass. Although advances of imagine techniques allows detection of relative small sizes of tumors in cancer patients and in experimental animal models, the early onset of metastatic processes remains unknown. As for lymphangiogenesis, there has been lacking appropriate and powerful *in vivo* assay systems that allow quantitatively study lymphangiogenesis. In this thesis work, we have: 1) developed a novel zebrafish model to study the early steps of the metastatic cascade. We take the advantage of the transparent nature of zebrafish embryos to visualize under normoxic and hypoxic conditions marked human or mouse tumor cell migration and invasion in association with tumor angiogenesis. We have found that tumor angiogenesis is essentially required for tumor cell invasion and dissemination. This study, for the first time, provides compelling evidence of tumor cell-tumor vessel interaction in promoting cancer cell dissemination to distal sites; 2) studied the interplay between FGF-2 and VEGF-C in promoting lymphatic metastasis. In the tumor microenvironment, various angiogenic factors often co-exist and they often cross-communicate with different signaling pathways. Although the individual factor-transduced vertical signals *via* their specific receptors are relatively well studied, their horizontal interplay with other signaling systems remains poorly characterized. We show that FGF-2-triggered lymphangiogenic signaling pathways synergistically promote lymphangiogenesis with the VEGF-C-VEGFR-3 system, leading to synergistic lymphangiogenic effects in various *in vivo* models. A clear lymphangiogenic synergism between FGF-2 and VEGF-C has been observed in the tumor microenvironment. Importantly, this synergistic lymphangiogenic activity leads to accelerated lymphatic metastasis in sentinel lymph nodes; 3) developed a unique *in vivo* model to study lymphangiogenesis induced by various factors. We take the advantage of the avascular nature of the mouse corneal tissue and implant various growth factors/cytokines alone or in combinations to quantitatively study lymphangiogenesis and lymphatic structures; and 4) studied the impact of clinical available antiangiogenic drugs on healthy vasculature and revealed potential sites for antiangiogenic drug-related side effects.

LIST OF PUBLICATIONS

I. Lee SL*, Rouhi P*, Dahl Jensen L, Zhang D, **Ji H**, Hauptmann G, Ingham P, Cao Y. Hypoxia-induced pathological angiogenesis mediates tumor cell dissemination, invasion, and metastasis in a zebrafish tumor model. Proc Natl Acad Sci U S A. 2009 Nov 17;106(46):19485-90

II. Cao R*, **Ji H***, Feng N, Zhang Y, Yang X, Andersson P, Sun Y, Tritsaris K, Hansen AJ, Dissing S, Cao Y. Collaborative interplay between FGF-2 and VEGF-C promotes lymphangiogenesis and metastasis. Proc Natl Acad Sci U S A. 2012 Sep 25;109(39):15894-9

III. Cao R, Lim S, **Ji H**, Zhang Y, Yang Y, Honek J, Hedlund EM, Cao Y. Mouse corneal lymphangiogenesis model. Nat Protoc. 2011 Jun;6 (6):817-26.

IV. Yang Y*, Zhang Y*, Cao Z*, **Ji H**, Wahlberg E, Länne T, Sun B, Li X, Liu Y, Cao Y. Vascular plasticity in response to anti-VEGF specific blockades. Submitted manuscript

RELATED PUBLICATION

Ji H*, Cao R*, Yang Y, Iwamoto H, Lim S, Zhang Y, Nakamura M, Andersson P, Yang X, Cao Y. TNFR1-mediates TNF-a-induced tumor inflammatory lymphangiogenesis and lymphatic metastasis by orchestrating the VEGF-C–VEGFR3 signaling system. Manuscript in preparation

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CONTENTS

LIST OF ABBREVIATIONS

Ang	Angiopoietins
ECs	Endothelial cells
EGFP	Enhanced green fluorescent protein
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
HA	Hyaluronan
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
LECs	Lymphatic endothelial cells
IFP	Interstitial fluid pressure
LLC	Lewis lung carcinoma
PDGF	Platelet growth factor
PDGFR	Platelet growth factor receptor
Prox-1	Prospero homeobox protein 1
RCC	Renal cell carcinoma
RT-PCR	Reverse transcriptase-Polymerase chain reaction
RTK	Receptor tyrosine kinase
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vSMCs	Vasular smooth muscle cells

1 INTRODUCTION

1.1 ANGIOGENESIS

Angiogenesis, the process of sprouting new microvessels from the pre-existing, has been recognized since 1971 by Dr. Judah Folkman who proposed that tumor growth is angiogenesis dependent[1]. Today, it is well established that tumor growth and metastasis are dependent on the development of new blood vessels and lymphatic vessels.

Sprouting angiogenesis occurs in several well-characterized stages: Angiogenic growth factors activate their cognate receptors present on endothelial cells (ECs) of pre-existing blood vessels; The activated ECs begin to release enzymes called proteases that degrade the basement membrane; ECs change morphology, proliferate, migrate and adhere tightly in tandem using adhesion molecules to form the new vessel wall of sprouts; The sprouts finally become a full-fledged vessel lumen to establish network of capillaries. Newly formed capillary sprouts are fragile. The supporting cells including pericytes for small capillaries and vascular smooth muscle cells (vSMCs) for larger vessels proliferate and migrate in parallel to the growth of the vascular sprout to solidify and stabilize the newly formed sprouts[1-4].

Angiogenesis is a complex balance process regulated by angiogenic factors and inhibitors[5-7]. The activation of angiogenesis requires up-regulation of angiogenic factors and/or down-regulation of endogenous inhibitors[7-10]. These angiogenic or anti-angiogenic factors may arise from various sources such as ECs, stromal cells, inflammatory cells, cancer cells or hypoxia. There are many angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), etc[2,11]. Although the list of angiogenic stimulators is constantly growing, VEGF family is the best characterized regulators of angiogenesis. VEGF is one of the key angiogenic factors that contributes to the onset and progression of many pathological conditions including cancers[12].

Hypoxia induces a complex response in a tissue, which aims at protecting the cells

against and counteracting the loss of oxygen. Sprouting angiogenesis is initiated in poorly perfused tissues when hypoxia in the tissue demands the formation of new blood vessels to satisfy the metabolic requirements of parenchymal cells (myocytes, hepatocytes, neurons, astrocytes, etc). Parenchymal cells respond to a hypoxic environment by secreting VEGF-A[13]. Hypoxia triggers an angiogenic response *via* the hypoxia inducible factor (HIF) 1-α- VEGF pathway[13-16].

1.1.1 Angiogenic stimulators

1.1.1.1 Vascular endothelial growth factor family

As we may know, VEGF family contains six structurally related growth factors, including VEGF-A, -B, -C, -D, -E, and placental growth factors (PlGF)[6]. The angiogenic activities of VEGF family are mediated through two structurally related tyrosine kinase receptors mainly expressed in ECs, VEGFR-1 and VEGFR-2[17-21]. Abundant experimental data demonstrate that VEGFR-2 is the primary functional receptor that transduces both angiogenic and vascular permeability signals, whereas VEGFR-1 may function as a decoy receptor[22,23].

In addition to VEGFR-1 and VEGFR-2, VEGFR-3, a lymphatic endothelial cell specific tyrosin kinase receptor, has been identified[24-27]. VEGF-C and VEGF-D can activate VEGFR-3 to induce lymphangiogenesis[28,29]. In addition to tyrosine kinase receptors, VEGF isoforms can interact with neuropilin-1 and -2, which serve as co-receptors of the VEGFR-1, VEGFR-2 and VEGFR-3 to modulate angiogenesis and lymphangiogenesis[22]. VEGFs and VEGF receptors signaling pathways are critical for inducing angiogenesis and lymphangiogenesis in physiological and pathological situations[30,31].

VEGF-A is an abundant endothelium-specific growth factor. It can stimulate proliferation, migration, sprouting and tube formation of ECs, as well as vascular integrity[32]. Moreover, VEGF-A has been reported to act as a chemoattractant for vSMCs, implicating a role for VEGF-A in blood vessel stabilization[5,33] VEGF-A is expressed in a wide variety of cell types, including embryonic fibroblasts, vSMCs, activated macrophages, keratinocytes and hepatocytes [20].

The expression of VEGF-A is markedly up-regulated in hypoxic conditions *via* HIF1-α/VEGF pathway[13-16.] VEGF transcription is activated and up-regulated in hypoxic condition[27,34].

PlGF was defined as an endothelial growth factor based on its sequence homology to VEGF. PlGF exclusively binds to VEGFR-1, which may function as a decoy receptor in regulation of VEGF-induced angiogenesis[35,36]. Although the direct role of PlGF still remains unknown under pathological conditions, PlGF has been shown to enhance VEGF-A-induced angiogenesis in part through a cross-talk between VEGFR-1 and VEGFR-2[35,36]. PIGF has been suggested to indirectly stimulate angiogenesis by binding VEGFR-1, thereby increasing the fraction of VEGF-A molecules available to activate VEGFR-2[37].

