From The Department of Microbiology, Tumor and Cell Biology,
Karolinska Institutet, Stockholm, Sweden

THE ROLE OF VEGF FAMILY IN ANGIOGENESIS, TUMOR GROWTH AND METASTASIS

Xiaojuan Yang

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THE ROLE OF VEGF FAMILY IN ANGIOGENESIS, TUMOR GROWTH AND METASTASIS
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By

Xiaojuan Yang

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Principal Supervisor:
Professor Yihai Cao
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Co-supervisor(s):
Doctor Kayoko Hosaka
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Opponent:
Doctor Jenny L Persson
Lund University
Department of Laboratory Medicine
Division of Experimental Cancer Research

Examination Board:
Doctor Charlotte Rolny
Karolinska Institutet
Department of Oncology-Pathology

Professor Laszlo Szekely
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Keiko Funa
University of Gothenburg
Sahlgrenska Cancer center
Institute of Biomedicine

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ABSTRACT

Tumor growth is dependent on angiogenesis, and cells in tumor tissues produce various angiogenic factors to induce neovascularization. Among tumor-derived angiogenic factors, members of the vascular endothelial growth factor (VEGF) family are most frequently and highly expressed in various solid tumors. VEGF-A, the prototype of VEGF, is the most powerful pro-angiogenic factor that binds to VEGF receptor-1 (VEGFR-1, also called FMS-Related Tyrosine Kinase-1/Flt-1) and VEGFR-2 (also called Kinase Insert Domain Receptor/KDR or Fetal Liver Kinase -1/Flk-1). While the VEGFR-2-transduced angiogenic signals, pathways, and functions are well characterized, the VEGFR-1-mediated functions are poorly understood. The angiogenic functions of placental growth factor (PlGF), which is a specific VEGFR-1-binding ligand, remain controversial. The role of VEGF-B in tumor angiogenesis is still unclear. In addition, the two other VEGF family members, VEGF-C and VEGF-D are the major lymphangiogenic factors that contribute to lymphatic metastasis.

The work contained in this thesis aimed to study the role of VEGF family members in angiogenesis, tumor growth and metastasis. Our work shows that PlGF exhibits a duality in modulation of angiogenesis and tumor growth in a VEGF-A-dependent manner. This is noted when the tumor cell-derived PlGF sensitizes the tumor to the anti-angiogenic and anti-tumor effects of anti-VEGF drugs. We also noted that anti-VEGF treatment induces various vascular alterations in mouse healthy tissues. Additionally, we revealed the collaborative interaction between FGF-2 and VEGF-C in promotion of lymphangiogenesis and metastasis.

In paper I, using two independent tumor models, we show that PlGF modulated tumor growth, angiogenesis, and vascular remodeling through a VEGF-dependent mechanism in either a positive or a negative manner. In the VEGF-A positive model, PlGF inhibited tumor growth and angiogenesis, leading to normalized tumor vasculature with dilated vessel lumens, infrequent vascular branches and increased perivascular cell coverage. Surprisingly, in the VEGF-A negative model, overexpression of PlGF resulted in the opposite phenotype to that seen in the VEGF-A positive model, namely accelerated tumor growth rates and abundant chaotic tumor vessels. Our data uncovered the molecular mechanisms underlying the complex interplay between PlGF and VEGF-A. These findings have conceptual implications for anti-angiogenic cancer therapy.

In paper II, we show that tumors from humans and mice with high levels of expression of PlGF were hypersensitive to anti-VEGF-A and anti-VEGFR-2 therapies. We then validated this finding with a loss-of-function experiment using *PLGF* shRNA in a human choriocarcinoma cell line. Down-regulation of PlGF significantly accelerated tumor growth rate and led to resistance to anti-VEGF drugs. We also show that VEGFR-2 and VEGFR-1 neutralizing antibodies displayed opposing effects on tumor growth and angiogenesis. These findings demonstrate that tumor-derived PlGF negatively modulates tumor angiogenesis and sensitizes treatment effect of anti-VEGF drugs in VEGF-A positive tumors, PlGF level in VEGF-A positive tumor may potentially be a predictive marker of anti-VEGF cancer therapy.

In paper III, we investigated vascular alteration in various organs after systemic treatment with anti-VEGF-A, anti-VEGFR-1 and anti-VEGFR-2 neutralizing antibodies. This study provides functional and structural mechanisms for anti-VEGF drug-induced adverse effects in patients.

In paper IV, we looked into the role of fibroblast growth factor-2 (FGF-2) and VEGF-C on angiogenesis, lymphangiogenesis and tumor metastasis. The results showed that FGF-2 and VEGF-C could both separately and collaboratively promote angiogenesis and lymphangiogenesis in the cornea of the mouse and in the mouse tumor tissue, resulting in pulmonary and lymph node metastases in animal models. By blocking VEGFR-3 and FGF receptor-1 (FGFR-1), we also revealed the fact that VEGFR-3-induced lymphatic endothelial cell (LEC) tip formation is a necessity for FGF-2-FGFR-1 signaling stimulated lymphangiogenesis. This study suggests that combined targeting of FGF-2 and VEGF-C might be an effective approach for cancer therapy and prevention of metastasis.

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I. **Xiaojuan Yang**, Yin Zhang, Yunlong Yang, Sharon Lim, Ziquan Cao, Janusz Rak, Yihai Cao. Vascular endothelial growth factor-dependent spatiotemporal dual roles of placental growth factor in modulation of angiogenesis and tumor growth. Proc Natl Acad Sci U S A. 2013 Aug 20;110(34):13932-7

II. Eva-Maria Eleonora Hedlund, **Xiaojuan Yang**, Yin Zhang, Yunlong Yang, Masabumi Shibuya, Weide Zhong, Baocun Sun, Yizhi Liu, Kayoko Hosaka, Yihai Cao. Tumor cell-derived placental growth factor sensitizes antiangiogenic and antitumor effects of anti-VEGF drugs. Proc Natl Acad Sci U S A. 2013 Jan 8;110(2):654-9.

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* Co-first author

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I. Hosaka K, Yang Yunlong, Seki Takahiro, Nakamura Masaki, Andersson Patrik, Rouhi Pegah, **Yang Xiaojuan**, Jensen Lasse, Lim Sharon, Feng Ninghan, Xue Yuan, Li Xuri, Larsson Ola, Ohhashi Toshio, Cao Yihai. Tumour PDGF-BB expression levels determine dual effects of anti-PDGF drugs on vascular remodelling and metastasis. Nat Commun. 2013;4:2129.

II. Ji Hong*, Cao Renhai*, Yang Yunlong*, Zhang Yin, Iwamoto Hideki, Lim Sharon, Nakamura Masaki, Andersson Patrik, Wang Jian, Sun Yuping, Dissing Steen, He Xia, **Yang Xiaojuan**, Cao Yihai. TNFR1 mediates TNF-α-induced tumour lymphangiogenesis and metastasis by modulating VEGF-C-VEGFR3 signalling. Nat Commun. 2014 Sep 17;5:4944.

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IV. **Xiaojuan Yang**, Yin Zhang, Kayoko Hosaka, Patrik Andersson, Jian Wang, Ziquan Cao, Yunlong Yang, Hideki Iwamoto, Sharon Lim, Yihai Cao. Vascular endothelial growth factor-B promotes cancer metastasis through a vascular endothelial growth factor-A-independent mechanism of vascular remodeling.
Submitted manuscript

* Co-first author

CONTENTS

LIST OF ABBREVIATIONS

AKT or PKB	Protein Kinase B, also known as AKT
Ang	Angiopoietin
CAFs	Carcinoma-Associated Fibroblasts
CCBE1	Collagen and Calcium Binding EGF Domain-containing protein 1
CTCs	Circulating Tumor Cells
CTX	Cyclophosphamide
Dll4	Delta-like 4
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
ECs	Endothelial Cells
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
ERK	Extracellular-Signal-Regulated Kinases
Fab domain	Fragment Antigen-Binding domain
FACS	Fluorescence-Activated Cell Sorting
Fc region	Fragment Crystallizable region
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FIGF	C-fos-Induced Growth Factor
Flk	Fetal Liver Kinase
Flt	FMS-Related Tyrosine Kinase
H&E staining	Hematoxylin and Eosin Staining
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
IFP	Interstitial Fluid Pressure
IGF	Insulin-Like Growth Factor
KDR	Kinase Insert Domain Receptor
LECs	Lymphatic Endothelial Cells
LLC	Lewis lung carcinoma

LRD	Lysine-Fixable Rhodamine Labeled Dextran
LYVE-1	Lymphatic Vessel Endothelial Hyaluronan Receptor-1
MAPK	Mitogen-Activated Protein Kinases
mCRC	Metastatic Colorectal Cancer
MMP	Matrix Metalloproteinase
NG2	Neuron-Glial Antigen 2
NRP	Neuropilin
PAI-1	Plasminogen Activator Inhibitor-1
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PlGF	Placental Growth Factor
Prox-1	Prospero Homeobox Protein 1
RNA	Ribonucleic Acid
shRNA	Small Hairpin RNA or Short Hairpin RNA
SMA	Smooth Muscle Actin
SMCs	Smooth Muscle Cells
sVEGFR	Soluble Vascular Endothelial Growth Factor Receptor
TAMs	Tumor-Associated Macrophages
TEM	Transmission Electron Microscope
TEM1	Tumor Endothelial Marker 1
TGF	Transforming Growth Factor
TIE	Tyrosine Kinases with Immunoglobulin-like and EGF-like Domains
TKIs	Tyrosine Kinase Inhibitors
TNF	Tumor Necrosis Factor
tPA	Tissue Plasminogen Activator
TSPs	Thrombospondins
uPA	Urokinase-type Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VPF	Vascular Permeability Factor

1 INTRODUCTION

1.1 TUMOR ANGIOGENESIS

1.1.1 Functions and architecture of vessels

In all vertebrates, vessels, including blood vessels and lymphatic vessels, comprise a transportation network for plasma, blood cells, oxygen, nutrition and metabolites throughout the whole body, which is essential for normal physiological functions and has implications in various pathological states[1]. The lymphatic vasculature is also involved in tissue fluid homeostasis, the absorption of dietary fat, and the functioning of the immune system[2].

Blood vessels are divided into three major types according to their function and structure: the arteries are responsible for carrying the oxygenated blood away from the heart; the veins return blood from the capillaries back to the heart and the capillaries are the smallest unit of blood vessels and are the sites of substance exchange between the blood and tissues[3].

Lymphatic vessels consist of two types of networks—the initial lymphatic capillaries that specialize in collection of the lymph from the interstitial fluid, and the larger lymph vessels that is responsible for the drainage of the lymph[3,4].

There are recognizable structural differences between typical blood capillaries and lymphatic capillaries. The endothelial cells (ECs) of blood vessels are connected with each other by tight junctions and are entirely covered by basement membrane together with contractive mural cells—pericytes and smooth muscle cells. Inversely, the lymphatic vessels start as small blind-ends, have no basal lamina, and the ECs are anchored to the adjacent matrix, which results in the vessels being open to the interstitial space[5]. When the interstitial fluid pressure (IFP) rises, while the blood capillaries may collapse, the lymphatic vessels open, allowing more interstitial fluid to return to the lymphatic system[2, 4, 5].