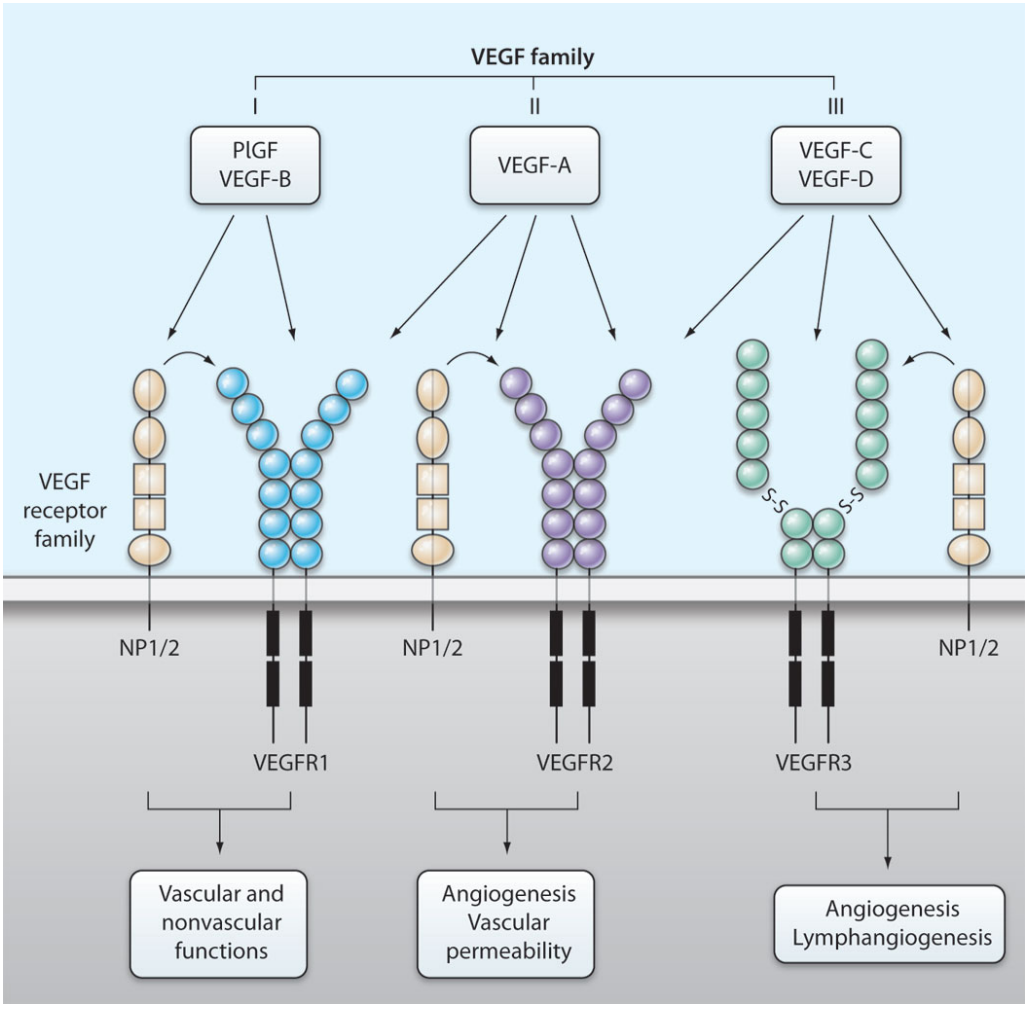

Figure 1. The VEGF family (adapted from reference 22)

VEGF-B is reported to be expressed in developing heart and skeletal muscles[38,39]. VEGF-E, a non-mammalian viral protein, is a selective agonist for VEGFR-2[40]. The precise physiological functions of VEGF-B and VEGF-E *in vivo* are still unknown[41,42]. In contrast to VEGF-A, VEGF-B plays a less pronounced role in angiogenesis. VEGF-B seems to bind to VEGFR-1 to play a role only in the maintenance of newly formed blood vessels under pathological conditions[39,43,44]

VEGF-C and VEGF-D can activate VEGFR-3 to induce lymphangiogenesis. Recent studies have shown that VEGF-C/ VEGF-D/ VEGFR-3-mediated signals are critical in the sprouting of the first lymphatic vessel from the developing veins in the embryo, suggesting that this signalling pathway is essential for differentiation of endothelial progenitor cells into the lymphatic lineage[24,42,45-49]. In addition to activating VEGFR-3, VEGF-C and VEGF-D also activate VEGFR-2 and induce angiogenesis[24,42,45-49]. VEGF-C and VEGF-D will be discussed further in details in the lymphangiogenic section (page10-12). In addition to VEGF-C and VEGF-D, VEGF-A has also been shown to act as a potent lymphangiogenic factor.

1.1.1.2 Fibroblast growth factor family

Fibroblast growth factors are involved in angiogenesis, wound healing and embryonic development. In humans, more than twenty members of the FGF family have been identified[50-52]. All FGFs are structurally related signaling molecules. FGF-1 and FGF-2 have been shown to promote angiogenesis, including ECs differentiation, proliferation, migration, integrin and cadherin receptor expression[24,53]. There are five fibroblast growth factor receptors: FGFR-1, -2, -3, -4, and FGFR-5[54,55]. FGFs bind to four structurally related tyrosine kinase receptors FGFR-1, -2, -3, -4, which present on many different cell types, including ECs[24,46,51,54,56-58]. The fifth receptor, FGFR-5, lacks intracellular tyrosine kinase domain, and its role is less understood[54,55,58].

During embryonic development, the expression patterns of the FGF receptors are distinct but overlapping. FGFs/FGFR-1 signalling is reported to play an important role in the development and maintenance of vascular network in the embryo[24,46,51,54,56-58]. FGFR-1 mutant mouse embryos are developmentally retarded or even die during gastrulation stage. In recent years, the existence of intimate cross-

talks between FGF-2 and members of the VEGF family, such as VEGF-C, during angiogenesis and lymphangiogenesis has been reported[59]. FGF-2 appears to induce angiogenesis by increasing VEGF expression in the ECs of forming capillaries[60,61].

FGF/FGFR signaling pathway plays an important role in normal organ, skeletal development and vascular formation. De-regulation of the FGF/FGFR signaling pathway through genetic modifications or overexpression of the receptors has been associated with many developmental disorders and promotion of disease progression, including induction of angiogenesis and lymphangiogenesis in cancers[24,46,51,54,56-58].

1.1.1.3 Other stimulators

Angiopoietins (Ang) bind to the Tie receptors expressing within the vascular endothelium to regulate blood vessel formation[62]. Tie receptors include Tie-1 and Tie-2, which play a critical role in embryonic development. Ang-1 is a Tie-2 agonist. During the early stage of vascular development, Ang-1 promotes subsequent vascular remodeling and stabilization after the vascular formation[63]. The actions of Ang-2 are complex. Ang-2 appears to play critical roles in vascular remodeling, vessel sprouting, and vessel regression[64].

The Insulin-like growth factor family (IGF) consists of two ligands, IGF-1 and IGF-2. IGF-1 and IGF-2 bind to two transmembrane receptors, IGF-1R and IGF-2R[65-67]. IGF-1 binds to IGF-1R expressed on ECs to directly stimulate ECs proliferation, migration and tubule formation. IGF-1/ IGF-1R signaling pathway has been shown to modulate angiogenesis by stimulating the production of VEGF-A[65-67].

There are four ligands (PDGF-A, -B, -C, -D) in PDGF family, and two receptor subtypes, PDGFR-α and PDGFR-β. PDGF-A and PDGF-B monomers can dimerize with each other to form functional dimers. PDGF-BB is mostly expressed on vascular endothelium, whereas PDGFR-β is mainly expressed in pericytes, vSMCs, and mesenchyme surrounding blood vessels. PDGFs may play an important role in regulating angiogenesis. Recent studies have provided experimental evidence for the role of PDGFs in tumor angiogenesis and metastasis. It has been reported that FGF2 and PDGF-BB can synergistically promote murine tumor neovascularization and

metastasis[59,68,69].

1.1.2 Angiogenic inhibitors

1.1.2.1 Bevacizumab

Bevacizumab (trade name Avastin**)** is a humanized VEGF neutralizing monoclonal antibody that has been widely used for treatment of various human cancers, including ovarian, lung, renal cell carcinoma (RCC) and glioblastoma[70-77]. Bevacizumab is the first clinically available angiogenesis inhibitor in the United States. Its main action is to inhibit the function of VEGF in angiogenesis. Bevacizumab binds directly to VEGF to form a protein complex of bevacizumab/VEGF complex to reduce available VEGF which is capable of further binding to VEGF receptor sites.

1.1.2.2 Ramucirumab

Ramucirumab is a fully humanized anti-human VEGFR-2 neutralizing monoclonal antibody being developed for the treatment of solid tumors. Ramucirumab directly binds to VEGFR-2 to work as a receptor antagonist blocking the binding of VEGF to VEGFR-2. Ramucirumab is being tested in several phase III clinical trials for the treatment of metastatic gastric adenocarcinoma, breast cancer and non-small cell lung carcinoma[18,78,79].

1.1.2.3 Sunitinib

Sunitinib is a small-molecule and multi-targeted receptor tyrosine kinase (RTK) inhibitor that is approved by the FDA for the treatment of solid tumors, including RCC and gastrointestinal stromal tumor. Sunitinib inhibits cellular signaling by targeting RTKs, including VEGFRs, to inhibit both tumor angiogenesis and tumor cell proliferation[80].

1.1.3 Hypoxia and angiogenesis

Oxygen availability is very important because most mammalian cells rely on aerobic metabolism to sustain energy production. Hypoxia is an effective driving force for angiogenesis, which represents a compensable mechanism against tissue ischemia. Sprouting angiogenesis is initiated in poorly perfused tissues when hypoxia in the tissue demands the formation of new blood vessels to satisfy the metabolic requirements of parenchymal cells. Hypoxia often triggers an angiogenic response mostly *via* the HIF signaling pathway. In hypoxic conditions, HIF prolyl-hydroxylase is inhibited and HIF1-α is stabilized, thus VEGF transcription is activated and up-regulated[81].

The HIF family comprises HIF1-α, HIF2-α, HIF3-α and HIF1-β or ARNT124. HIF1-α is important for acute responses to hypoxia. In clinics, many tumors are constantly hypoxic due to poorly perfused and low quality blood vessels because tumors express high levels of VEGF that significantly contributes to high degrees of leakiness and tortuosity of the tumor vasculature. Tumor hypoxia plays an important role in tumor cell dissemination and distant metastasis. However, the molecular mechanisms and detailed processes underlying hypoxia-associated metastasis remain poorly understood[13-16,82-84].

1.2 LYMPHANGIOGENESIS

Lymphangiogenesis is the process of the growth of lymphatic vessels. Whereas the mechanisms of angiogenesis have been studied extensively, relatively little is known about the molecular mechanisms regulating lymphangiogenesis. The research on lymphangiogenesis was limited maybe due to the lack of immunohistochemical, accurate and specific markers to detect lymphatic endothelial cells (LECs). This limitation was improved recently with the identification of novel markers specifically expressed on LECs, such as VEGFR-3, lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), prospero related homebox gene-1 (Prox-1) and podoplanin[85-89].

1.2.1 Specific markers of lymphatic vessels

1.2.1.1 Vascular endothelial growth factor receptor-3

VEGFR-3 was the first specific marker for lymphatic vessels in both normal and pathological tissues including tumors. VEGFR-3 is activated by VEGF-C and VEGF-D, both members of the VEGF family[28,48]. In adults, VEGFR-3 is expressed on the inner surface of lymphatic vessels. However, VEGFR-3 is also expressed in embryonic blood vascular endothelium. The expression of VEGFR-3 is up-regulated in blood vessels during pathological conditions, including inflammation, wound healing and tumor growth[90]. VEGFR-3 is still expressed in other tissues, such as endocrine glands, monocytes, macrophages and dendritic cells.

1.2.1.2 Lymphatic vessel endothelial hyaluronan receptor-1

LYVE-1, a CD44 homolog, is expressed in a subset of ECs in the large central veins and currently provides the first indicator of lymphatic endothelial competence. LYVE-1 is one of the most specific and widely used lymphatic endothelial markers[91,92]. The expression of LYVE-1 is largely restricted to lymphatic endothelium. LYVE-1 is expressed on both the lumenal and ablumenal surfaces of lymphatic endothelium, and also on hepatic blood sinusoidal endothelia. Potential roles of LYVE-1 have been suggested in hyaluronan (HA) transport and turnover, or in promoting HA localization to the surfaces of lymphatic endothelium. HA is a key mediator of cell migration during embryonic development. It is also important in adult processes such as wound healing and tumor metastasis[93]. However, further studies are required to explore the function of LYVE-1 in HA-transport and tumor metastasis.

1.2.1.3 Prospero related homebox gene-1

Prox-1 is specific for lymphatic vessels in the vascular system. Although Prox-1 was also found expressed in non-endothelial cells of the heart, liver and nervous system, the expression of Prox-1 persists in adult lymphatic endothelium[94]. Prox-1 has been

reported to be a transcription factor involved in the budding and elongation of lymphatic vessels sprouts[89,95].

1.2.1.4 Podoplanin

Podoplanin is a glomerular podocyte membrane mucoprotein required for lymphatic development[96,97]. Podoplanin is expressed strictly in lymphatic endothelium, but not in the blood vasculature[98]. In the lymphatic system, podoplanin is expressed in small lymphatic capillaries lined by a single layer of LECs. Podoplanin knockout mice have defects in lymphatic vessel and die at birth due to respiratory failure[99].

1.2.2 Functions of the lymphatic vascular system

The lymphatic vascular system comprises a tree-like hierarchy of capillaries, collecting lymphtic vessels, and the right and left ducts[100]. Lymphatic capillaries are blind ended, irregularly shaped, and larger in diameter than blood capillaries, lined by a single layer of non-fenestrated LECs[101]. Unlike blood capillary endothelium, LECs have poorly developed junctions with large frequent inter-endothelial gaps. LECs lack continuous basement membranes, and adjacent cells lack junctions and instead overlap at their edges. Lymphatic capillaries harbour discontinuous or completely absent basement membranes, and are not invested by pericytes or vSMCs. The abluminal surfaces of LECs are attached to the extracellular matrix *via* elastic anchoring fibers. These elastic anchoring fibers keep the lymphatic vessels from collapsing and also promote their dilation to allow the flow of interstitial fluid to the lymphatics[102].

The lymphatic capillaries are responsible for the uptake of lymph, which consists of interstitial fluid, macromolecules and cells. The physiological function of lymphatic vascular system is to drain excess fluid, and then transport them to the blood vessels for circulation. When fluid accumulates and interstitial pressure increases, the anchoring fibers separate the LECs, generating gaps between adjacent cells and allowing for fluid drainage into the lymphatic vessel. From the capillaries, the lymph is transported to the collecting lymphatics, then ultimately into the venous circulation

via the thoracic duct[103-105].

The lymphatic system is also concerned with immune functions by filtering lymph through a chain of lymph nodes before entering the venous circulation. Lymph nodes are located at intervals along the lymphatic system. Several afferent lymphatic vessels bring in lymph, which percolates through the substance of the lymph node, and is drained out by an efferent lymphatic vessel[103.]

Lymphangiogenesis occurs during both normal development and pathological conditions, including inflammation, lymphedema and metastasis of tumors[106-108]. The lymphatic vessels serve as a major route for metastatic spread of tumor cells from the primary site to regional lymph nodes, then possibly to distant organs[86,88,109-118]. However, little is known about possible structural and functional differences between healthy lymphatic vessels and tumoral lymphatic vessels. It is well known that tumor blood vessels usually consist of disorganized, leaky and tortuous vasculatures. Recent studies have demonstrated that peritumoral and intratumoral lymphatic vessels also consist of disorganized and leaky microvessels that might lack drainage function[108]. The structural irregularity and leaky features of tumoral lymphatic vessels might make them more susceptible for invasion by malignant cells[119].

1.2.3 Lymphangiogenic factors

VEGF-C and VEGF-D were the first identified growth factors to activate LECs and stimulate lymphangiogenesis[120,121]. Recently, several new lymphangiogenic factors have been found. These lymphangiogenic factors appear to be functionally important for the development of the lymphatic system.

1.2.3.1 Vascular endothelial growth factor family

Among all known lymphangiogenic factors, VEGF-C and VEGF-D are the most potent lymphangiogenic factors, which play essential roles in regulation of physiological and pathological lymphangiogenesis. VEGF-C is a member of the VEGF family that are critical mediators of angiogenesis and lymphangiogenesis[122].

VEGF-C directly stimulates the migration and proliferation of LECs *in vitro*, and stimulates lymphatic vessel growth *in vivo*. VEGF-C contributes to formation and maintenance of lymphatic vascular systems. During early embryogenesis, VEGF-C plays an important role in the development of the lymphatic system. It is expressed along with its receptor VEGFR-3 in regions where the initial lymphatic vessels sprout and develop.

VEGF-C appears to play a role in inflammatory responses. It has been shown to induce lymphatic vessel growth in response to pro-inflammatory cytokines[123]. Both VEGF-C and VEGFR-3 are prominently expressed by activated macrophages[122].

VEGF-D binds to the same receptors as VEGF-C, VEGFR-2 and VEGFR-3. During early embryogenesis, VEGF-D is mostly expressed in the developing lung and skin, where it is thought to play a modifying role in lymphangiogenesis during embryonic development. In adults, VEGF-D is expressed in numerous tissues, including lung, heart and skeletal. VEGF-D appears to promote solid tumor growth and lymph node metastasis. It is a poor prognostic marker for many cancers in patients, such as colorectal, ovarian, prostate and lung cancers[124-127]. In experimental tumor models, VEGF-D induces growth of intratumoral lymphatics and promotes lymphatic metastasis[128]. Both VEGF-C and VEGF-D have direct effects on LECs *in vitro,* and have been shown to induce tumoral lymphangiogenesis and promote lymphatic metastasis when these factors are expressed at high levels in various human cancers[124-127,129-133].

VEGFR-3 is the LECs specific tyrosine kinase receptor which activates lymphangiogenic signals. In adults, VEGFR-3 expresses mainly on lymphatic endothelium[28]. VEGFR-3 was originally thought to be expressed specifically on the lymphatic endothelium[134], but further studies demonstrated that VEGFR-3 is up-regulated in the neovasculatures of tumors[120]. Both VEGF-C and VEGF-D directly bind to VEGFR-3 acting on LECs to induce cell proliferation and migration. It is reported that VEGFR-3 can form heterodimers with VEGFR-2 upon binding of VEGF-C and VEGF-D, which may lead to unique combinatorial signals by the intracellular domains of the two receptors[135].

The VEGF-C/-D/VEGFR-3 signaling system is involved in the formation of the first lymphatic vessels in the embryonic development. The VEGF-C/-D/VEGFR-3 signaling pathway plays a central role in regulation of physiological and pathological lymphangiogenesis[136]. Several indirect lymphangiogenic factors might induce lymphangiogenesis *via* activation of the VEGF-C/-D/VEGFR-3 signaling pathway[45,108].

VEGF-A is a key angiogenic factor over-expressed in most of human cancers. VEGF-A can bind to VEGFR-2 to promote angiogenesis. VEGF-A has recently been found to stimulate lymphangiogenesis in various animal models[137]. VEGF-A inducing lymphangiogenesis suggests that this factor may not only have a major impact on tumor progression and angiogenesis, but also on tumor lymphatic metastasis. VEGF-A-overexpressing tumors generally have a very fast growth rate due to the robust angiogenic response induced[138].

VEGF-A appears to promote new lymphatic sprouts from the pre-existing limbal lymphatic vessel in the lymphangiogenesis model. In the mouse corneal model, VEGF-A expressing viral vectors promoted the growth of functional lymphatic networks[24].

VEGF-A might promote lymphangiogenesis *via* both direct and indirect effects on LECs. VEGF-A downstream signaling cascade is mediated principally *via* VEGFR-2. VEGFR-2 was previously considered to be expressed exclusively on vascular endothelium. However, it has recently been shown that lymphatic endothelium also expresses VEGFR-2[139]. VEGF-A promotes survival, proliferation and migration of LECs *via* VEGFR-2[24].