ECs are the indispensable component of all vessels as they line the interior surface of the vessels and depart the fluid phase from the tissues. The ECs located in different organs participate in various bioactivities, for example, hemostasis, coagulation (thrombosis and fibrinolysis), fluid filtration, arteriosclerosis blood vessel tone, inflammatory recruitment and infiltration, hormone trafficking, and most importantly, they play a very important role in angiogenesis by migration, proliferation and secretion of certain angiogenic factors[6, 7, 8].

The mural cells are another basal structure of the vessel wall. Pericytes are the mural cells of capillaries and venules; smooth muscle cells are the mural cells of other blood vessels. Beneath the ECs, the pericytes line the interior of the basal membrane, and discontinuously cover the vessel wall. They communicate with the ECs by paracrine signaling or direct cell junction, and strengthening the barrier between capillary well and surrounding tissues[9, 10, 11]. Gap junctions between pericytes and ECs allow the exchange of ions and small molecules. Interestingly, cell-cell contact appears necessary for vessel formation and maintenance[12, 13]. The second class of mural cells, smooth muscle cells (SMAs), which are usually located

exterior of the basal membrane, control the vasoconstriction and vasodilation together with pericytes[14].

As a part of tumor microenvironment, the properties of capillaries in tumor tissue contribute to every stage of tumor progression and metastasis[15].

1.1.2 Angiogenesis

More than 220 years ago, the Scottish surgeon Dr. John Hunter first proposed that there is an equilibrium between vascularity and metabolic needs in both healthy and diseased states, which means that the vasculature changes along with the physiological and pathological status of the human body[16]. This concept was revisited in 1971 by Dr. Judah Folkman who stated the study of angiogenesis by hypothesizing that tumor growth is angiogenesis-dependent and describing interactions between ECs and tumor cells via tumor-angiogenesis factor[17]. From then on, the field of angiogenesis research was established and studied by more and more scientists from all medical and biological fields.

Throughout life, there are two ways of blood vessel formation—"vasculogenesis" and "angiogenesis" [18]. Vasculogenesis indicates new vessel formations when there are no pre-existing vessels, whereas, angiogenesis refers to the process of new vessels sprouting from pre-existing vessels[19, 20]. Vasculogenesis and angiogenesis are indispensable in embryonic development, tissue regeneration, and the female reproductive system, for example, during the menstrual cycle and development of corpus luteum. It also contributes to many pathological disorders, such as cancer, age-related macular degeneration, obesity, asthma, and arthritis[7, 10, 21, 22].

Until now, four different bio-mechanisms of angiogenesis have been defined and all of these can be found in tumor development: sprouting, intussusception, mimicry and cooption.

1.1.2.1 Sprouting

Sprouting angiogenesis includes the oriented filopodia extension of the tip cells and proliferation of the stalk cells induced by certain angiogenic factors including vascular endothelial growth factor-A (VEGF-A), angiopoietin-1 (Ang-1), fibroblast growth factor (FGF-2), delta-like 4 (Dll4) [23, 24, 25, 26, 27] (See 1.1.4). Gerhardt et al. observed that the retina vessel sprouting is controlled by agonistic activity of VEGF-A whereby the tip cells migration is correlated with an increased concentration of VEGF-A, and in the meantime, the stalk cells proliferate upon reaching a concentration threshold of VEGF-A[23].

1.1.2.2 Intussusception

Intussusceptive angiogenesis, also called vascular splitting denotes vascular network formation by protrusion of interstitial tissue columns (tissue pillars or posts) into the vascular lumens, and further transluminal pillars are formed, resulting in new vessel lumens formation[28, 29]. Intussusceptive angiogenesis not only depends on angiogenic growth factors[30,

[31, 32, 33] but is also regulated by intravascular blood-flow patterns or intravascular shear stress which is a potent trigger of intussusception[34].

1.1.2.3 Mimicry

Vascular mimicry describes how highly aggressive tumor cells organize or mimic the vessel-like structure with basal membrane and extracellular matrix (ECM) but without presence of ECs and mural cells[35]. Vascular mimicry has been observed in several tumor types, and was initially found in malignant melanoma by Dr. Hendrix [36], followed by its discovery in breast cancer by Dr. Wakasugi [37]. Vascular mimicry reminds us that aggressive tumor cells exhibit high plasticity and pluripotency, and mimicry is strongly associated with poor prognosis[38].

1.1.2.4 Cooption

Holash et al. first defined vessel cooption when they found that tumor cells migrated toward host blood vessels in well-vascularized organs or in a metastatic site to support blood vessel-dependent tumor growth instead of triggering angiogenesis. These vessels then regress because of apoptosis mediated by Ang-2 expressed by the coopted ECs. Later on, angiogenesis began at the periphery of the growing tumor mass[39]. Vessel cooption happens during the early stage of tumor development and is dependent on the site of the tumor, often in the brain and lung[40, 41].

1.1.2.5 Vasculogenesis

Vasculogenesis is the mechanism by which mesoderm-derived angioblasts (endothelial precursor cells) migrate and differentiate into ECs to form primitive blood vessels. Vasculogenesis mainly occurs during embryonic development of the cardiovascular system and some researchers also found it in adult pathological conditions, for example during hemangioma formation[42].

1.1.2.6 Lymphangiogenesis

The cellular processes of lymphangiogenesis are considered to be similar to that of hematoangiogenesis—the lymphatic vessels inside or surrounding the tumor mass undergo sprouting and enlargement contributed by migration and proliferation of lymphatic endothelial cells (LECs). These patterns of lymphatic remodeling have been found in various primary human cancers[43]. Studies on tumor lymphatic vessels mainly focus on the ability of lymphatic capillaries to facilitate the entry and transport of tumor cells[5, 43, 44, 45, 46, 47].

1.1.3 Tumor vessels

Angiogenesis is a prerequisite for the process of solid tumor growth and metastasis. When the tumor size reaches to several millimeters in diameter, the tumor will be deprived of oxygen and nutrition since molecules cannot reach to the center of tumor by diffusion. The hypoxic environment in the tumor will trigger tumor angiogenesis. However, compared to healthy tissue, tumor vessels have numerous special properties that may facilitate cancer progression and tumor cell intravasation, as well as the development of resistance to anti-angiogenic

treatment[21, 48, 49]. ECs, pericytes, and the vascular basement membrane in tumor vessels are all structurally and functionally abnormal, which causes problems when it comes to identification of the tumor vessels[50, 51]. Structurally, tumor vessels do not have a typical arteriole–capillary–venule hierarchy that is seen in normal tissue. Instead, abundant endothelial sprouting branches are widespread, and some ECs are partially detached or form incomplete layers[50, 52]. Tumor blood vessels are often irregular, chaotic, tortuous, lack of mural cell coverage and have a relatively higher density compared to normal vasculature. Pericytes of tumor vessels associate with ECs loosely and can even detach from the ECs[53]. Functionally, tumor vessels are poorly perfused and with greater leakiness, some ECs just sprout in a haphazard manner or form a vessel lumen without blood flow. Conversely, some routes of blood flow are not lined by ECs. These features of tumor vessels endow the tumor microenvironment with high IFP and hypoxic level[17, 54, 55].

1.1.4 Angiogenic stimulators

Angiogenesis depends a complex set of cellular events, including proliferation, sprouting, migration and tube formation. The series of cellular events in hematoangiogenesis and lymphangiogenesis may be similar and some blood vessel regulators are involved in lymphatic development as well. In this section, I will briefly introduce several principal pro-angiogenic factors and their roles in tumor angiogenesis, excluding VEGF family members.

1.1.4.1 Fibroblast growth factor-2 (FGF-2)

FGF-2 is a heparin-binding protein that was shown to be a potent pro-angiogenic factor[56]. It promotes neovascularization by stimulating proliferation and migration of ECs, as well as degradation of the ECM through up-regulation of proteases, e.g. matrix metalloproteases (MMPs) and urokinase-type plasminogen activator (uPA)[57, 58]. FGF-2 is highly expressed by ECs, infiltrated inflammatory cells and various tumor cells, such as prostatic cancers, hematological malignancies, melanoma and pancreatic tumors[56, 59, 60, 61, 62]. This indicates that FGF-2 may act on all these components via autocrine or paracrine signaling. Under these conditions, FGF-2 may facilitate cancer progression not only by promoting angiogenesis but also by acting directly on tumor cells and tumor-associated macrophages[62, 63, 64].

FGF-2 was also shown to be a very strong lymphangiogenic factor through activations of FGF receptor-1 (FGFR-1) and FGFR-3. It has been shown that FGF-2 can also stimulate lymphangiogenesis through up-regulation of VEGF-C[65, 66, 67].

In paper IV, we uncovered the mechanism that lymphangiogenic factors, VEGF-C and FGF-2, collaboratively stimulate lymphangiogenesis via VEGF receptor-3 (VEGFR-3) and FGFR-1 transduced signaling pathways.

1.1.4.2 Platelet-derived growth factor-BB (PDGF-BB)

The role of PDGF-BB and PDGF receptor-β (PDGFR-β) in angiogenesis is to promote recruitment, migration and proliferation of mural cells[68, 69, 70]. In one of our published works,

in addition to abundant PDGF-BB stimulated angiogenesis, we surprisingly found bidirectional modulation of pericytes recruitment to the tumor vessel by PDGF-BB. Tumor cell-derived PDGF-BB detached the pericytes from ECs, whereas, ECs attracted the pericytes by producing PDGF-BB[71].

Additionally, PDGF-BB induced LECs proliferation via activation of mitogen-activated protein kinases (MAPK) as well as enhancing cell motility, thus resulting in intratumor lymphangiogenesis and lymph node metastasis in a mouse tumor model[72]. Later, a clinical study demonstrated that PDGF-BB expression level correlated with lymphatic invasion of human esophageal carcinoma[72]. Recently, prospero homeobox protein 1 (Prox-1) was found to be the responsible transcription factor for maintenance of PDGFR-β[73].

1.1.4.3 Neuropilins (NRPs)

NRPs were originally isolated from the nervous system of Xenopus[74]. They are the major receptors of class 3 semaphorins, which play a critical role in several physiological processes including acting as anti-angiogenic factors[75, 76]. They also participate in angiogenesis as co-receptor of the VEGF family. It has been shown that, VEGF-B and placental growth factor (PlGF) binds to NRP-1, and VEGF-A, VEGF-C as well as VEGF-D can bind to both NRP-1 and NRP-2[77]. NRP-1 is considered critical for vascular formation since *Nrp1*$^{-/-}$ transgenic mice die *in utero* at E13.5 due to severe angiogenic defects[78].

NRP-2, modulates the VEGF-C signaling pathways as a co-receptor of VEGFR-3[79]. Genetic deletion or antibody neutralization of NRP-2 can block VEGF-C induced lymphatic vessel sprouting *in vivo*[80].