1.2.3.2 Fibroblast growth factor family

FGF family involves in angiogenesis and lymphangiogenesis. FGF-2 has been shown to promote LECs proliferation, migration and assembly into capillary-like tube structures *in vitro*. *In vivo,* FGF-2 has been reported to stimulate lymphatic vessel growth indirectly *via* up-regulation of VEGF-C expression in vascular endothelial and perivascular cells in the cornea assay[24,46,140]. Blockage of VEGFR-3 signalling

suppresses FGF-2-induced lymphangiogenesis demonstrating that FGF-2 might stimulate lymphangiogenesis in dependent on VEGFR-3 signalling pathway *in vivo* [24,46,140].

FGFs/FGFR-1 plays an important role in the development and maintenance of a mature vascular network in the embryo. FGFR-1-mutant mouse embryos are developmentally retarded or even die during gastrulation stage. In recent years, some intimate cross-talk exists between FGF-2 and members of the VEGF-family during vasculogenesis, angiogenesis and lymphangiogenesis have been reported[24,46,140]. In paper II, we found that co-implantation of micropellets containing VEGF-C plus FGF-2 resulted in angiogenic synergism in the corneal tissue, suggesting that VEGF-C and FGF-2 collaboratively promote corneal angiogenesis and lymphangiogenesis. FGF-2 directly promoted LEC proliferation and migration via activation of the FGFR-1–mediated signaling pathway *in vitro*, which demonstrated that FGF-2 is a direct lymphangiogenic factor. Interestingly, this FGF-2–induced lymphangiogenesis was completely inhibited by VEGFR-3 blockade *in vivo.* We found that the tip cell formation at the leading front of growing lymphatic vessels induced with FGF-2 could be completely inhibited by VEGFR-3 blockade (paper II).

1.2.3.3 Other lymphangiogensis factors

IGF family has been demonstrated to induce lymphangiogenesis in several systems. For example, IGF-1 and IGF-2 induce lymphangiogenesis in a mouse cornea assay[141]. Interestingly, IGF-1R signalling was recently shown to positively regulate the expression of VEGF-A, VEGF-C and FGF-2[142]. This suggested that the IGF family might induce lymphangiogenesis, at least through an indirect mechanism. The IGF-1R is expressed in most tissues, including vascular ECs. Both IGF-1 and IGF-2 act *via* IGF-1R to stimulate EC proliferation, migration and tube formation[143]. IGF-1R-activation might indirectly induce intratumoral lymphatic vessel growth and thereby promote lymphatic metastasis.

In PDGF family, PDGF-AA, -AB and -BB are able to promote lymphangiogenesis, while PDGF-BB is the most potent lymphangiogenic factor within this family[69]. PDGF-BB induces cell migration of LECs, suggesting its direct role in

lymphangiogenesis. Overexpression of PDGF-BB tumor tissues stimulates the growth of both intra- and peritumoral lymphatic vessels, resulting in increased lymphatic metastasis. The PDGFR-β has been detected in cancer patients with lymphangiomatosis, suggesting the clinical relevance of the PDGF/PDGFR signalling pathway in lymphatic malignancy and metastasis[144].

Ang-1 and Ang-2 were recently suggested to play the roles in the development of functional lymphatic vessels. Both Ang-1 and Ang-2 bind to tie-2 receptor expressed on LECs, indicating direct effects of Ang-1 and Ang-2 on lymphangiogenesis. Ang-2 knockout mice display defects in the patterning and function of the lymphatic vasculature[64].

Hepatocyte growth factor (HGF) is reported to promote lymphangiogenesis[145]. C-Met is the receptor of HGF expressed in the newly formed lymphatic vessels of inflammatory tissues although quiescent lymphatics do not express this receptor. HGF involved in inducing the dilation of peritumoral lymphatics and lymphatic metastasis[146-149].

1.2.4 Lymphangiogenic inhibitors

Current development of lymphangiogenic inhibitors is mainly focused on the VEGF-C/VEGF-D/VEGFR-3 signalling pathway, including neutralization of VEGF-C and VEGF-D with soluble VEGFR-3[150], and anti-receptor antibodies to block VEGFR-3[151]. In this thesis, we showed that both FGF-2– and VEGF-C– induced lymphangiogenesis were completely inhibited by VEGFR-3 blockade, an anti-receptor antibody, in mouse models. In addition, it was reported that several drugs, such as celecoxib (Celebrex; Pfizer) and rofecoxib (Vioxx; Merck), are powerful inhibitors of lymphangiogenesis[119]. However, the therapy of lymphangiogenesis needs to be further studied.

1.2.5 Inflammation and lymphangiogenesis

Inflammation is associated with many physiological and pathological conditions,

including wound healing and tumor development. There is increasing evidence that inflammation contributes to angiogenesis, lymphangiogenesis and tumor metastasis[152-158]. Whereas the role of inflammation in angiogenesis is well studied, less is known about how acute and chronic inflammation contributes to lymphangiogenesis.

It has been reported that several inflammatory cytokines might induce VEGF-C and VEGF-D expressions in several cell types via the downstream of NF-κB pathway. Inflammatory cells, such as macrophages attracted by inflammatory cytokines in several mouse models, have been shown to produce high level of lymphangiogenic factors, including VEGF-C and VEGF-D, to induce lymphangiogenesis. Several causative factors, such as interleukin-1 beta, transforming growth factor-beta, tumor necrosis factor-alpha and macrophage colony-stimulating factor, may be actively involved in macrophage-induced lymphangiogenesis[157,159-161]. There is growing evidence that macrophages play an important paracrine role in the growth of lymphatic vessels. The paracrine role of macrophages involves their secretion of VEGF-C and VEGF-D in stimulating lymphangiogenesis from pre-existing lymphatics. Lymphangiogenesis is also associated with chronic inflammation, such as Crohn's disease or rheumatoid arthritis[162].

Inflammatory responses in tumor tissues have been associated with malignant progression and lymphatic metastasis. Infiltration of inflammatory cells into tumor tissues might induce the growth of intratumoral lymphatic vessels, which in turn might promote lymphatic metastasis[157,159-161].

Alteration of macrophage phenotype and function has profound the effect on the development and progression of inflammation and malignancy. Macrophage depletion for controlling lymphangiogenesis may provide a novel approach for prevention and treatment of lymphatic-associated diseases. Moreover, the inhibitors of inflammatory pathways might suppress lymphangiogenesis and lymphatic metastasis in tumor. Anti-inflammatory drugs, such as COX-2 inhibitors and non-steroid anti-inflammatory drugs have been reported to have potent anti-lymphangiogenesis activity in tumor models[163].

1.3 CANCER

More than 35 years ago, Dr. Judah Folkman proposed that tumor growth and spread are dependent on angiogenesis. Today, angiogenesis is well known to promote growth, development and metastasis of tumors. It is widely accepted that in the absence of blood vessels tumors can not grow beyond the size of a few mm^3 or metastasise to distant organs[5,7,18]. In addition to hemangiogenesis, various types of tumors often contain lymphatic vessels, which may facilitate lymphatic metastasis. However, the role of tumor lymphatic vessels in promoting metastasis remains poorly understood. Cancer metastasis employs complex processes that are collectively termed as the metastatic cascade, which involves multiple-step defined mechanisms. A clinical detectable metastatic mass represents the ultimate consequence of the complex metastatic cascade that includes dissemination of tumor cells from the primary site; intravasion of tumor cells into the circulation or lymphatic system; transport of tumor cells along blood circulation or lymphatic system to distal tissues or organs; extravasation of tumor cells from the circulation of lymphatic system; formation of the primary metastatic niche in distal tissues; manipulation of metastatic microenvironment; and regrowth of metastatic nodules to a visible metastatic mass.

1.3.1 Tumor angiogenesis

Angiogenesis is well known to promote growth, development and metastasis of tumors. Metabolism and growth of cells require adequate oxygen and nutrition supply, as well as removal of metabolites. Tumor-derived angiogenic factors act in a paracrine manner to recruit blood vessels from the surrounding stroma into the tumor to supply oxygen and nutrition, allowing survival and further growth of the tumor[13]. VEGF is one of the key angiogenic factors that contributes to the onset and progression of tumors. In tumor environment, ischemia and hypoxia in tumor tissue result in marked production of VEGF-A followed by a new robust angiogenic response.

The vasculature in tumor tissue is structurally and functionally abnormal, highly disorganized, tortuous, dilated and leaky[164]. In addition, ECs within tumor vessels are irregularly shaped and disorganized. Tumor vessels have widened interendothelial junctions, numerous openings in their walls, and a discontinuous or even totally

absent basement membrane, resulting in the intravasion of tumor cells into the circulatory system, and thus allow metastatic dissemination of invasion-competent cells[165].

In clinics, many tumors are constantly hypoxic due to poorly perfused and low quality blood vessels because tumors express high levels of VEGF. Then VEGF might induce high degrees of leakiness and tortuosity of the tumor vasculature[166]. In paper I, we showed a hypoxic zebrafish model to monitor tumor cell dissemination, invasion and metastasis in living fish at the single cell level. We found that tumor hypoxia played an important role in tumor cell dissemination and distant metastasis.

1.3.2 Tumor lymphangiogenesis

In addition to angiogenesis, various types of tumors often contain lymphangiogenesis, which may facilitate lymphatic metastasis. A clinically detectable cancer metastasis in lymph nodes employs complex processes, including the dissemination of tumor cells from the primary site to the lymphatic vessels, the transport of tumor cells along lymphatic system, the extravasation of tumor cells from the lymphatic system to the lymph nodes, the settlement of tumor cells in lymph nodes, and the growth of the metastatic lesion to a detectable mass.

Regional lymph node metastasis represents the first step of tumor dissemination for cancers, which is reported in breast cancer, colon cancer and prostate cancer. Regional lymph nodes metastasis of malignant tumors is one of the early signs of cancer spread in patients. In clinics, the extent of lymph node metastasis is a major determinant for the staging and the prognosis of cancer patients, which often guides therapeutic decisions. It is reported that the density of tumoral lymphatic vessels correlates with the incidence of lymph node metastasis and poor prognosis in some human cancers[167-176]. It is known that tumor cells can gain access to the lymphatic system either by inducing the growth of intratumoral lymphangiogenesis or by inducing the growth and dilation of peritumoral lymphangiogenesis during tumor expansion *via* growth factor production, such as VEGF[88,125,142,177,178].

However, the role of tumor lymphangiogenesis in facilitating metastasis remains

poorly understood. Recently, the improved lymphatic-specific markers such as VEGFR-3, lymphatic LYVE-1, Prox-1 and podoplanin have made it possible to study the formation of tumor-associated lymphatic vessels, and to investigate the contributions and relationship of different factors in inducing lymphangiogenesis and lymphatic metastasis.

It is well known that VEGF-C and VEGF-D bind to VEGFR-3 to induce tumor lymphangiogenesis and promote the formation of lymph node metastasis[179,180]. This VEGF-C/-D/VEGFR-3 signaling pathway plays the central role in regulation of lymphangiogenesis and lymphatic metastasis[136]. The other indirect lymphangiogenic factors maybe induce lymphangiogenesis *via* activating the VEGF-C/-D/VEGFR-3 signaling pathway. Conversely, inhibitors of the VEGF-C/-D/VEGFR-3 signalling pathway, such as VEGFR-3 blocking antibodies, may have the potential to block lymphatic tumor spread[119].

In the tumor microenvironment, tumor cells, stromal cells and inflammatory cells produce multiple lymphangiogenic factors that might stimulate tumoral lymphangiogenesis. 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, PDGF family and IGF family. Various angiogenic factors often co-exist in the tumor microenvironment and they often cross-communicate with different signaling pathways. Although the individual roles of various angiogenic factors in promoting angiogenesis and lymphangiogenesis are relatively well studied, the interplay between them in the tumor environment remains poorly understood. In the paper II of this thesis, we chose FGF-2 and VEGF-C, two commonly expressed potent angiogenic factors, to investigate the interplay between them in promoting lymphangiogenesis and lymphatic metastasis (Figure 2).

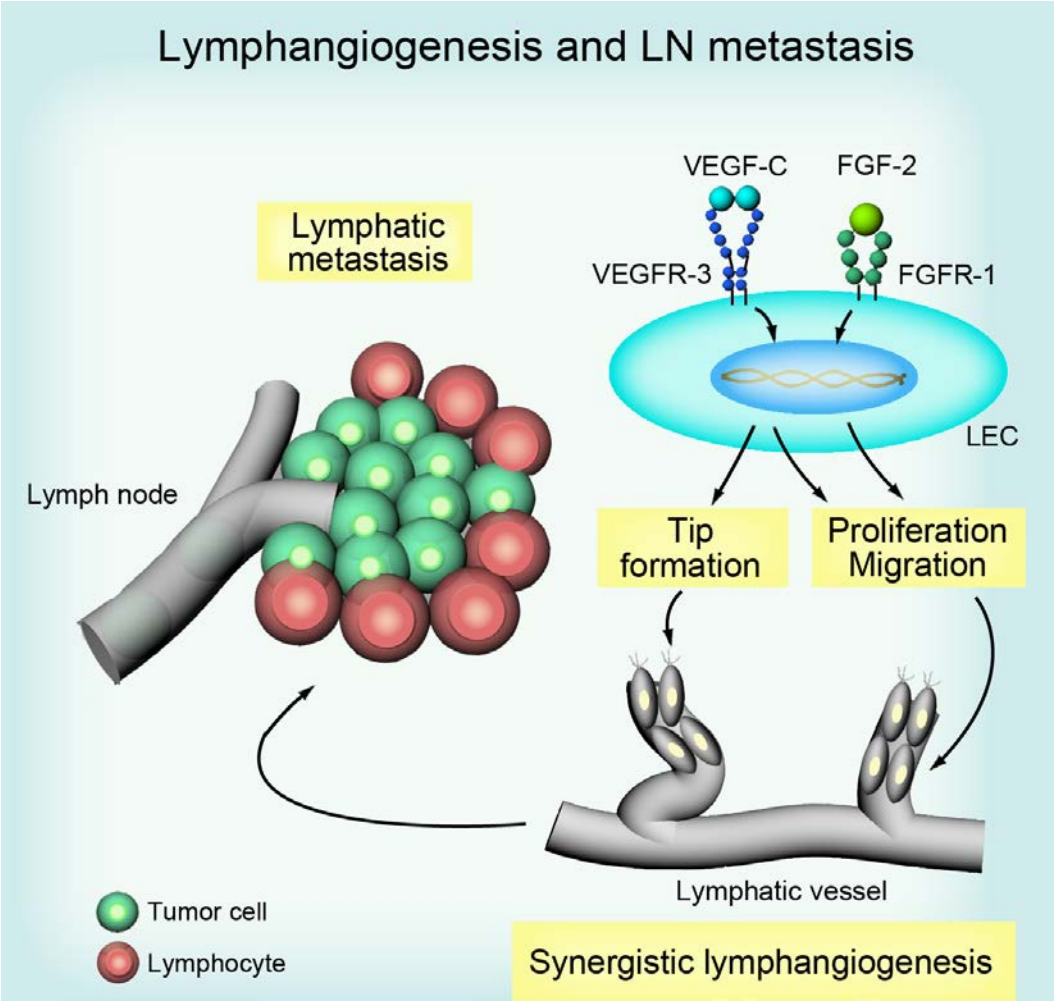

Figure 2. FGF-2 and VEGF-C collaboratively promote tumor lymphangiogenesis and lymphatic metastasis. FGF-2 activates FGFR-1 on LECs to stimulate LEC proliferation and migration. VEGF-C activates VEGFR-3 receptor on LECs, leading to LEC tip formation, proliferation and migration. VEGFR-3–triggered tip cell formation is a prerequisite for FGF-2–induced lymphangiogenesis. (adapted from paper II).

Although it is well known that induction of tumor angiogenesis in solid tumors is to supply oxygen and nutrients for tumor cells and removes metabolic waste products, it is poorly understood how and why tumors induce lymphangiogenesis. Compared to the blood vascular system, the lymphatic system appears to be more accessible for tumor cell dissemination. Unlike blood capillaries, lymphatic capillaries consist of a single, thin LECs layer. LECs have poorly developed junctions with frequent large inter-endothelial gaps. LECs lack a continuous basement membrane, and adjacent cells lack junctions and instead overlap at their edges. In addition, lymphatic capillaries are not coated with pericytes or vSMCs. These structural features of

lymphatics make them more accessible for intravasion of tumor cells into the lymphatic system and extravasation of tumor cells from the lymphatic system to regional lymph nodes to establish metastatic niche in distal tissues. In addition, many solid tumors have increased interstitial fluid pressure (IFP). The lymphatic system may lower IFP in solid tumors to increase blood perfusion to facilitate tumor growth. Furthermore, the interaction between LECs and tumor cells has been shown to facilitate tumor-cell invasion into the lymphatics. LECs produce cytokines that can guide the tumor cells toward the lymphatic vessels. For example, CCL21 produced by the LECs appears to attract some tumor cells that express its receptor CCR7 into lymphatic vessels[181-184]. Lymphangiogenesis might contribute to tumor growth, invasion and metastasis by several mechanisms. The biology and mechanism of tumor lymphangiogenesis and lymphatic metastasis need to be studied further.

2 AIMS

I. To develop a novel zebrafish model to study the early steps of the metastatic cascade (Paper I).

II. To study the interplay between FGF-2 and VEGF-C in promoting lymphatic metastasis (Paper II).

III. To develop a unique *in vivo* model to study lymphangiogenesis induced by various factors (Paper II -III).

IV. To study the impact of clinical available antiangiogenic drugs on healthy vasculatures and revealed potential sites for antiangiogenic drug-related side effects (Paper IV).

3 METHODS

3.1 Zebrafish tumor model

In paper I, we develop a zebrafish metastatic model to take the advantage of the transparent nature of zebrafish embryos to study molecular mechanisms of tumor cell invasion, dissemination and metastasis in association with angiogenesis and hypoxia in single cell level *in vivo*. The EGFP positive vasculature of transgenic *Tg(fli1:EGFP)* zebrafish embryos is very clear under microscope. Morpholino was used for reverse genetics to silence host gene functions. We developed a semi-closed system where the water was pre-calibrated to a particular oxygen level to established hypoxic conditions to the zebrafish. We implanted murine T241 fibrosarcoma cells, Lewis lung carcinoma (LLC), or human tumors cells into the perivitelline cavity of two days old fish embryos of transgenic *Tg(fli1:EGFP)* zebrafish under normoxic or hypoxic conditions. The embryos were incubated with water containing 0.2 mM 1-phenyl-2-thio-urea (Sigma) to prevent pigmentation. The two days old zebrafish embryos were anesthetized and injected with glass capillaries needles in microinjector. The tumor cells were labeled with the red fluorescent dye DiI before injection. Injected embryos were examined every other day for monitoring tumor growth and invasion using a fluorescent microscope (Nikon Eclipse C1, Japan).

3.2 Mouse tumor model

Different kinds of mice models have been used to investigate tumor growth and metastasis. In paper II, tumors were grown syngeneically to study the interplay of FGF-2 and VEGF-C on tumor growth, tumor-associated angiogenesis, lymphangiogenesis and metastasis. Approximately 1×10^6 tumor cells were subcutaneously implanted in the central position along the dorsal midline of each mouse. Half the numbers of FGF-2– and VEGF-C–tumor cells were mixed for co-implantation experiments (six mice per group). Tumor growth was measured every other day and tumor volumes were calculated. Then the subcutaneously grown primary tumors located at the middle dorsum were surgically removed at the size of 1.5 cm^3 under anesthetic conditions (within the ethical limit). Open wounds were

sutured and mice were given painkiller in two consecutive days (Temgesic, 0.1 mg/kg, twice a day). Typically, two weeks after tumor removal, visible sentinel lymph node metastases were detectable in bilateral subaxillary lymph nodes of VEGF-C– or FGF-2 plus VEGF-C tumor-bearing mice. At this time point, tumor-bearing mice were sacrificed and various organs, including lymph nodes, lung, liver, spleen, kidney and brain were collected for histological analysis.