Moreover, there are some other angiogenic factors binding to NRPs, such as PDGF-BB and FGF-2[81, 82].

1.1.4.4 Angiopoietins (Angs)

To date, four isoforms of Ang have been identified and all of them bind to tyrosine kinases with immunoglobulin-like and EGF-like domains 1 (TIE-1) and TIE-2 receptors[39]. The Angs and TIE receptors are essential to development and maturation of the vascular system. Knockout of any one amongst Ang-1, Ang-2, TIE-1 and TIE-2 are embryonic lethal or result in early postnatal death due to impaired vessel formation[83, 84, 85, 86]. TIE receptors are mainly expressed on ECs, however, they can be detected on myocardium and on hematopoietic stem cells[87, 88]. During angiogenesis, Angs-TIEs axes are responsible for vessel formation and stabilization through a complicated equilibrium among all members: Ang-1 strongly activates TIE-2 signal transduction, while Ang-2 acts as an antagonist of Ang-1, however, Ang-2 has very weak stimulatory effect on TIE-2 compared to Ang-1. In addition, TIE-1 may regulate function of TIE-2 by forming heterodimers with TIE-2[89,10, 84].

Lymphangiogenesis is stimulated by Ang-1 via both direct and indirect mechanisms: Direct mechanism is due to activation of TIE-2 expressed on LECs, and indirect stimulation is due

to up-regulated VEGFR-3 expression[90]. Interestingly, one knockout and rescue experiment indicated that Ang-2, considered as an antagonist of TIE-2 during angiogenesis, actually acts as an agonist in lymphangiogenesis[85].

1.2 VEGF FAMILY IN TUMOR DEVELOPMENT

Among all the pro-angiogenic factors, VEGF family members play the central roles in tumor angiogenesis. In this section, I will discuss more about the VEGF family, the ligands, the receptors, their structures, the signaling transductions, the biological function and especially how they affect tumor angiogenesis and contribute to tumor growth and metastasis.

VEGF-A is the major representative of the VEGF family, usually in the literatures, "VEGF" points to both "VEGF family" and "VEGF-A". To avoid confusion upon the terms, in this thesis, the "VEGF" only refers to "VEGF family" but not "VEGF-A".

1.2.1 Introduction of VEGF family

Up to date, in total 7 members of the VEGF family have been identified, including five ligands from the mammalian genome: VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF, one analogue ligand from Orf virus—VEGF-E (Orf-VEGF)[91], and one analogue from Trimeresurus flavoviridis—T. f. svVEGFs (VEGF-Fs)[92]. In this thesis, I will focus on the five factors coded by mammalian genes, which are all crucial during embryonic development. The genesis of new blood vessels and lymphatic vessels is controlled by these ligands activating three tyrosine kinase receptors—VEGFR-1, VEGFR-2 and VEGFR-3.

According to their receptor binding pattern and functions, we classified these five VEGF family members into three subgroups. PlGF and VEGF-B belong to subgroup as they bind exclusively to VEGFR-1. Generally, VEGFR-1 is widely expressed on blood ECs but not found on the LECs. The second subgroup consisting of VEGF-A, binds to both VEGFR-1 and VEGFR-2, VEGFR-2 is expressed mainly on blood ECs but weakly on LECs of collecting lymphatic vessels. The last subgroup includes VEGF-C and VEGF-D, both of which can activate principally VEGFR-3 as well as VEGFR-2. VEGFR-3, as a marker of LECs and is the main lymphangiogenesis related receptor that is structurally different from VEGFR-2 and VEGFR-1[93, 94, 95].

As typical tyrosine kinase receptors, activation of VEGFRs requires ligand dimer to bind to and dimerize two adjacent VEGFR monomers, leading to phosphorylation of tyrosine residues in the intracellular tyrosine kinase domain. This phosphorylation leads to the formation of a binding site for specific proteins, further initiating the signal transduction cascade[96].

1.2.2 VEGF-A

VEGF-A (formerly known as vascular permeability factor/VPF) was identified separately by Senger et al. in 1983 and Ferrara et al. in 1989[97, 98]. It is a glycoprotein capable of undergoing dimerization forming two subunits which are connected by disulfide bonds. The human

VEGFA gene expresses several different isoforms of VEGF-A: VEGF-A121, VEGF-A145, VEGF-A165, VEGF-A189, and VEGF-A206. The highly homologous rodent VEGF-A proteins lack one amino acid compared to the human orthologs[99]. Owing to various splice variants of the heparin-binding domain, different VEGF-A isoforms have different levels of solubility. For example, among three major isoforms, VEGF-A121 is completely soluble, VEGF165 is moderately soluble, but VEGF-A189 is almost insoluble[100]. The most important isoform *in vivo* is the VEGF-A165, which is expressed in a variety of cells in the body. Isoform-specific mutant mice bearing either VEGF-A120 or VEGF-A188, but not VEGF-A164, were embryonic lethal due to severe defective vessel development, indicating that the isoform VEGF-A164 is essential and sufficient for the normal development of the circulatory system[101, 102]. Additionally, VEGF-A164 binds to NRP-1 that is a co-receptor for the collapsin/semaphorin family mediating neuronal cell guidance[103, 104]. VEGF-A is essential for embryonic and early postnatal development. The single allele knockout of *Vegfa* leads to embryonic lethality[105, 106].

VEGF-A is the main stimulator of angiogenesis. As a strong mitogen of ECs, it controls proliferation, survival, and migration of the ECs by activating VEGFR-2 and VEGFR-1. VEGF-A can be secreted by tumor cells as well as normal tissue cells, and diffuses to the surrounding area, eventually binding to VEGFRs on the ECs[107]. Abundant vessels prompted by VEGF-A alone are tortuous and leaky just like the typical tumor vessels described in section 1.1.3.

It is still controversial when it comes to the lymphangiogenic effect of VEGF-A. Kubo et al. showed in their experiments that VEGF-A could only induce blood angiogenesis not lymphangiogenesis using a mouse cornea assay. However, Dr. Hirakawa detected VEGFR-2 level tumor-associated lymphatic vessels, under his experimental conditions, VEGF-A promoted proliferation of LECs, resulting in tumor metastasis to the sentinel and distant lymph nodes[65, 108].

VEGF-A significantly increases vascular permeability through the activation of VEGFR-2[109]. Some studies also revealed that VEGF-A has immunosuppressive properties, e.g. induces the accumulation of immature dendritic cells[110]. Additionally, VEGF-A is involved in hemodynamics by controlling the vasoconstriction and vasodilation[111, 112].

It is well known that the tumor possesses a hypoxic core with nearby necrotic when the tumor grows to a critical size. Hypoxia is a very potent stimulator of hundreds of genes, and it is central to the induction of VEGF-A. On one hand, hypoxia can stabilize hypoxia inducible factor 1-α (HIF1-α) and HIF2-α which bind to HIF hypoxia-responsive element of the *Vegfa* promoter, inducing the transcription of DNA. In addition to direct transcription, hypoxia can also lead to the stabilization of VEGF-A mRNA[113]. Thereafter, VEGF-A can be secreted by various hypoxic cells, and diffuse to the surrounding zone, to promote new vessel formation and relieve the tissue from hypoxia[107, 113, 114].

1.2.3 VEGF-B

VEGF-B was first isolated in 1996, and two isoforms were identified, VEGF-B186 and VEGF-B167[115]. Both VEGF-B isoforms bind to VEGFR-1 and the co-receptor—NRP-1. The isoform VEGF-B167 possesses the heparin-binding domain, whereas VEGF-B186 does not contain the heparin-binding domain[116, 117]. *Vegfb*$^{-/-}$ mice developed normally except for minor defects during cardiovascular formation and revascularization[118, 119].

Studies on the role of VEGF-B in angiogenesis mainly focus on heart and brain tissue revascularization. When it comes to tumor vessel angiogenesis, limited research has been conducted. It is reported that VEGF-B increased the mRNA level of uPA and Plasminogen activator inhibitor-1 (PAI-1), but not tissue plasminogen activator (tPA) activity in certain ECs *in vitro*[116], however, VEGF-B did not show pro-angiogenic activity in our tumor bearing mice model and many other studies *in vivo*[120]. More and more clinical studies show that the VEGF-B levels in tumor tissue correlated with tumor progression and a worse prognosis[121, 122, 123, 124]. Recently, a study on biological activities of VEGFR-1 demonstrated that, unlike PlGF, VEGF-B may not efficiently induce downstream signaling of VEGFR-1[125]. whereas, it has been shown that VEGF-B is able to form heterodimers with VEGF-A *in vitro*, thus VEGF-B may be able to regulate the activity of VEGF-A[126].

1.2.4 VEGF-C and VEGF-D

In the VEGF family, there are two members that has been shown experimentally to contribute to lymphangiogenesis—VEGF-C and VEGF-D. These are the most well-known lymphangiogenic stimulators that activate VEGFR-2 and VEGFR-3. Activation of VEGFR-3 stimulates protein kinase C-dependent activation of the extracellular-signal-regulated kinase 1 (ERK1) or ERK2 signaling cascade and phosphorylation of protein kinase B (PKB, also known as AKT), leading to survival, proliferation and migration of LECs[39, 40, 127], as well as lymphatic smooth muscle cells alteration at the collecting lymphatic vessels[128].

People used to think that lymphatic capillaries cannot grow into the tumor due to their thin and are structurally weak. Several studies showed that VEGF-C and VEGF-D promoted lymphatic metastasis by inducing intratumoral and peripheral lymphatic vessels growth via binding to VEGFR-3[129, 130, 131].

Joukov et al. isolated *VEGFC* cDNA from prostatic cancer and determined that VEGF-C binds to tyrosine kinase receptor VEGFR-3 and VEGFR-2[132, 133]. The homozygous *Vegfc* knockout mice aborted between E15.5 and E17.5 due to severe swelling caused by the absence of lymphatics, and whilst the heterozygous can survive, they show hypoplasia of cutaneous lymphatics[127].

VEGF-D was initially termed the c-fos-induced growth factor (FIGF) when it was isolated for the first time, and was found to be a mitogenic factor for fibroblasts[134]. VEGF-D has a very similar protein structure to VEGF-C and has been shown to bind to the same receptors.

Due to these similarities, in a zebrafish model, *Vegfd* could rescue loss of *Vegfc* and compensate for its function in lymphatic sprouting[135].

1.2.5 PlGF

PlGF exclusively binds to VEGFR-1. As the name suggests, PlGF is mainly expressed in the placenta, however, it is also detectable in the heart, lung and skeletal muscle at lower levels. The gene for PlGF in humans code for four isoforms, they are PlGF-1, PlGF-2, PlGF-3 and PlGF-4, whereas, only PlGF-2 exists in the mouse[136].

Genetic ablation of PlGF does not affect vessel structure during physiological development[137] but it dose alter angiogenesis during pathological conditions such as hypoxic brain, myocardial ischemia, and wound healing[137, 138]. Studies examining the effects of PlGF on tumor angiogenesis and progression were controversial. On one hand, it is demonstrated that PlGF suppressed tumor growth in mouse models of LLC and fibrosarcoma, and led to the dilation of the tumor vessels and inhibited vessel branching[139]. On the other hand, overexpression of PlGF in mouse melanoma increased the tumor growth and pulmonary metastases through activation of MMPs and tumor angiogenesis[140].

Additionally, since VEGFR-1 is involved in recruitment of monocytes and macrophages, thus PlGF may promote angiogenesis indirectly by attracting inflammatory cells that secret pro-angiogenic factors[141]. It has been reported that PlGF reconstituted hematopoiesis by recruiting VEGFR-1^+ stem cells from bone marrow.

In paper I, we uncovered the mechanism by which PlGF regulates VEGF-A-VEGFR-2 signaling in both a negative and a positive manner by heterodimerization with VEGF-A and competitive binding to VEGFR-1. In paper II, we demonstrated that PlGF in tumor cells led to sensitization of the tumors to anti-VEGF therapy.

1.2.6 VEGFR-1

The function of VEGFR-1 is uncertain and quite controversial. When both alleles of *Vegfr1* were deleted, the mice embryos showed hyper-proliferation of ECs and are aborted early in development[142]. Whereas, if only the tyrosine kinase domain from VEGFR-1 was deleted, the mice developed normally[143], however, isolated monocytes showed impaired migration *in vitro* under stimulation from VEGF-A and PlGF[144]. Downstream signaling pathway of VEGFR-1 is still an enigma until today[145].

1.2.7 VEGFR-2

In contrast to VEGFR-1, the role of VEGFR-2 in angiogenesis is relatively well elucidated. After receptor undergoes dimerization and autophosphorylation of the tyrosine kinase residues on the intracellular domain, it is capable of activating of various pathways, primarily: (1) The phospholipase C (PLC)- γ /protein kinase C (PKC) pathway, leading to the beginning of the c-Raf-MEK-MAPK cascade and cell proliferation. (2) The PI3K-Akt signal

transduction pathway, contributing to cell survival. (3) The p38 MAPK signaling pathway, resulting in cell migration and, (4) eNOS activation for vascular permeability[109, 146, 147].

1.2.8 VEGFR-3

VEGFR-3 is the major receptor for VEGF-C and VEGF-D and is essential for the development of the lymphatic vessels. Primitive lymphatic vessels are transdifferentiated from the Prox-1^+ embryonic veins, [148], the Prox-1^+ cells undergo differentiation and form the lymphatic vessels when they were exposed to VEGF-C which activates the VEGFR-3 on these cells. The VEGFR-3 is eventually shifted from the embryonic veins to the lymphatic vessels and became a specific marker of lymphatic vessels[149, 150]. Despite this, VEGFR-3 can also be expressed on blood capillaries in tumor angiogenesis or during wound healing[151, 152]. Similar to *Vegfc* knockout mice, complete deletion of the *Vegfr3* gene causes early embryonic death due to defective blood vessel development in embryos—the large vessels' lumens develops abnormally, leading to serious edema and cardiovascular failure at E9.5[153, 154].

1.3 ANTI-VEGF CANCER THERAPY

Based on Dr. Judah Folkman's hypothesis that all solid tumor growth is dependent on angiogenesis, anti-angiogenic therapy could be considered to be an efficient way of inhibiting tumor growth. The aim of anti-VEGF treatment is to prevent the activation of VEGFR and the downstream signaling pathways, thus hindering the neovascularization in tumor tissue, and eventually resulting in tumor vessel regression and markedly reduced tumor growth.

Some possible ways of inhibiting VEGF signals include: (1) Neutralizing antibodies that block the VEGF family ligands. (2) Neutralizing antibodies that block the VEGFRs, thus obstructing activation of the receptors. (3) Soluble receptors with the ligand-binding domain of membrane receptors, acting as decoy for the ligands before they bind to the receptors on the cell surface. (4) Small molecules that block ligand-binding sites on VEGFRs. (5) Tyrosine kinase inhibitors (TKIs)—small molecules with the ability of crossing the cell membrane that bind to the tyrosine kinase domain of the VEGFRs to inhibit the activation of downstream signaling pathways. (6) RNA interference technology.

1.3.1 Neutralizing antibodies

In the past 20 years, a few non-human antibodies were developed and their efficiency were validated using mice model in preclinical experiments. These heterogenic antibodies may induce the human immune response if they are directly introduced into patients, therefore humanization of the antibodies is required. Usually, the standard way of humanizing an antibody is to transfer the specific binding region to a human antibody framework[155].

Nowadays, anti-VEGF-A antibodies are the most well-developed blockades among the anti-angiogenic treatments since the function of VEGF-A is well understood. Dr. Ferrara made the first anti-VEGF-A antibody called A4.6.1[156]. Later, A4.6.1 was humanized into bevacizumab (Avastin, Genentech/Roche) which is the first monoclonal antibody approved to treat malignant cancer. Recently, ranibizumab (Lucentis, Genentech), a smaller neutralizing

antibody containing only the fragment antigen-binding (Fab) domain of IgG was derived from bevacizumab, it has 140 times higher affinity for VEGF-A than bevacizumab[157], but until now, ranibizumab is only approved for macular diseases.

Early this year, a human VEGFR-2 antagonist (ramucirumab) developed by Eli Lilly and Company was approved by the Food and Drug Administration (FDA) for treatment of advanced gastric cancer or gastro-esophageal junction adenocarcinoma, as a single-agent after prior fluoropyrimidine- or platinum-based chemotherapy[158].

1.3.2 Soluble receptors

Regeneron Pharmacerticals generated a decoy receptor that is a fusion protein of domain 2 from VEGFR-1, domain 3 from VEGFR-2 and an IgG Fc. This VEGF trap blocks not only VEGF-A but also PlGF and VEGF-B[159]. It is approved for metastatic colorectal cancer (mCRC) that is resistant to or has progressed following an oxaliplatin-containing regimen, and is marketed under the commercial name ZALTRAP[160].

In addition, tumor lymphangiogenesis is clearly associated with the lymphatic metastasis. To block the lymphagiogenesis pathway, soluble VEGFR-3 was developed by fusing the VEGFR-3 domain to an IgG fragment crystallizable (Fc) region. This fusion product inhibited lymphangiogenesis and lymph nodes metastasis in a murine tumor model[161] and ablated lymphangiogenesis in embryonic mice[162].

2 AIMS

The overall aim of this thesis was to investigate the role of VEGF family members in tumor angiogenesis, tumor growth, and metastasis.

The specific aims included:

To explore the mechanism of VEGF-A dependent PlGF induced tumor vessel remodeling.

To elucidate the role of PlGF in anti-VEGF drug resistance and sensitivity.

To study the systemic effects of VEGF targeting drugs.

To understand the effects of two lymphangiogenic factors VEGF-C and FGF-2 on lymphangiogenesis and tumor metastasis, and the interplay between VEGF-C and FGF-2.

3 METHODS

In this section, I will highlight the details in the methods used in our research that require extra attention or discussion. The details of each protocol can be found in the constituent papers.

3.1 ANIMAL MODEL

It is difficult to relate conclusions from an animal experiment directly to clinical practice since there are individual variances amongst people as well as many biological differences between animals and humans. Despite not being able to relate the situation seen in animals directly to humans, in most preclinical studies, animal models are necessity. Laboratory animals have identical and homogeneous biological backgrounds, thus it is easier to exclude individual variability from a group of model animals in comparison with a human population, and therefore a smaller sample size is needed for equal statistical power. Additionally, there is better accessibility to a larger sample size when it is required. To manage and collect samples from a group of animals is more feasible. The animal models are also able to take over the majority of risks during scientific research, for example, the toxicity of a new compound or a new therapeutic protocol. These points are important given the *in vitro* experiments can currently not mimic the systemic reaction of a whole organism, or many complex interactions seen in the human body.

3.1.1 Murine tumor xenograft model

To validate the anti-tumor therapeutic protocol, we have to perform animal experiments. In this thesis, we inoculated tumor cells subcutaneously into the mid line dorsal area to be able to observe the tumor size accurately in real-time, meanwhile, to avoid influences from interscapular and inguinal adipose tissue that are highly vascularized organs. If such close observation of the tumor size is unnecessary, the orthotopic tumor model is preferred, because it provides an appropriate tumor microenvironment and produces accurate phenotypes.

We measured the tumor size with vernier calipers. And the volume of tumor was calculated according to a formula: tumor size = length × width^2 × 0.52 if the tumor grew into hemispheric mass. If the tumor was not hemispheric, we compensated by measuring the dissected tumor mass. The dissected tumor mass was obtained for the JE-3 tumor in paper II and the metastatic lymph nodes in paper IV.

When one treats the mice with antibodies or small molecules, one should consider the following issues. Firstly, the best route of drug administration must be decided upon. These can be oral (p.o.), intravenous injection (i.v.), or intraperitoneal injection (i.p.). If possible, we first select the same route of administration as the clinical application of the drug. An example of this would be sunitinib as it is given orally in paper II. Secondly, the pharmacological properties should be taken into consideration, for example when a drug needs to be cleaved into its functional metabolite by low pH, we have to choose p.o. to

transport the drug into stomach. Additionally, we tend to choose the easiest route of administration to minimize suffering of the mice. The second point of consideration is to optimize the dosage. Every species has specific metabolic rates and other features that affect pharmacokinetic parameters, moreover, the sensitivities to the drug also alter in different animals. So one should consider the species, age, gender and size of the animal when one calculates the dosage, and a pilot experiment using different dosages is often required.

Finally, we need to sacrifice the mice using different methods according to the ethical permit and the purpose of the experiment. If we want to collect blood by heart puncture or the mice need to be perfused through heart injection, CO_2 inhalation is the best approach. Otherwise, cervical dislocation is a good way to prevent undue pain during euthanasia.

3.1.2 Metastasis assay

Nowadays, several *in vivo* metastasis models are frequently used to evaluate every steps of distal metastasis. In order to evaluate the capability of tumor cell intravasation, we can check the number of fluorescently labeled circulating tumor cells (CTCs) using Fluorescence-activated cell sorting (FACS) or by blood culture assay[71]. If tumor cells were injected via the tail vein, by observation of the formation of metastatic nodules in distal organs, we can obtain information on tumor cell extravasation[163]. In paper IV, we examined the pulmonary metastasis to assess the hematogenous metastasis as the dorsal subcutaneous tumors have the tendency to metastasize to the lung. To evaluate lymphatic metastasis, we examined the subaxillary lymph nodes, as these are the sentinel lymph nodes for our tumor bearing mice model. Recently, we developed a new zebrafish metastasis model with which we can ascertain the aggressiveness of a tumor cell line efficiently[164].