A number of histological techniques are used to investigate angiogenesis and lymphangiogenesis in tumor tissue, such as H&E staining for general morphology, CD31 staining for blood vessel density, and LYVE-1 staining for lymphatic vessel density.

3.4 Mouse corneal model

We developed a mouse corneal model to study lymphangiogenesis induced by various factors. The steps of this study include: preparation of micropellets, corneal implantation, immunohistochemistry and data analysis. Firstly, we created a micropocket in the cornea of each mouse. Then we inserted a micropellet into the corneal micropocket. Corneal angiogenic responses were detected on day 5 or 6 after implantation. Unlike blood vessels, lymphatic vessels are not perfused with red blood cells and thus remain invisible under light microscopy. So we used immunohistochemical staining of corneal tissues with lymphatic-specific markers, such as LYVE-1, to study the fine structure of lymphatic microvessels. After careful dissection, the entire corneal tissue should be used for whole-mount staining with double or triple immunostaining using combinations of several antibodies specific for blood and lymphatic vessels, to study the relationships between blood vessels and lymphatic vessels. At last, the stained corneal tissues should be analyzed using a multichannel confocal microscope that detects positive signals in different colors.

3.5 Confocal microscopy of whole-mount specimens

In this thesis, we mainly used a combination of CD31 and LYVE-1 antibodies in whole mount staining to detect blood- and lymphatic vessels in mouse corneas (paper II, III) and tumor tissues (paper II). To further confirm lymphatic vessel identity, we

have co-stained the corneal tissue with other specific markers such as VEGFR-3 (paper III).

Laser Scanning Confocal Microscopy is an advanced microscopic technique routinely used for generating high resolution images and three-dimensional reconstructions of vasculars. Immunostained positive signals and EGFP positive tumor cells were detected using a Nikon C1 Confocal microscope (Nikon) or a Zeiss Confocal LSM510 microscope (Carl Zeiss). For 3D images of each dataset, scanning five to six layers were assembled using a confocal microscope software program (EZ-C1).

4 RESULTS

4.1 HYPOXIA-INDUCED ANGIOGENESIS STIMULATES METASTASIS IN A ZEBRAFISH TUMOR MODEL (PAPER I)

Tissue hypoxia is known to significantly contribute to tumor invasion and metastasis[185-188]. Hypoxia is also an effective driving force for angiogenesis which is not only essential for primary tumor growth but also facilitates tumor invasion and metastasis. However, the mechanisms and detailed processes underlying hypoxia-associated metastasis remain poorly understood.

Our aim was to take the advantage of the transparent nature of zebrafish embryos to visualize under normoxic and hypoxic conditions marked human or mouse tumor cell migration and invasion in association with tumor angiogenesis. This zebrafish metastatic model allows us to monitor dissemination of single tumor cells from primary sites in the living body to study the role of hypoxia in tumor invasion and metastasis.

In this study, we implanted murine T241 fibrosarcoma cells into the perivitelline cavity of two days old fish embryos of transgenic *fli1:EGFP* zebrafish under normoxia or hypoxic conditions. The cells were labeled with the red fluorescent dye DiI before injection. After exposing the embryos to 7.5 % air saturation for three days, we observed significant numbers of tumor cells disseminated from primary sites, invaded into neighboring tissues, and metastasized to distal parts of the fish body under hypoxic conditions in contrast to normoxic conditions. In addition, we found that hypoxia significantly stimulated neovascularization and tortuosity of the tumor vasculature.

Similarly, overexpression of VEGF-A by the transfected tumor cells, which is well-correlated with increased neovascularization and tortuosity of the tumor vasculature, also led to tumor cells dissemination and metastasis. Furthermore, we found that inhibition of VEGF receptor signaling pathways by sunitinib or VEGFR-2 morpholinos abrogated the VEGF-induced tumor cell dissemination and metastasis. All these results were similarly repeated with another murine tumor cell line, LLC.

Finally, we used this zebrafish model to study the role of hypoxia-induced angiogenesis in mediation of tumor cell dissemination and metastasis. We used sunitnib to inhibit VEGF receptors under hypoxia, and found that VEGFR blockade effectively blocked hypoxia-induced tumor angiogenesis and metastasis.

We also used this tumor model to study the early events of dissemination and metastasis of human tumor cells. We chose the low metastatic human ovarian carcinoma cell line OVCAR 8 and highly metastatic human MDA MB 231 breast cancer cell line. Our results showed that most of the injected MDA MB 231 cells disseminated whereas OVCAR 8 mostly grew in situ. The different capacities of dissemination resulted in difference of metastatic potentials between these two cell lines.

These findings demonstrate that hypoxia- and VEGF-induced pathological angiogenesis can promote tumor dissemination, invasion and metastasis in the zebrafish model. Furthermore, this zebrafish model might be used to discriminate high and low metastatic potentials of human cancers and to predict prognosis, and provide compelling evidence on the beneficial effects of clinically available anti-VEGF drugs for cancer therapy.

4.2 THE INTERPLAY BETWEEEN FGF-2 AND VEGF-C IN PROMOTING LYMPHANGIOGENESIS AND LYMPHATIC METASTASIS (PAPER II)

In addition to hemangiogenesis, various types of tumors often contain lymphatic vessels, which may facilitate lymphatic metastasis. The entire metastatic process is tightly linked to the intimate interactions between tumor cells and hemvascular and lymphvascular systems.

Tumors can produce various angiogenic factors to stimulate angiogenesis and lymphangiogenesis. Various angiogenic factors often co-exist in the tumor microenvironment and they often cross-communicate with different signaling pathways. Although the individual roles of various angiogenic factors in promoting angiogenesis, lymphangiogenesis and tumor metastasis are relatively well studied, the

interplay between them in the tumor environment remains poorly understood. To study interplay between various angiogenic factors in promoting lymphangiogenesis, we chose FGF-2 and VEGF-C, two commonly expressed angiogenic factors for our study.

Both VEGF-C and FGF-2 are potent angiogenic factors commonly expressed in various tumor tissues. The expression levels of VEGF-C and FGF-2 have been correlated with tumor growth, progression and metastasis[189]. In this study, we investigated the interplay between FGF-2 and VEGF-C in promoting lymphangiogenesis and metastasis in various *in vivo* models.

We established a corneal lymphangiogenesis assay to study the factor-induced lymphangiogenesis and vascular structures in the avascular corneal tissue. In brief, FGF-2, VEGF-C, or FGF-2 plus VEGF-C with a slow-release polymer composed of sucralfate and hydron, were implanted into the mouse cornea. Then the corneas were dissected at day 6 and double immunostained with CD31 and LYVE-1 antibodies.

As expected, both VEGF-C and FGF-2 were also able to stimulate corneal angiogenesis and lymphangiogenesis. Interestingly, co-implantation of micropellets containing VEGF-C plus FGF-2 resulted in angiogenic synergism in the corneal tissue, which suggested that VEGF-C and FGF-2 collaboratively promote corneal angiogenesis and lymphangiogenesis. In contrast, implantation of the slow-release polymer soaked in PBS, as the negative control, did not induce angiogenesis or lymphangiogenesis, and the only vessels detected in these negative control corneas were the pre-existing lymphatics of the limbus.

We used LECs to do *in vitro* proliferation and migration assays to study the cellular basis of lymphangiogenic synergism between FGF-2 and VEGF-C. Both VEGF-C and FGF-2 potently stimulated human LECs proliferation alone, whereas FGF-2 plus VEGF-C significantly increased cell proliferation. RT-PCR analysis demonstrated that FGFR-1 expressed in LECs is a crucial receptor that mediates the FGF-2-induced lymphangiogenesis, as VEGFR-3 is the crucial receptor of VEGF-C-induced lymphangiogenesis. Our findings also suggested that there was additive lymphangiogenic activity when both FGF-2 and VEGF-C are co-exposed to LECs, which is caused by the significant up-regulation of fgfr-1 and vegfr-3 expression

stimulated by the two factors. The VEGFR-3 signaling system or the FGFR-1 signaling system was blocked by siRNA or anti–VEGFR-3 and anti-FGFR-1 neutralizing antibodies.

These potent anti-lymphangiogenic activities were also observed *in vivo*. FGFR-1 neutralizing antibody significantly inhibited FGFR-2 plus VEGF-C–induced angiogenesis and lymphangiogenesis in corneas. As expected, VEGFR-3 neutralizing antibody completely blocked VEGF-C–induced lymphangiogenesis. Interestingly, VEGFR-3 neutralizing antibody also completely blocked FGF-2–induced lymphangiogenesis in corneas. We were very surprised because the same VEGFR-3 blockade did not significantly affect FGF-2–induced LECs proliferation and migration *in vitro*.

Recently, some groups have reported that the formation of tip endothelial cells is an essential process in angiogeneisis and lymphangiogenesis[151,190]. In our study, we found that both FGF-2 and VEGF-C were able to induce tip formation at the leading front of the growing lymphatics, and coimplantation of these two factors resulted in an additive effect on the tip formation. Additionally, we found that VEGFR-3 blockade completely inhibited all the lymphatic tip formation in the VEGF-C–, FGF-2–, or FGF-2 plus VEGF-C–induced tip formation at the leading front of the lymphatics. These findings suggested that VEGFR-3–induced tip formation is a prerequisite for lymphangiogenesis.