3.1.3 Mouse corneal model

In order to understand the mechanisms behind angiogenesis or examine the capabilities of an angiogenic factor to exert an effect, proper biological assays need to be established. In the past, a series of *in vitro* and *in vivo* assays have been developed and utilized to investigate angiogenesis[165, 166]. The *in vivo* assays allow us to observe the exact outcomes in a sophisticated physiological tumor microenvironment, which can never be reproduced *in vitro*. Amongst all the *in vitro* assays available, we chose the mouse corneal micropocket assay to study angiogenesis and lymphangiogenesis stimulated by FGF-2 and VEGF-C in paper IV based on the following considerations: (1) Compared with the chick embryo chorioallantoic membrane (CAM) assay and transparent zebrafish embryos, mice have less species difference from human beings. (2) Unlike the fragile CAM, the cornea is naturally exposed to the air and will not be disturbed by the atmospheric gas levels or unregulated pH. (3) The new vessel formation in the cornea is considered to be the most reliable evidence of neo-angiogenesis, because the mouse cornea is a completely avascular tissue and can avoid influence from pre-existing vessels[167]. (4) According to our previous data, the mouse corneal micropocket assay is reliable, stable and quantitative.

The stimulators are not placed in the center of the mouse cornea, the new vessels usually grow unevenly. In order to compare the quantified data in a reproducible and equitable way we divided the whole flat-mounted cornea into four circular sectors and named them according to their location relative to the pellets, for example, 1.5-4.5h. After this we can compare the vessel area of the whole cornea from each groups, or the vessel area in the same sector from different groups.

3.2 EXPERIMENTAL METHODS *IN VITRO*

3.2.1 Cell proliferation assay

In paper I, II, and IV, we measured the cell proliferation rate using the MTT assay. The basic rationale of MTT assay is to detect the formazan product from reducing MTT, which is used as an approximation of the amount of MTT taken up by living cells. This is a quick and convenient assay, but is not a suitable way of comparing proliferation rates of different cell types. This is due to variability of different cell types to reduce MTT. Additionally, the value from this assay does not give the exact number of cells in each sample but just provides an impression of the relative cell density. The MTT reagent is sensitive to light, so the MTT assay should be performed in the dark. Nowadays, other tetrazolium salts have been developed to replace MTT, e.g. XTT[168], MTS, WSTs[169].

3.2.2 Cell migration assay

To study the ability of cells to migrate or evaluate chemotaxic strength of an attractor on a certain type of cell, target cells and agents were usually assembled into a 3D culture system where the cells can be co-cultured with the other cells or factors. The Boyden chamber assay is one of the classical ways for studying cell chemotaxis—cell motility toward increasing concentrations of soluble attractants. In paper IV, we used the Boyden chamber assay to measure LEC migration, as our purpose was to assess VEGF-C and FGF-2 stimulated cell migration regardless of the effect on cell proliferation and cell-cell interactions. In this chamber, ECs generally go through the porous membrane within a few hours, a much shorter time than is required for cell division. The other well established systems for evaluating of cell migration include the *in vitro* wound healing assay, the tube formation assay, and the cell mobility assay in matrigel[170, 171].

3.3 IMAGING ANALYSIS

Imaging is an important method to evaluate neo-angiogenesis. The vasculature can be distinguished from the surrounding tissues by perfusion reagents, specific vessel markers and by cell conformation[41, 51]. In this thesis, we imaged the vessels with a stereomicroscope (Paper IV), a confocal microscope (all papers) and a transmission electron microscope (TEM, Paper III).

3.3.1 Labeling of targets

In order to detect a certain group of cells to be able to analyze their location, number or morphological features, we label them with specific cell markers. Usually the same marker can be shared by different groups of cells while one cell can express several markers, therefore we may identify the target cells with more than one markers.

3.3.1.1 Blood endothelial markers

CD31 is a widely used immunohistological marker for ECs, therefore it was chosen for this thesis. CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is a pan marker for blood ECs. CD31 is not only highly expressed on the surface of adult and embryonic vascular ECs but also expressed on bone marrow stem cells, platelets, megakaryocytes, myeloid cells, T lymphocytes, trophoblasts[172], and even human brown adipocytes[173]. CD34 is also another frequently used EC marker and is mainly expressed on early hematopoietic and vascular-associated tissue. Additionally, endomucin, VEGFR-2, and Dll4 amongst others also serve as endothelial markers in diverse studies[166, 174].

3.3.1.2 Lymphatic endothelial markers

Various proteins have been used as LEC markers, including VEGFR-3[149], LYVE-1[175], podoplanin[176], Prox-1[148, 177] and NRP-2[4]. In paper IV, LYVE-1 was used for immunofluorescence staining to distinguish lymphatic vessels from blood vessels in tumors. It is generally expressed on LECs of lymphatic capillaries but not on LECs from collecting lymphatics[175].

3.3.1.3 Mural cell markers

The detection of pericytes is often dependent on certain angiogenic stage of the tissue or in a tissue-specific manner[12, 13]. Neuron-glial antigen 2 (NG2), also known as melanoma-associated chondroitin sulfate proteoglycan, is the most widely used pericytes surface pan-marker (Paper I and II). Interestingly, NG2 binds to angiogenic regulators such as FGF-2, PDGF-AA and angiostatin[178]. As the name suggests, NG2 is expressed in the central nervous system by oligodendroglial precursor cells and pericytes[179]. PDGFR-β, also a cell-surface protein, is the critical receptor controlling the recruitment of pericytes. It is expressed in pericytes progenitors and pre-mature pericyte, however in the mature pericytes, PDGFR-β is expressed in lower levels[180]. Alpha-smooth muscle actin (αSMA) is wildly expressed by the smooth-muscle cell lineage and myofibroblasts (Paper I)[181]. Similarly, desmin is usually expressed in muscle cells, including skeletal muscle cells, smooth muscle cells and myocardial cells[182]. Both Desmin and αSMA are normally used as markers of contractive mural cells. Additionally, by gene expression profiling, new pericyte markers were identified from PDGF-BB deficient embryos[183]. Regulator of G-protein signaling 5 was found in pericytes and smooth muscle cells. Its expression is further induced during angiogenesis. Interestingly, Kir6.1 is considered as another specific pericytes marker, but it is detectable in special tissue, for example, brain and pancreas[69, 183].

3.3.2 Imaging

In this thesis, several microimaging techniques were used to observe alterations in angiogenesis. In paper IV, under the stereomicroscope, we saw the gross new blood vessel structures but not the lymphatic vessels due to the presence of red blood cells. In paper III, TEM allowed us to see the endothelial fenestrations and caveoli directly without labeling as it was possible to distinguish the capillary ECs according to their special ultrastructure and cellular organelles.

Confocal microscope imaging is a robust technique to acquire 3D reconstructions of the vasculatures. It is also the most frequently used method in our studies. Before scanning, we perform a whole mount staining procedure to label the samples with fluorescein and specific cell markers. Even though whole mount staining requires fresh tissues and consumes more time and reagents, the confocal imaging allows us to: (1) reconstruct the whole 3D structure of the vessels, (2) see the deep layer of the sample, (3) obtain precise positional information of each component of the tissue, (4) observe the details of the cell in a 3D pattern and (5) localize the labeled molecules in the cell[184, 185].

4 RESULTS AND DISCUSSION

The constituent papers in this thesis describe the angiogenic effects of VEGF family members on the tumor vasculature (paper I, II and IV), the results from the remodeling of tumor vessels including impacts on distant metastasis (paper IV) and therapeutic outcomes of anti-VEGF treatment (paper II). Finally, paper III illustrated the systemic consequences of anti-VEGF treatment.

4.1 PLGF REGULATES THE TUMOR VASCULATURE AS EITHER A PRO-ANGIOGENIC OR AN ANTI-ANGIOGENIC FACTOR DEPENDING ON ITS HETERODIMERIZATION WITH VEGF-A (PAPER I)

Despite numerous studies, the role of PlGF in tumor growth and angiogenesis is still controversial[186]. Up-regulation of PlGF has been reported to be a promoter of mouse melanoma angiogenesis, growth and metastasis[187]. However, in another tumor model, overexpression of PlGF showed the opposite effects[188].

Why does this debate exist? Bjorndahl et al. have reported that PlGF and VEGF-A heterodimerize with each other[189]. We propose that the different experimental results come from the complicated cross-talk of PlGF, VEGF-A, VEGFR-1 and VEGFR-2.

To simplify the study, we removed the key factor—VEGF-A from this system by choosing a VEGF-A-null tumor cell line as the major subject. This mouse fibrosarcoma tumor cell line, 528ras, was derived from VEGF-A deficient mice embryos, the Ras oncogene was transfected into the non-tumorigenic fibroblasts to give rise to a highly tumorigenic and fibrosarcoma[190]. Another mouse fibrosarcoma tumor cell line T241 was used as VEGF-A-positive control.

To investigate the function of PlGF on VEGF-A negative and VEGF-A positive tumors, *PLGF* cDNA was cloned into 528ras and T241 cells to yield overexpression of PlGF. However, the overexpression of PlGF had no impact on tumor cell proliferation rates *in vitro* (Figure 1A). We then checked the levels of various dimers inside and outside the tumor cells by performing a sandwich ELISA assay using cell lysate and cell culture conditioned medium. The ELISA data verified that the PlGF/PlGF dimers were markedly up-regulated in *PLGF* transfected cells and the majority of PlGF homodimers were secreted into the conditioned medium. Despite this, VEGF-A/PlGF heterodimers were significantly increased in the medium of *PLGF* transfected VEGF-A positive cells. Interestingly, the secretion of VEGF-A/VEGF-A homodimers was partially prevented by formation of VEGF-A/PlGF heterodimers (Table S1).

We wondered what would happen in tumor vasculature based on the changes of various dimers detected *in vitro*, so we implanted tumor cells subcutaneously into C57BL/6 mice. Using the s.c. tumor-bearing mouse model, we monitored the tumor development by palpation and measured the tumor size with vernier calipers. Usually, the tumor masses under

the skin grew in a hemispherical manner, the volume of tumor was calculated according to a formula mentioned above.

First, we investigated the angiogenic effect of PlGF in VEGF-A positive tumors. One million T241 Vector or T241 PlGF tumor cells were injected into each mouse. It took 16 days for T241 Vector tumors to grow to 1 cm^3. Within the same period, the average volume of T241 PlGF tumors only reached 0.5 cm^3 (Figure 1B lower chart). High levels of PlGF suppressed primary tumor development in VEGF-A positive tumor cells.

We then examined the tumor vessel structure using immunohistofluorescent staining with an EC marker—CD31 and a pericyte specific marker—neuron-glial antigen 2 (NG2). Analysis of 3D images of intratumoral vessels showed that the average number of vessels per field dramatically reduced and the vessels were noticeably more dilated in the *PlGF* transfected tumors. Compared with the T241 vector tumor, T241 PlGF tumor vessels were smoother with less branching points. The NG2 signal increased in the *PlGF* transfected tumors, showin they possessed better pericyte coverage (Figure 1C and D).

Besides the vessel structure, we also explored the functional changes that resulted from PlGF-induced tumor vessel remodeling, including perfusion and leakage of vasculature in the tumors. For this purpose, we perfused the tumor bearing mice with two forms of a hydrophilic polysaccharides dextran weighing either 2000 kDa or 70 kDa through the tail vein just before euthanasia of the animals. The dextran was labeled with a fluorescent dye and conjugated to lysine residues that can subsequently be treated with paraformaldehyde for fixation. The dextran perfusion assay showed that improved pericyte coverage in PlGF tumor reduced extravasated 70 kDa dextran, and increased the number of 2000 kDa dextran perfused vessels due to more organized vasculature in PlGF tumor (Figure 1E and F).

To validate the phenomena we saw, especially when theses data were inconsistent with results from some previous literature, we established another stable high PlGF expressing cell line, the Lewis lung carcinoma (LLC) PlGF. We saw the same trend of tumor growth and vessel remodeling as in the previous experiment using the T241 PlGF tumors. The growth of high PlGF tumors was slower compared to vector tumor and in high PlGF tumors, the vessels were dilated and the vascular sprouting was suppressed, resulting in less branch points and a greater coverage of pericytes (Figure S1).

Next, we investigated the angiogenic effect of PlGF in VEGF-A negative tumors. The VEGF-A-null tumor cells with or without PlGF overexpression were inoculated subcutaneously into immune deficient SCID mice subcutaneously. The same experimental procedures were performed and the same parameters were checked and recorded as in the T241 tumor cell experiments. Surprisingly, genetic deletion of VEGF-A from the tumor cells completely reversed the PlGF induced inhibition of tumor growth and remodeling of tumor vasculatures. Compared to the vector control tumors, in VEGF-A-null PlGF tumors we firstly saw that tumor growth rate was accelerated by more than 50% (Figure 1B upper panel). Secondly, the number of CD31 positive vessels were tripled and the number of branch points

doubled. Additionally, the α-SMA marked smooth muscle cell coverage was decreased to about 40% (Figure 1C and D). For the vessel function indexes, the extravasated 70 kDa dextran was dramatically increased, which indicated very leaky vessels, while the 2000 kDa perfused vessel number was significantly decreased(Figure 1E and F).

The question remained as Why PlGF played a pro-angiogenic role in this tumor model. Did the PlGF itself change VEGF-A levels in the tumor? The enzyme-linked immunosorbent assay (ELISA) and quantitative real-time PCR data demonstrated that both protein and mRNA levels of VEGF-A were not altered by overexpression of PlGF in VEGF-A-null tumors (Figure S2C and D). We also examined the tumor-associated macrophages (TAMs) in tumor tissue. Our results differed from another study that showed there was an increase in the switch from M2-like macrophages to M1 macrophages when PlGF was down-reguated[191]. Our study showed that transfection of PlGF led to a decrease in the number of $CD206^+$ M2-like macrophages in VEGF-A-null tumors.

In order to answer whether the angiogenic promotion in the VEGF-A-null PlGF tumor was dependent on host-derived VEGF-A, we blocked VEGF-A with an anti-VEGF-A neutralizing antibody. We administered the anti-VEGF-A neutralizing antibody (also called VEGF blockade in the paper) through intraperitoneal injection, so VEGF-A from all sources should be blocked. As a result, the vessel density of VEGF-A-null PlGF tumors was brought down to the same level as that of VEGF-A-null vector tumor (Figure 2). These data showed that the host-derived VEGF-A was driving factor of angiogenesis in VEGF-A-null PlGF tumors.

Furthermore, we treated the tumor bearing mice with a VEGFR-2 specific neutralizing antibody (DC101, also called VEGFR-2 blockade in the paper) to define the function of VEGFR-2 transduced signaling on PlGF stimulated tumor angiogenesis. Comparable to VEGF-A blockade, anti-VEGFR-2 treatment inhibited tumor growth and vascular sprouting in VEGF-A-null PlGF tumor (Figure 3). This finding verified the critical role of the VEGF-A-VEGFR-2 signaling pathway in tumor angiogenesis. Interestingly, unlike the data from VEGF-A positive tumors, both VEGF-A and VEGFR-2 inhibition did not altered the average vessel diameter in VEGF-A-null tumors (Figure 2 and 3).

4.2 THE RESPONSE TO ANTI-VEGF THERAPY DEPENDS ON THE LEVEL OF PLGF IN THE TUMOR (PAPER II)

The first paper demonstrateed that the interaction between VEGF-A and PlGF affected vascular on remodeling. In addition to those findings, the anti-VEGF-A and anti-VEGFR-2 treatment outcomes gave an idea that the PlGF high expression tumors still respond to anti-VEGF therapy even without tumor cell-derived VEGF-A.

With the purpose of uncovering the contribution of the complex interplay between VEGF-A and PlGF to the therapeutic efficiency of anti-VEGF treatment, we treated both human and mouse PlGF-expressing tumors with several anti-VEGF agents.

In order to screen for an appropriate human tumor cell line, we performed ELISA assay to measure intrinsic PlGF levels in the conditioned medium of different human tumor cell lines. Among all the studied tumors, JE-3 choriocarcinoma expressed the highest level of PlGF, which is not surprising because JE-3 choriocarcinoma is a trophoblastic cancer from placenta (Table S1). We further looked at the vessel structure in all of these tumor tissues by detecting vascular ECs using an anti-CD31 antibody, as expected, the microvessels in the JE-3 tumors had normal properties compared with other human tumors, namely a very smooth vessel contour line with fewer branching points (Figure S1). We made a JE-3 tumor bearing immune deficient SCID mouse model and treated the mice with different anti-VEGF drugs: the anti-VEGF-A neutralizing antibody, the anti-VEGFR-1 neutralizing antibody (MF1, also called VEGFR-1 blockade in the paper), and the anti-VEGFR-2 neutralizing antibody.

Our data demonstrated that JE-3 tumors are hypersensitive to anti-VEGF-A and anti-VEGFR-2 antibodies treatment, but did not respond to anti-VEGFR-1 treatment. At first, the tumor growth curve showed that anti-VEGF-A and anti-VEGFR-2 antibodies dramatically suppressed tumor growth, while the anti-VEGFR-1 antibody did not change the tumor growth rate (Figure 1A and D). The measurement of tumor weight at the experimental endpoint showed the same trend. When we examined the tumor vasculature under confocal microscope, consistent with the growth curve, fewer vessels or even avascular areas were frequently found in anti-VEGF-A and anti-VEGFR-2 treated tumor. In contrast, VEGFR-1 blockade surprisingly increased the tumor vessel density and endothelial cell sprouting. All anti-VEGF agents also resulted in reduced pericyte coverage on the tumor microvasculatures (Figure 1B and E). These findings validate the observation that PlGF-expressing JE-3 tumors were highly sensitive to VEGFR-2 blockade.

In order to know whether the hypersensitivity of JE-3 tumors to anti-VEGF-A and anti-VEGFR-2 blockades is linked to PlGF, we down-regulated PlGF expression in JE-3 tumor cells by *PLGF*-shRNA transfection. Compared to *PLGF*-shRNA transfected cells, quantitative real time PCR showed that the relative *PLGF* mRNA level dropped to 40% while the *VEGFA* mRNA level remained constant (Figure 2C), as did the cell proliferation rate *in vitro* (Figure S3B).

We then treated the control-shRNA JE-3 and *PLGF*-shRNA tumor bearing mice with VEGFR-2 blockade, and measured the growing tumor size every two days. Under these conditions, we were able to judge the drug sensitivity by calculating efficiency of inhibition, which is presented by T/C value (tumor volume of treated group / tumor volume of control group) at both the endpoint and the same-tumor-size point: the smaller the T/C value is, the more sensitive the tumors are to the drugs. As shown in figure 2, knockdown of PlGF in the JE-3 choriocarcinoma not only accelerated the tumor growth *in vivo*, but also led to acquired drug resistance as determined by a rise in the T/C value.

To further explore the mechanism of PlGF mediated drug sensitivity to anti-VEGF treatment, we established a PlGF-expressing T241 fibrosarcoma cell line, and analyzed various VEGF and PlGF dimers levels in cell culture conditioned medium. As expected, T241 PlGF tumor

cells secreted a large amount of PlGF/PlGF homodimers and VEGF-A/PlGF heterodimers (Table 2). Certainly, as seen previously in our research group, the T241 PlGF tumor grew slower than the vector control tumor. In order to exclude the possibility that PlGF directly slowed down tumor cell proliferation, we also tested tumor cell growth *in vitro* and found no difference between these two groups. Additionally, the VEGFR-1 and VEGFR-2 mRNA levels in tumor cells were also monitored by RT-PCR under the positive control of ECs, but neither were detectable. As an extra control, we stimulated the T241 cells with PlGF and VEGF-A protein *in vitro*, and did not see any difference in the rate of cell proliferation (Figure S3).

The T241 tumor bearing mice were then treated with 3 anti-VEGF agents, including VEGF-A blockade, MF1 and DC101, as well as one tyrosine kinase inhibitor (TKI)—sunitinib. Except for VEGFR-1 blockade, all the treatment on T241 tumor gave rise to suppression of tumor growth (sunitinib treated data not shown) and inhibition of tumor vessel angiogenesis (Figure 3 and 4). Similar to what we have seen in the JE-3 tumor model, high levels of PlGF in T241 tumors increased tumor sensitivity to VEGF-A and VEGFR-2 blockade (Figure 3).

Since PlGF is a VEGFR-1 specific ligand, we were curious about the role of VEGFR-1 in PlGF induced tumor growth inhibition and vessel normalization. Therefore, we investigted the T241 PlGF tumor vasculature remodeling in Flt1 $TK^{-/-}$ mice in which the tyrosine kinase domain of VEGFR-1 was genetically knocked out. Compared with the wild type littermate, tumors on Flt1 $TK^{-/-}$ mice had higher vessel density in both T241 vector and T241 PlGF group, indicating that Flt1 may transduce a negative signal of angiogenesis. In addition, in Flt1 $TK^{-/-}$ mice, T241 PlGF tumors had relatively smoother and fewer dilated vessels than the T241 vector tumors, which demonstrate the same trend of normalization as seen in wild type mice (Figrue 4E and F). This data pointed out that PlGF induced vessel normalization is dependent not only on VEGFR-1 but also on the other signaling pathways. One possible explanation may be that this phenotype is a result of the complicated modulation of VEGF-A by PlGF mentioned in paper I.

4.3 VEGF-DEPENDENT PLASTICITY OF VASCULATURE IN HEALTHY TISSUE (PAPER III)

Based on the hypothesis proposed by Dr. Judah Folkman in 1971, several approaches have been developed to block or modulate tumor angiogenesis. Tumor angiogenesis is a sophisticated procedure involving great number of gene products and different cell types. However, VEGF and its signaling pathway is the most important angiogenic factors, thus blockades of VEGF signaling are perhaps the best validated anti-angiogenic approaches. It has been 10 years since the first specific VEGF-A monoclonal antibody—bevacizumab was approved by FDA in the USA to treat certain cancers[192, 193]. In clinical practice, the side effects caused by anti-VEGF agents attract more and more attention nowadays. In order to explore the mechanism behind of anti-VEGF agents related side effects, we performed a series of preclinical experiments on healthy mice using systemic drug administration.