We also used a mouse tumor metastatic model, in which tumors located subcutaneously at the middle dorsum were surgically removed at the size of 1.5 cm^3, to study the angiogenic and lymphangiogenic synergisms of FGF-2 and VEGF-C. The transfected murine fibrosarcoma cell lines which expressed FGF-2 or VEGF-C were subcutaneously implanted in immune deficient SCID mice. Both FGF-2 and VEGF-C significantly promoted tumor growth, hemangiogenesis and lymphangiogenesis. Interestingly, implantation of the same number of tumor cells consisting of 50% FGF-2 and 50% VEGF-C cells to the mice resulted in a markedly accelerated tumor growth, a higher density of disorganized vascular networks including intratumoral lymphatic vessels, and highly dilated peritumoral lymphatics. Additionally, we found more EGFP tumor cells in the dilated peritumoral lymphatics induced by FGF-2 plus VEGF-C.

Furthermore, we studied collaborative potentials of FGF-2 and VEGF-C in promoting hematogenous and lymphatic metastasis. We checked the lungs and sentinel lymph nodes of these tumor-bearing mice. More than 70% of FGF-2 plus VEGF-C tumor-bearing mice had pulmonary metastases, wheares no pulmonary metastasis was detected in FGF-2 tumor-bearing mice, and only less than 40% of VEGF-C tumor-bearing mice had pulmonary metastases. On the other hand, 100% of FGF-2 plus VEGF-C tumor-bearing mice carried metastatic sentinel lymph nodes, and both weights and volumes of these metastatic lymph nodes were significantly bigger than the other groups. Collectively, our results demonstrated that FGF-2 and VEGF-C collaboratively stimulate hematogenous and lymphatic metastasis.

4.3 MOUSE CORNEAL MODEL IN STUDYING LYMPHANGIOGENESIS (PAPER III)

As for lymphangiogenesis, the formation of new lymphatic networks is a multistep process that involves LECs proliferation, migration, lymphatic tube formation, maturation and remodeling, all of which are tightly regulated by lymphangiogenic factors and inhibitors[24,69,191,192]. Studying the inhibition of lymphangiogenesis might potentially offer a new opportunity for the treatment of cancer metastasis because the intratumoral and peritumoral lymphangiogenesis have been associated with cancer metastasis. However, although there are several *in vitro* and *in vivo* assays to study LECs proliferation, migration and tube formation, there has been lacking appropriate and powerful *in vivo* assay systems that allow us to study lymphangiogenesis quantitatively.

In this study, we developed a unique *in vivo* model to study lymphangiogenesis induced by various factors. In comparison with other *in vivo* lymphangiogenesis models, the corneal tissue is avascular for both blood vessels and lymphatic vessels. This model offers us a unique opportunity to study lymphatic vascular formation, structure, stability and remodeling. The avascular feature of the corneal tissue makes this model useful for a quantitative assessment of lymphangiogenesis. We took the advantage of the avascular nature of the mouse corneal tissue and implant various growth factors/cytokines alone or in combinations to quantitatively study

lymphangiogenesis and lymphatic structures. We have described this model in studying the interplay between FGF-2 and VEGF-C in promoting lymphangiogenesis and metastasis (paper II)

As the details described in the methods part of my thesis, the steps of this study include: preparation of micropellets, corneal implantation, and immunohistochemistry and data analysis. This protocol describes a unique *in vivo* animal model which allows us to investigate the lymphangiogenesis in an ideal condition in which the amount of lymphangiogenic factor, the size of the micropellet, the implantation site and the time point of responses are all well defined.

4.4 ANTI-VEGF DRUG-INDUCED VASCULAR ALTERATION IN HEALTHY TISSUES (PAPER IV)

Humanized VEGF neutralizing monoclonal antibodies, such as bevacizumab, have been widely used for treatment of various human cancers, including lung cancer, glioblastoma and RCC. However, systemic impacts of anti-VEGF or anti-VEGFR drugs in host healthy vasculatures remain poorly understood. In paper IV, we chose VEGF and VEGFR specific blockades to study the impact of antiangiogenic drugs on healthy vasculatures and to reveal antiangiogenic drug related side effects.

We used three specific anti-VEGF agents to block VEGF-induced biological activities and study the impact of VEGF specific blockades on vasculatures in various healthy tissues: a rabbit anti-mouse neutralizing monoclonal antibody (BD0801)[193]; a rat anti-mouse VEGFR-1 neutralizing monoclonal antibody (MF-1)[194-196]; and a rat anti-mouse VEGFR-2 neutralizing monoclonal antibody (DC101)[194-196].

We first analyzed vasculatures in endocrine organs, such as thyroid, adrenal cortex and pancreatic islets that are known to express relatively high levels of VEGF. We observed significant reduction of vascular density in thses endocrine organs in response to both anti-VEGF and anti-VEGFR-2 blockades. These data demonstrate that VEGF/VEGFR-2 signaling pathway plays a pivotal role in maintenance of vascular homeostasis in these endocrine organs.

We also studied the impact of antiangiogenic drugs in other healthy tissues. We found

that VEGF and VEGFR-2 specific blockades significantly decreased vascular density in gastrointestinal tracts and the female reproductive system, whereas VEGFR-1 increased vascular density in this tissue. In kidney, liver and pancreatic acini area, anti-VEGF and anti-VEGFR-2 blockade produced a similar vascular regressive phenotype, but anti-VEGFR-1 blockade did not affect the vessel density in these organs.

Interestingly, we found that anti-VEGF-induced thyroid vascular regression was completely reversible after discontinuation of anti-VEGF treatment. The vascular density and architecture returned to nearly the same levels as the untreated animals after two weeks cessation of VEGF blockade.

It is well known that vascular fenestrations are crucial for maintenance of endocrine organ functions[1,197-199]. Therefor, we next investigated the impact of anti-VEGF drugs on alteration of vascular fenestrations and endocrine functions in thyroid gland. As expected, VEGF blockade completely suppressed the formation of endothelial fenestrations in thyroid vessels. This result suggested that VEGF acts as a homeostatic factor for maintenance of vascular fenestrations in thyroid. We next treated the mice with VEGF blockade for a prolonged period of four weeks to further study the functional impact of anti-VEGF treatment in modulation of thyroid gland functions. We found that the circulating level of the predominant thyroid hormone free thyroxine (T4) was significantly decreased in the VEGF blockade-treated mice. The endocrine function of thyroid gland was impaired after prolonged treatment with VEGF blockade.

5 DISCUSSION

5.1 METASTATIC CASCADE STUDIED IN ZEBRAFISH MODEL (PAPER I)

Metastatic cascade consists of multiple-step defined mechanisms, including dissemination of tumor cell from the primary site; intravasion of tumor cells into the circulation; transport of malignant cells *via* blood circulation or lymphatic systems to distal tissues or organs; extravasation of tumor cells from the circulation of lymphatic system; formation of the primary metastatic niche in distal tissues; manipulation of metastatic microenvironment; and regrowth of metastatic nodules to a visible metastatic mass. Although advances of imagine techniques allows detection of relative small sizes of tumors in cancer patients and in experimental animal models, the early onset of metastatic processes remains unknown. It is known that hypoxia is an effective driving force for angiogenesis, but the mechanisms and detailed processes underlying hypoxia-associated metastasis remain poorly understood. It is important to establish an ideal animal model to investigate early steps of tumor cell dissemination and metastasis under hypoxic condition.

We develop a zebrafish metastatic model that allows us to take the advantage of the transparent nature of zebrafish embryos to monitor dissemination of single tumor cells from primary sites in the living body under different conditions, such as normoxic and hypoxic conditions, to study tumor cell migration and invasion in association with tumor angiogenesis.

We have found that tumor angiogenesis is essentially required for tumor cell invasion and dissemination. Hypoxia and VEGF significantly contribute to early events of the metastatic cascade. Hypoxia significantly increases tumor cell dissemination, invasion and metastasis by activation of VEGF, and its receptor-mediated signaling pathway. It is known that VEGF induces disorganized, leaky and tortuous vasculatures, which we also observed clearly in our zebrafish model. The disorganized vasculatures induced by VEGF are susceptible for malignant cell invasion. Additionally, outgrowth of blood vessels in tumors and perfusion of VEGF-induced vessels could act as an attractants for tumor cell dissemination, invasion and metastasis.

Utilizing the the zebrafish model, our study provides evidence of hypoxia- and VEGF-induced pathological angiogenesis in promoting tumor cell dissemination, invasion and metastasis to distal sites at the single cell level. These findings further provide evidence on the beneficial effects of clinically available anti-VEGF drugs for the treatment of human cancers.

5.2 FGF-2 AND VEGF-C COLLABORATIVELY PROMOTE LYMPHANGIOGENESIS AND LYMPHATIC METASTASIS (PAPER II)

Tumors can produce various angiogenic factors to stimulate angiogenesis and lymphangiogenesis. In contrast to the extensive studies on tumor-associated angiogenesis, little is known about the mechanisms by which tumor stimulates lymphangiogenesis, and how tumor cells gain entry to the lymphatic system. Various angiogenic factors often co-exist in the tumor microenvironment and they often cross-communicate with different signaling pathways. Although the individual roles of various angiogenic factors in promoting angiogenesis are relatively well studied, the interplay between them in the tumor environment remains poorly understood.

In this study, we chose FGF-2 and VEGF-C, two commonly expressed potent angiogenic factors, to investigate the interplay between them in promoting lymphangiogenesis and lymphatic metastasis.

There is no doubt that VEGF-C is a critical lymphangiogenic factor contributing to lymphangiogenesis. Both VEGF-C and FGF-2 induce proliferation and migration of LECs *in vitro* and lymphangiogenesis *in vivo*. FGF-2 directly promotes LECs proliferation and migration *via* activating the FGFR-1–mediated signaling pathway *in vitro*, which demonstrats that FGF-2 is a direct lymphangiogenic factor. But this FGF-2–induced lymphangiogenesis is completely inhibited by VEGFR-3 blockade *in vivo*. The tip cell formation at the leading front of growing lymphatic vessels induced with FGF-2 could be completely inhibited by VEGFR-3 blockade. Our results have suggested that LEC tips formation is an essential process for lymphatic vessel growth, and this process maybe tightly controlled by the VEGFR-3 signaling system. VEGFR-3 signaling system may play a pivotal role in lymphangiogeneisis triggered by other lymphangiogenic factors. However, there are still some questions included in

this case. For example, how does VEGFR-3 become activated? Does FGF-2 induce VEGF-C/-D expression or transactivate the VEGFR-3 signaling in the absence of these ligands? Do other factors induce lymphangiogenesis *in vivo* depending on the VEGFR-3 signaling system?