The mice were randomly divided into two groups and treated with a control IgG, or a specific anti-VEGF-A IgG. In order to know which receptor was the major receptor responsible for vessel alteration from VEGF-A blockade, two more groups of mice were given MF1 or DC101. The dosages we applied were previously seen to be able to modify tumor angiogenesis. The vessels in all organs were checked under the confocal microscope after being marked with an anti-CD31 antibody. Notably, even though the drugs were delivered systemically, different tissues reacted to different extents, suggesting vessels in various tissues had a distinctive sensitivity to agents blocking the VEGF pathway. (See the table below, comparison of vessel density of anti-VEGF drugs treated tissue with that of vehicle treated tissue.)

Impact of Anti-VEGF Blockades on Vasculature of healthy mice

	Endocrine organs			Gastrointestinal tracts												Brain					Muscle					
	Thyroid	Adrenal cortex	Pancreatic islet	Gastric wall	Small intestine wall	Small intestine villi	Colon wall	Ovary	Uterus	Liver	Pancreatic acini	Thymus	Renal cortex	Glomerulus	Olfactory bulb	Cerebral cortex	Hypothalamus	Cerebellum	Medulla oblongata	Myocardium	Intrinsic lingual	Skeletal muscle	Bone marrow	Retina	Adrenal medulla
Anti-VEGF-A	***	***	***	**	**	**	*	*	***	*	***	**	***	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Anti-VEGFR2	***	***	**	*	**	*	*	*	**	*	*	**	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Anti-VEGFR1	ns	ns	ns	ns	ns	ns	ns	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*: P<0.05; **: P<0.01; ***: P<0.001; ns: no significant difference

In most tissues, the vasculatures regressed in response to VEGF-A and VEGFR-2 blockades. In the endocrine organs that are physiologically more active, the vessels were inhibited the most. An example of this would be in the thyroid, nearly 60% reduction of vascular density was observed (Figure 1).

Generally, VEGFR-1 blockade slightly increased the vessel area but did not change the vessel density significantly except in the ovaries and uterus. Certainly, as shown by hematoxylin and eosin (H&E) staining, there were no detectable structural changes in any tissue. (Figure 1-4)

Interestingly, tissues in five body compartments, including brain, muscle, bone marrow, retina and adrenal medulla did not respond to any of these three anti-VEGF drugs, at least when the mice were treated with the doses that we gave. (Shown in the supplementary data)

In order to further understand the effect of anti-VEGF-A therapy on healthy tissues, we focused on the most sensitive organ—thyroid, and investigated further. By using the hypoxia probe pimonidazole and examining levels of hypoxia in thyroid tissue, we saw the treated thyroid tissue were more hypoxic (Figure 5G). In addition, the immunohistochemical staining of cleaved caspase-3 illustrated that a higher percentage of vessel ECs underwent apoptosis in the treated thyroid (Figure 5A). We also performed a drug withdrawal experiment to see how stable the anti-VEGF-A induced vessel regression was. The vessel density returned to untreated level approximately14 days after drug cessation (Figure 5B and C).

As mentioned above, the thyroid vessels numbers were dramatically reduced by anti-VEGF-A treatment, so we also determined the extent by which the function of thyroid could be affected. We checked the thyroid hormone level in mouse serum and saw that the free thyroxine (T4) level was significantly impaired after only two weeks of treatment (Figure 5F). Based on this, we wondered whether the change in thyroid function was due to the dysfunctional thyroid vessels. We used dextran perfusion assay described above, to determine the health of treated thyroid vessels. The perfusion assay showed less general perfusion due to fewer vessels in the treated thyroid, even through the average perfusion of each vessel was not altered in response to VEGF-A blockade, the treated thyroid showed less blood perfusion because of the lower density of vessels (Figure 5E). Additionally, according to TEM imaging, the endothelial fenestration of blood ECs were completely suppressed by anti-VEGF-A, leading to an increased number of endothelial caveoli (Figure 5H).

4.4 FGF-2 PROMOTES LYMPHANIGIOGENESIS AND TUMOR METASTASIS VIA VEGFR-3 INDUCED LEC SPROUTING (PAPER IV)

It is well known that VEGF-C is one of the most important growth factors that controls lymphangiogenesis and promotes lymphatic metastasis[130, 133, 152], and in LECs, VEGFR-3 is the major receptor of VEGF-C. FGF-2 was also identified as a pro-lymphangiogenesis factor in previous published papers[65, 194]. One study has shown that the VEGFR-3 blockade inhibited FGF-2-induced lymphangiogenesis in a mouse cornea model, but the mechanism underlying this result were not investigated[65]. In this paper, our data revealed the mechanism behind the collaborative interplay between FGF-2 and VEGF-C in promoting angiogenesis, lymphangiogenesis and tumor metastasis.

In order to understand the cellular and molecular effects of VEGF-C or FGF-2 on LECs, we treated LECs with VEGF-C, FGF-2, or VEGF-C plus FGF-2. Both VEGF-C and FGF-2 could independently stimulate proliferation and migration of LECs (Figure 2A, H and I). Furthermore, the mRNA levels of FGFR-1 and VEGFR-3 in LECs were increased by both VEGF-C and FGF-2 separately (Figure 2E and F). The protein level of phosphorylated intracellular signaling molecules, including p-Akt, p-ERK1/2 and p-rpS6, were also increased by both VEGF-C and FGF-2 stimulation (Figure 2B and C). In addition, in order to validate that FGFR-1 was responsible for FGF-2 stimulated LECs activation, DNA of all FGFRs were detected by PCR, the data showed that *Fgfr1* was expressed in LEC (Figure 2D) but *Fgfr2*, *Fgfr3*, and *Fgfr4* reminded very week or undetectable. To confirm this data, we performed siRNA knockdown of *Fgfr1*. knockdown of *Fgfr1* abolished FGF-2 stimulated LEC proliferation (Figure 2G).

Using a mouse cornea model, we put a tiny piece of polymer pellet containing VEGF-C or FGF-2, VEGF-C plus FGF-2, into the artificial micropocket on the avascular cornea. Six days later, the corneas were collected to check for neovascularization. Visual inspection of the corneas showed remarkable new blood vessel formation in all treated groups (Figure 1A). The analysis of LYVE-1^+ lymphatic vessels and $CD31^+$ blood vessels showed that either VEGF-C or FGF-2 strikingly promoted angiogenesis and lymphangiogenesis, especially the

FGF-2 seemed to have stronger angiogenic effect than VEGF-C. Additionally, implantation of micropellets containing both FGF-2 and VEGF-C led to synergistic hematoangiogenesis and lymphangiogenesis (Figure 1C, G and H). If one looked closely at the LECs, dramatic tip formations were triggered by both FGF-2 and VEGF-C (Figure 3A).

From all the experiments above, we saw very similar effects of FGF-2 and VEGF-C. This fact gave rise to the hypothesis that FGF-2 and VEGF-C may share the same signaling pathway to initiate the similar outcomes.

To further discern the major role of FGFR-1 or VEGFR-3 on hematoangiogenesis and lymphangiogenesis, we tried to block these two signaling pathways with neutralizing antibodies—anti-FGFR-1 and anti-VEGFR-3. The data from *in vitro* experiments exhibited that on one hand, anti-FGFR-1 treatment inhibited the proliferation and migration of LEC induced by FGF-2 but not VEGF-C. On another hand, anti-VEGFR-3 treatment could only inhibit LEC proliferation and migration promoted by VEGF-C but not FGF-2. This result demonstrated that FGF-2 and VEGF-C could independently stimulate LEC via their own receptors. We then performed an antibody blockade experiment *in vivo*. Consistent with the *in vitro* assay, anti-FGFR-1 inhibited FGF-2 induced lymphangiogenesis as well as hematoangiogenesis in mouse cornea (Figure 2K). Meanwhile, anti-VEGFR-3 treatment reduced cornea lymphangiogenesis and the number of LEC filopodia promoted by both FGF-2 and VEGF-C, indicating that VEGFR-3-mediated LEC tip formation was required for both FGF-2 and VEGF-C induced lymphangiogenesis (Figure 3B). Notably, anti-VEGFR-3 did not affect hematoangiogenic activity of either FGF-2 or VEGF-C (Figure 3C).

In order to explore whether collaboration of FGF-2 and VEGF-C synergistically promote tumor hematoangiogenesis and lymphangiogenesis, we established T241 fibrosarcoma cell lines which stably overexpress FGF-2 or VEGF-C by gene transfection. T241 FGF-2 or VEGF-C cells were separately injected into mice or mixed together at ratio of 1:1 prior inoculation.

As expected from our previous finding, both FGF-2 and VEGF-C overexpression significantly accelerated tumor growth. Co-existence of these two factors led to the fastest growth rate (Figure 4A). Consistent with the mouse cornea assay, FGF-2 and VEGF-C dramatically stimulated tumor angiogenesis as well as intratumoral lymphatic vessel formation (Figure 4B and 5A). We then wanted to know how much the high level of vascularization contributed to tumor metastasis. We removed the primary tumors when they reached to 1.5cm^3, and kept the mice for extra 2 weeks. At the endpoint, all mice were sacrificed and examined for subaxillary lymph nodes and lung metastases. We checked under the microscope and found there were no metastatic nodules found in the lungs of T241 FGF-2 tumor bearing mice. Around 40% of VEGF-C tumor-bearing mice got lung metastases. And up to 70% of T241 FGF-2 plus T241 VEGF-C tumor-bearing mice development pulmonary metastases (Figure 4D and E). Examination of sentinel lymph nodes revealed that less than 40% of T241 FGF-2 tumor-bearing mice had enlarged lymph nodes, while all the mice in the other two groups developed subaxillary lymph nodes metastasis (Figure 5D). Compared with

the VEGF-C group, metastatic lymph nodes in FGF-2 plus VEGF-C tumor bearing mice were larger and had a greater mass (Figure 5C, E and F). Analysis of green fluorescent protein(GFP) signal in tumor cells and H&E staining validated the lung and lymph node metastases (Figure 4F and 5G). Taken together, these data demonstrate that FGF-2 and VEGF-C collaboratively accelerate pulmonary and lymph node metastases by stimulating hemato- and lymph-angiogenesis.

As shown in figure 5H, VEGF-C bind to VEGFR-3 on LECs and stimulate LEC proliferation, migration and tip formation, while FGF-2 independently triggers LEC proliferation and migration by activating FGFR-1. However, VEGF-C-VEGFR-3 initiated LEC tip formation is the necessity for FGF-2–induced lymphangiogenesis. Simultaneously, both VEGF-C and FGF-2 promote hematoangiogenesis via signal transduction by VEGFR-2 or FGFRs on blood vessel ECs. Therefore, VEGF-C and FGF-2 collaboratively facilitate tumor metastasis through both blood vessels and lymphatic vessels.

5 CONCLUSION AND PROSPECTIVE

5.1 DUAL ROLES OF PLGF ON TUMOR ANGIOGENSESIS

In VEGF-A positive tumor and VEGF-A-null tumor, overexpression of PlGF resulted in different alteration on tumor growth and vessel remodeling. Both sets of results can be found from previous studies in different research groups. To uncover the underlying mechanisms of these conflicting phenomena will answer many questions existing in the field.

In order to explain the dual roles of PlGF, we should exam the existing research that has been conducted in the field. Firstly, it is known that the VEGF-A-VEGFR-2 signaling pathway is the major trigger of new vessel formation, This is despite VEGF-A having a higher binding affinity to VEGFR-1, which can only be activated very weakly. Activation of VEGFR-1 has an uncertain effect on angiogenesis. Secondly, it has been reported that VEGF-A and PlGF heterodimerize with each other intracellularly and bind preferentially to VEGFR-1 instead of VEGFR-2. Due to the formation of heterodimers, the level of tumor cell secreted VEGF-A homodimers are decreased, thus reducing activation of VEGFR-2, and resulting in reduced angiogenesis and tumor growth rates. Conversely, when overexpressing PlGF in VEGF-A negative tumors, all the excess PlGF molecules are secreted as homodimers and have no chance to alter the number of host derived VEGF-A homodimers. Furthermore, numerous PlGF homodimers occupy more binding sites on VEGFR-1, thus saturating this receptor and pushing VEGF-A homodimers to bind to VEGFR-2, leading to enhanced angiogenesis, vascular permeability, and tumor growth signals (Paper I, Figure 4).

5.2 SIGNALING MODULATION BY CROSS COMMUNICATION BETWEEN PROTEINS

Data from paper I and paper II showed that PlGF-VEGFR-1 negatively modulated tumor angiogenesis when coexpressed with VEGF-A in the same cell. Based on previous research, PlGF may suppress tumor angiogenesis through the following mechanisms. The first mechanism may be due to activation of VEGFR-1 by PlGF. Knockout of *Vegfr1* in mice causes excessive proliferation of embryonic ECs and vessel formation[142]. This hints that VEGFR-1 activation may be responsible for PlGF induced angiogenic inhibition. In paper II, we have further evidence of VEGFR-1 transduced negative signaling—if we block VEGFR-1 downstream signal by genetic deletion of the tyrosine kinase domain, the inhibition of angiogenesis by PlGF overexpression became slightly weaker. The second mechanism may involve trapping VEGF-A on VEGFR-1. Knockout of Tyrosine kinase domain on *Vegfr1* or *Plgf* did not result in hyper-vascularization shown in *Vegfr1*$^{-/-}$ mice[143,138], suggesting that the binding sites of VEGFR-1 are sufficient for suppressing excessive angiogenesis without VEGFR-1 signaling capability. Meanwhile, it has been shown that the signal transduced by VEGF-A-VEGFR-1 binding is very weak even though the binding affinity of VEGF-A to VEGFR-1 is about 10 times of that of VEGF-A binding to VEGFR-2[195]. These data suggest the VEGFR-1 may serve as a decoy receptor for VEGF-A to reduce the promotion of angiogenesis. The final possible mechanism may be due to reduction of VEGF-A

homodimers. Dr. Eriksson reported that PlGF antagonized VEGF-induced angiogenesis and tumor growth by formation of PlGF-1/VEGF heterodimers[196]. Later, another publication verified this initial finding by Dr. Eriksson that overexpression of PlGF in tumor cells reduced secretion of VEGF-A homodimers. In addition to this confirmation, it was also seen that even if all PlGF was trapped in the cells by endoplasmic reticulum retention signal peptide preventing its secretion from cells, overexpression of PlGF still can inhibit tumor angiogenesis[139]. Taken together, this evidence shows that PlGF and VEGFR-1 can interact with VEGF-A and interfere with VEGF-A-VEGFR-2 induced angiogenesis at different levels, including affecting VEGF-A production, VEGF-A-VEGFR-2 binding and by negative angiogenic signaling from activation of VEGFR-1 upon ligand binding.

Actually, there are other elegant signaling control systems capable of regulating angiogenesis. One such example is Angs and TIE receptors system(see 1.1.4.4).

5.3 TARGETING TREATMENT

In Paper III, VEGF neutralizing antibodies resulted in systemic vessel reduction in most of normal tissue and caused abnormal organ functions. To avoid such a wide range of side effects, a novel anti-angiogenic drug specially targeting the tumor vessels would be the best solution. To develop this kind of anti-angiogenic agent with strong specificity to the tumor, specific molecular signitures of the tumor vasculature need to be identified. These specific cell signatures have to be expressed on cell surface in order to guide the targeted anti-tumor agent to the tumor.

To date, several cell surface molecules that are specifically overexpressed on tumor vessels have been found, such as, tumor endothelial marker 1 (TEM1), TEM5, TEM7 and TEM8[197, 198, 199]. Notably, these cell markers highly or specially expressed in the tumor also participate actively in physiological process[200], therefore, more details on the differences between physiological angiogenesis and pathological angiogenesis are still required.

5.4 COMBINED TREATMENT OF MALIGNANT CANCER

The side effects of anti-VEGF drugs are still challenging, however, the therapeutic outcomes obtained from anti-VEGF treatment have been validated in different models. We have seen the following benefits: (1) The immature vessels could not survive, leading to vessel regression[201]. (2) The tumor IFP was reduced and the delivery of other anti-tumor drug, macromolecules and oxygen was increased, therefore the efficiency of chemotherapy and radiotherapy was improved[202, 203, 204]. (3) The permeability of tumor vessels was reduced as well as the risk of tumor cell intravasation and distal metastasis[49, 205, 206].

Another way to enhance anti-tumor efficacy and reduce drug toxicity is to combine different therapies and minimize the dose of each drug. As mentioned above, the vessel modification by anti-VEGF treatment may increase the curative effect of conventional chemotherapeutics or radiotherapy. Our recent study has demonstrated that anti-angiogenic TKIs—such as

sunitinib (commercially named as Sutent, Pfizer) significantly improved tumor bearing mice survival by reducing chemotoxicity of cyclophosphamide (CTX) and carboplatin[207].

In another study, Dr. Bruns[208] et al. combined DC101 with gemcitabine to treat pancreatic tumors and found increased cell death and decreased cell proliferation. One research added VEGF-TRAP to paclitaxel treatment and reduced ovarian tumor burden[209]. Zips and colleagues delayed the squamous carcinoma growth by combination of vatalanib (PTK787, Bayer Schering and Novartis) and radiotherapy[210]. Moreover, combination of anti-hematoangiogenesis and anti-lymphangiogenesis agents showed better therapeutic outcomes in controlling tumor development and metastasis then a single anti-angiogenic approach[211, 212].

5.5 BIOMARKERS FOR TREATMENT OUTCOMES AND PROGNOSIS

In clinical practice, we not only choose the better therapeutic regimen for the patients according to indications and contraindications, but also select treatment of patients by certain biomarkers that can did prediction of the therapeutic efficacy and prognosis. With some level of prediction, the treatment may result in very modest benefits and cause harmful side effects. A perfect biomarker should have the following features: (1) Sensitivity: its levels should change consistently together with the particular phenotype. (2) Feasibility: it should be easy to be detected the samples and the samples should be easy to obtain. The detection method should be quick, accurate and economical. (3) Specificity: it should be specific for a particular phenotype.

It has been reported that the use of anti-PIGF on anti-VEGF drug resistant tumors led to inhibition of tumor growth[213]. This is in contrast to another study that did not show inhibition of angiogenesis and tumor growth in various tumors treated by PlGF blockade, except on a VEGFR-1 overexpression tumor[214]. Under these conditions, the expression level of VEGFR-1 in a tumor may be a potential biomarker for anti-PlGF treatment.

In paper II, mouse and human tumors with high PlGF expression were more sensitive to anti-VEGF drug than the low PlGF expressing tumors. In this case, the expression level of PlGF in a tumor may serve as a potential biomarker for anti-VEGF treatment.

Increasing evidences including our unpublished data, show that VEGF-B level in tumor is correlated with cancer metastasis and survival[121, 215], thus VEGF-B can be a potent predictor for prognosis.

Another candidate is FGF-2. Many researchers put efforts on validating the prognostic ability of FGF2 levels in urine or serum of cancer patients, however, the results are still ambiguous[59, 216, 217]. Notably, FGF-2 has no signal peptide for secretion, subsequently, expression level of FGF-2 in cells does not necessarily correlate with the amount of FGF-2 outside the cells. Yet, cell damage and exocytosis has been confirmed as the potent alternative ways of releasing the growth factor to the ECM[218].

Plenty of well-designed clinical studies are required to validate the potential of a new biomarker. Before that, we can do a preliminary assessment by checking and analyzing data from the clinical databases.

5.6 VEGFR-3 PLAYS THE CRUCIAL ROLE IN LYMPHANIOGENESIS

In paper IV, anti-VEGFR-3 treatment blocked FGF-2 stimulated lymphangiogenesis in mouse corneas by preventing LEC tip formation This means that VEGFR-3 activation is indispensable in lymphangiogenesis even though FGF-2 can independently induce LEC proliferation and migration *in vitro*. Upon reviewing the literature, more similar cases can be found. One of our recent paper uncovered that Tumor necrosis factor alpha (TNF-α) promoted lymphangiogenesis was blocked by VEGFR-3 neutralizing antibody which led to reduction in the number of the LEC tips[219]. Another study reported that hepatocyte growth factor (HGF) induced lymphangiogenesis was partly blocked by a soluble VEGFR-3[220]. All these results suggest that VEGFR-3 may play a crucial and irreplaceable role in inihibition of LEC tip formation during lymphangiogenesis under the control of many different factors. Certainly, there are still some questions that need to be answered. What is the active ligand for VEGFR-3 in FGF-2 induced angiogenesis? Is it possible that FGF-2 can directly activate VEGFR-3? Did FGF-2 lead to an increased expression of VEGFR-3 and VEGF-C or VEGF-D? In future, we first can measure the expression level of VEGFR-3, VEGF-C and VEGF-D upon FGF-2 stimulation. Furthermore, we need to check which cell type is responsible for the production of VEGFR-3, VEGF-C and VEGF-D. The VEGF-C and VEGF-D blockade treatment can provide the information whether VEGF-C and VEGF-D are the only activator of VEGFR-3.

5.7 ANIMAL MODELS IN PRECLINICAL STUDIES

Because there are a few similarities in physiological and pathological states between humans and the other species, various types of animal models have been built up with the purpose of understanding the progression of human diseases. However, more and more translational studies revealed that the therapeutic effects seen from animal models could not be reproduced in clinical practice. We shall be careful when translating laboratory experimental data to clinical situations. Considering the differences between a group of experimental animals and a group of clinical patients, we can see why the difficulties exist. Firstly, genetic divergence of evolutionarily between creatures means that different species certainly do not react exactly the same way to different pathogenic situations or treatments. Secondly, there are many more individual variations among the humans than experimental animals, that are genetically identical and live under controlled environments. Nevertheless, animal models are still required for preclinical studies. Importantly, we should always set the appropriate control groups to minimize the interference and error. In addition, extra attentions should be paid to the “three R” principle when performing the animal experiment, reduce, refine and replace.

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