FGF-2 and VEGF-C also display marked additive activity in stimulation of hemangiogenesis. These activitions are completely insensitive to VEGFR-3 blockade. Although the tip cell formation at the leading edge of angiogenic vessels is probably also essential for hemangiogenesis, this process does not seem to require activation of VEGFR-3, which is not prominently expressed in blood vessels.

Lymphatic vessels provide one of the main routes for tumor cell dissemination, especially to regional lymph nodes. We have found that FGF-2 plus VEGF-C in the tumor microenvironment leaded to increased bloodstream and lymphatic metastasis. The intratumoral lymphatic vessels are highly disorganized and premature, which are susceptible features for facilitating tumor-cell dissemination and intravasation into lymphatics. Furthermore, peritumoral lymphatic vessels are highly dilated. These disorganized intratumoral lymphatic vessels and the dilated peritumoral lymphatic vessels may substantially contribute to lymphatic metastasis. Once metastasized to sentinel lymph nodes, the growth of metastases is further dependent on hemangiogenesis. Then the tumor cells can be further spread to other tissues and organs, and become metastatic masses in distal organs.

In this study, we have showed that FGF-2 and VEGF-C collaboratively stimulate lymphangiogenesis *in vivo*, probably by enhancing the VEGF-C/VEGFR-3–induced LEC tip cell formation and FGF-2–triggered proliferative signals. It is important to develop therapeutic agents that interfere with the interplay between various tumor angiogenic factors.

5.3 CONTRIBUTION OF MOUSE CORNEAL MODEL IN LYMPHANGIOGENESIS (PAPER III)

In contrast to the extensive studies on angiogenesis, less is known about the mechanisms of lymphatic system. The formation of new lymphatic networks involves

LECs proliferation, migration, lymphatic tube formation, maturation and remodeling. This process is tightly regulated by lymphangiogenic factors and inhibitors.

In this protocol, we have described an *in vivo* mouse corneal model which is used to study lymphangiogenesis induced by various factors. The main advantage of this corneal lymphangiogenesis model is that it allows us to study the lymphatic vessel formation in an avascular tissue for both blood and lymphatic vessels, thus avoiding any pre-existing vascular background. The avascular feature of the corneal tissue makes this model useful for a quantitative assessment of lymphangiogenesis. We take the advantage of this avascular nature of the mouse corneal tissue and implant various growth factors to quantitatively study lymphangiogenic stimulus–induced newly formed lymphatic vessels[107,200]. The amount of lymphangiogenic factor, the size of the micropellet, the implantation site and the time point of responses can be well defined in the absence of pre-existing vascular background in this mouse corneal model. So the structure, formation and architecture of lymphatic vessels induced by various factors can be studied and compared accurately.

Furthermore, this corneal lymphangiogenesis model allows the investigation of the joint effects of various promotors or anti-lymphangiogenic inhibitors on lymphangiogenesis. For example, this model allows us to study the interplay between FGF-2 and VEGF-C in lymphangiogenesis and metastasis (paper II). Thus, we could use this corneal lymphangiogenesis model not only for the investigation of structural and functional aspects of lymphatic vessels, but also for therapeutic assessment of anti-lymphangiogenic compounds.

5.4 THE IMPACTS OF ANTI-VEGF DRUGS IN HEALTHY TISSUES (PAPER IV)

Bevacizumab, the humanized VEGF neutralizing monoclonal antibody, has been widely used for treatment of various human cancers, such as lung cancer, glioblastoma and RCC since it was approved by US FDA in 2004[70-76]. FDA also approved other antiangiogenic drugs in the treatment of human cancers such as RCC. Tyrosine kinase inhibitors that block VEGFRs such as sunitinib, sorafenib and pazopanib are commonly used in clinics. Additionally, bevacizumab in combination

with chemotherapy has become the first-line standard care for treatment of several cancers, including RCC, non-small cell lung carcinoma and gastrointestinal stromal tumors[73,74,201-203].

On the other hand, it has been known that these anti-VEGF drugs in treatment of cancer patients have a broad impact on vasculatures in healthy tissues and organs which induces a range of adverse effects such as hypertension, renal vascular injury, or congestive heart failure. However, systemic impacts of these anti-VEGF or anti-VEGFR drugs in healthy vasculatures and healthy tissues and organs remain poorly understood. In paper IV, we chose VEGF and VEGFR specific blockades to study the systemic impact of anti-VEGF drugs in mice healthy vasculatures and healthy tissues.

We have observed significant reduction of vascular density in endocrine organs, gastrointestinal tracts and the female reproductive system. VEGF/VEGFR-2 signaling pathway plays a pivotal role in maintenance of vascular homeostasis in these healthy tissues and organs. VEGF may display two main functions: maintenance of endothelial cell survivals and vascular fenestrations in the healthy tissues.

We also have found that anti-VEGF-induced thyroid vascular regression is completely reversible in a relatively short time (approximate two weeks) after discontinuation of anti-VEGF treatment. The vascular density and architecture return to nearly the same levels as the untreated animals after two weeks cessation of VEGF blockade. One possible explanation is that anti-VEGF drugs may increase VEGF expression levels in healthy tissues.

Another clinical relevant finding is that anti-VEGF drugs completely suppressed the formation of endothelial fenestrations in thyroid vessels, which suggested that VEGF acts as a homeostatic factor for maintenance of vascular fenestrations in thyroid. It is well known that vascular fenestrations are crucial for maintenance of endocrine organ functions[76,198]. We have found the endocrine function of thyroid gland is impaired because there is low-level production of thyroxine after prolonged treatment with VEGF blockade.

In paper IV, our findings reveal the functions of VEGF in maintenance of vascular homeostasis in various healthy tissues and organs. The anti-VEGF drugs induced

adverse effects in mice are in general concordance with clinically manifested adverse effects caused by these drugs in cancer patients. Thus, it is very important to understand the impacts of these anti-VEGF drugs in healthy tissues and organs in clinical treatment.

6 CONCLUSIONS AND PERSPECTIVES

Hem- and lymph- angiogenesis have gained more attentions recently. Hemangiogenesis, the process of sprouting new microvessels from the pre-existing blood vasculature, is known to promote tumor growth and metastasis. In addition to hemangiogenesis, various types of tumors often contain lymphatic vessels, which may facilitate lymphatic metastasis. Metastasis of malignant tumors to regional lymph nodes is one of the early signs of cancer spread in patients. In clinics, the extent of lymph node metastasis is a major determinant for the staging and the prognosis of cancer patients, which often guides therapeutic decisions. Since the recent discovery of the specific lymphatic vessel markers, the isolation of LECs, the establishment of lymphangiogenesis animal models, and the identification of the VEGF-C/VEGF-D-VEGFR-3 signaling pathway, great developments have been made at the molecular understanding of lymphangiogenesis.

Understanding the biology of angiogenesis and lymphangiogenesis and how they are connected to tumor growth and tumor cells spread are fundamentally important in understanding the concept of hem- and lymph- angiogenesis in cancer metastasis. Although advances of imaging techniques allow detection of relative small sizes of tumors in cancer patients and in experimental animal models, the early onset of metastatic processes remains unknown. As more angiogenic and lymphangiogenic factors are discovered, the molecular interplay among these factors will probably become more complex. As for lymphangiogenesis, there has been lacking appropriate and powerful in vivo assay systems that allow us to study lymphangiogenesis quantitatively.

In this thesis work, we have: 1) developed a novel zebrafish model to study the early steps of the metastatic cascade. We took the advantage of the transparent nature of zebrafish embryos to visualize under normoxic and hypoxic conditions marked human or mouse tumor cell migration and invasion in association with tumor angiogenesis. We have found that tumor angiogenesis is essentially required for tumor cell invasion and dissemination. This study, for the first time, provide compelling evidence of tumor cell-tumor vessel interaction in promoting cancer cell dissemination to distal sites; 2) studied the interplay between FGF-2 and VEGF-C in

promoting lymphatic metastasis. In the tumor microenvironment, various angiogenic factors often co-exist and they often cross-communicate with different signaling pathways. Although the individual factor-transduced vertical signals via their specific receptors are relatively well studied, their horizontal interplay with other signaling systems remains poorly characterized. We show that FGF-2-triggered lymphangiogenic signaling pathways synergistically promote lymphangiogenesis with the VEGF-C/VEGFR-3 system, leading to synergistic lymphangiogenic effects in various *in vivo* models. This synergistic lymphangiogenic activity leads to accelerated lymphatic metastasis in sentinel lymph nodes; 3) developed a unique in vivo model to study lymphangiogenesis induced by various factors. We take the advantage of the avascular nature of the mouse corneal tissue and implant various growth factors/cytokines alone or in combinations to quantitatively study lymphangiogenesis and lymphatic structures; and 4) have also studied the impact of clinical available antiangiogenic drugs on healthy vasculatures and revealed potential sites for antiangiogenic drug-related side effects.

In the field of cancer treatment, an extensive effort has been made to identify potent antiangiogenic agents, and some antiangiogenic drugs have already used in clinics or entered late stages of clinical trials. To anti-lymphangiogenesis treatment, maybe it is very important to develop anti-lymphangiogenesis therapeutic agents to block the pivotal signalling pathway of lymphangiogenesis that might be triggered by various growth factors. Understanding the molecular mechanisms of angiogenesis and lymphangiogenesis in cancers will help us to design therapeutic agents to target blood vessels and lymphatic vessels in cancer treatment.

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