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Karolinska Institutet, Stockholm, Sweden

MECHANISMS OF TUMOR MICROENVIRONMENT IN PROMOTING METASTASIS

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Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by E-Print AB

ISBN 978-91-7676-474-9

Mechanisms of tumor microenvironment in promoting metastasis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Atrium, Nobels väg 12B

Måndagen den 28 November, 2016, kl 09:30

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'In the middle of difficulty lies opportunity'

Albert Einstein

ABSTRACT

Tumor tissues contain diverse cell populations that relentlessly cross-communicate with each other in the tumor microenvironment. In addition to malignant cells, infiltration of other host cells including inflammatory cells, fibroblasts, and cells in the vessel walls in tumors significantly contribute to tumor growth and metastasis. The diversity of cell populations in the tumor microenvironment determines the production of various growth factors and cytokines, which are often upregulated. Although these signaling molecules interact with their specific receptors to trigger signaling events in the targeted cells, they often crosstalk to each other to elicit additive or synergistic effects in the tumor tissue. This thesis work provides several examples of such complex interactions between various cellular and signaling components in the tumor microenvironment in promoting metastasis. We particularly focused our research on angiogenesis-related signaling events to identify molecular mechanisms underlying tumor metastasis. In paper I, we show that expression levels of PDGF-BB in tumor cells can serve as a surrogate marker for drug response. One of the most surprising findings is that high levels of tumor cell-derived PDGF-BB ablates pericytes from the tumor microvasculature. Mechanistically, tumor cell-derived PDGF-BB attracts pericytes from the vessel wall toward tumor cells, leaving the endothelium unprotected. Ablation of pericytes leads to exposure of primitive microvessels susceptible for tumor cell intravasation. As a result, inhibition of the PDGF-BB-PDGFR signaling in high PDGF-BB-producing tumors prevents tumor cell intravasation and metastasis. Conversely, inhibition of the PDGF-BB-PDGFR signaling in PDGF-BB negative tumors ablates pericytes from the tumor microvasculature and promotes tumor metastasis. Therefore, PDGF-BB levels may serve as a potential surrogate marker for predicting anti-PDGF therapeutic outcomes. In paper II, we uncover a novel mechanism of pericytes in promoting tumor metastasis. In PDGF-BB-activated pericytes, genome-wide profiling shows that IL-33 is the most upregulated gene among all genes. ST2 as a receptor for IL-33 is abundantly expressed in macrophages. In various *in vitro* and *in vivo* experimental settings, IL-33 promotes the polarization of macrophages to an M2 subtype. Gain- and loss-of-function experimental data show that IL-33-activated macrophages promote tumor metastasis. Together, this work reveals a previously unknown mechanism underlying pericyte-mediated tumor metastasis and targeting the PDGF-PDGFR-IL-33-ST2 signaling axis provides a novel therapeutic option for treatment of cancer patients. Paper III identifies VEGF-B; a VEGFR-1 exclusive binding ligand, as a promoter of tumor metastasis through a VEGF-A-independent mechanism. VEGF-B remodels tumor vessels to become pseudonormalized and highly leaky by ablating pericytes from tumor vessels. The highly leaky tumor vessels permit tumor cell intravasation into the circulation and facilitate metastasis. Importantly, high expression levels of VEGF-B in cancer patients correlate with poor prognosis. In the last paper, we show that FGF-2 and VEGF-C collaboratively promote lymphangiogenesis. For the first time, we show that the VEGFR3 signaling is crucial for non-VEGF-C-induced lymphatic networks. Importantly, FGF-2 and VEGF-C synergistically promotes metastasis. Altogether, this thesis work uncovered several novel mechanisms underlying tumor metastasis and targeting these signaling pathways may offer new opportunities for effective treatment of cancer patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är en sjukdom som drabbar allt fler människor världen över och där dödligheten är fortsatt väldigt hög. Den främsta orsaken till att patienter dör, är att sjukdomen spridits till flera organ i kroppen via en process som kallas metastasering. Idag finns det ingen effektiv behandling för patienter som utveckar metastaser. En målsättning med den här avhandlingen är att med ny kunskap kunna utveckla bättre behandling av dessa patienter. En tumör uppstår på grund av okontrollerad tillväxt av celler som överlever trots att dom inte borde. Dessa växande celler klarar sig dock inte på egen hand, utan behöver hjälp från andra typer av celler som tillsammans utgör den så kallade mikromiljön. Här finner man celler som bygger upp blodkärl och lymfkärl, stödceller, celler i bindväven, samt immunceller. Utöver celler, spelar även lösliga faktorer stor roll för uppbyggnaden av tumörer. Det sker intensiv kommunikation och samarbete mellan alla dessa delar och det påverkar hur tumörer växer, sprider sig, samt hur väl dom svarar på behandling. Idag har vi endast begränsad kunskap om detta samspel. I det beskrivna arbetet har vi lyckats samla information om sådana samspel. Den växande massan bestående av tumörceller behöver konstant inflöde av syre och näring, vilket sker med hjälp av blodkärl. Man vet att tillväxt av nya blodkärl i tumörer är avgörande för tumörens överlevnad. Spridning av tumörceller kan ske lokalt till närliggande vävnader, men vanligtvis sker det via kärlsystemen. Blodkärl kan ses som ett rör med flera skyddande lager på utsidan. Ett av dessa skyddande lager utgörs av så kallade stödceller som har i uppgift att skydda kärlen och förhindra läckage. Tumörceller har väldigt svårt att bryta detta lager och kan normalt sett inte ta sig in i blodkärlen. Det finns dock olika sätt som detta skyddande lager kan försämras och ge tumörcellerna en chans att ta sig in blodkärlen och vidare sprida sig till andra organ. Vi har påvisat hur tumörceller direkt kan producera lösliga faktorer som leder till ett försämrat skyddande lager. Som ett resultat, fann vi att tumörer började växa i lungor och i lever. Tumörceller är som fabriker där allt möjligt tillverkas. Celler i omgivningen påverkas av olika saker som produceras. Vi har funnit hur stödcellerna runt blodkärl kan utbildas av tumörceller till att aktivt bidra till att underlätta spridning av tumörceller. Som i många fall när det kommer till cancer, så är förklaringen till ett visst beteende väldigt komplicerat. Ha i åtanke att det, som tidigare nämnt, sker intensiv kommunikation mellan olika delar i mikromiljön. I stora drag:

Tumörceller påverkar mängden och kvalitén på blod- och lymfkärl → tumörceller har lättare tillgång till blodkärl → metastaser

Tumörceller påverkar immunceller → immunceller hjälper tumörceller att sprida sig via blodkärl → metastaser

Tumörceller kommunicerar med stödceller runt blodkärl → stödceller påverkar immunceller → immunceller hjälper tumörceller att sprida sig via blodkärl → metastaser

Genom den kunskapen vi bidrar till att utöka, kan vi hitta nya sätt för behandling av cancerpatienter. Samt underlätta valet av behandling som bör ges till en viss patient utifrån olika kännetecken hos den enskilde individens tumör.

LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. Hosaka K, Yang Y, Seki T, Nakamura M, **Andersson P**, Rouhi P, Yang X, Jensen L, Lim S, Feng N, Xue Y, Li X, Larsson O, Ohhashi T, Cao Y.
Tumour PDGF-BB expression levels determine dual effects of anti-PDGF drugs on vascular remodelling and metastasis.
Nature Communications 2013;4:2129.

II. Yang Y*, **Andersson P***, Hosaka K, Zhang Y, Cao R, Iwamoto H, Yang X, Nakamura M, Wang J, Zhuang R, Morikawa H, Xue Y, Braun H, Beyaert R, Samani N, Nakae S, Hams E, Dissing S, Fallon PG, Langer R, Cao Y.
The PDGF-BB-SOX7 axis-modulated IL-33 in pericytes and stromal cells promotes metastasis through tumor-associated macrophages.
Nature Communications 2016;7:11385. *equal contribution

III. Yang X, Zhang Y, Hosaka K, **Andersson P**, Wang J, Tholander F, Cao Z, Morikawa H, Tegnér J, Yang Y, Iwamoto H, Lim S, Cao Y.
VEGF-B promotes cancer metastasis through a VEGF-A-independent mechanism and serves as a marker of poor prognosis for human cancer patients.
Proc Natl Acad Sci U S A. 2015;112(22):E2900-9.

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

LIST OF RELATED SCIENTIFIC PAPERS, NOT INCLUDED IN THE THESIS

I. Yang Y*, Zhang Y*, Iwamoto H, Hosaka K, Seki T, **Andersson P**, Lim S, Fischer C, Nakamura M, Abe M, Cao R, Skov PV, Chen F, Chen X, Lu Y, Nie G, Cao Y.
Discontinuation of anti-VEGF cancer therapy promotes metastasis through a liver revascularization mechanism.
Nature Communications 2016;7:12680 *equal contribution

II. Ji H*, Cao R*, Yang Y, Zhang Y, Iwamoto H, Lim S, Nakamura M, **Andersson P**, Wang J, Sun Y, Dissing S, He X, Yang X, Cao Y.
TNFR1-mediates TNF-α-induced tumor lymphangiogenesis and metastasis by orchestrating the VEGF-C–VEGFR3 signaling.
Nature Communications 2014;5:4944. *equal contribution

III. Lim S*, Zhang Y*, Zhang D, Chen F, Hosaka K, Feng N, Seki T, **Andersson P**, Li J, Zang J, Sun B, Cao Y.
VEGFR2-mediated vascular dilation as mechanism of VEGF-induced anemia and bone marrow cell mobilization.
Cell Reports 2014;9(2):569-80. *equal contribution

IV. Jensen LD, Cao Z, Nakamura M, Yang Y, Bräutigam L, **Andersson P**, Zhang Y, Wahlberg E, Länne T, Hosaka K, Cao Y.
Opposing effects of circadian clock genes Bmal1 and Period2 in regulation of VEGF-dependent angiogenesis in developing zebrafish.
Cell Reports 2012;2(2):231-41.

CONTENTS

LIST OF ABBREVIATIONS

Ang	Angiopoietin
CML	Chronic myeloid leukemia
CSF	Colony stimulating factor
CTC	Circulating tumors cell
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
HIF	Hypoxia inducible factor
IFN	Interferon
IL	Interleukin
IL-1RAcP	IL-1 receptor accessory protein
LEC	Lymphatic endothelial cell
Mφ	Macrophage
MMP	Matrix metalloproteinase
NHL	Non-Hodgkin’s lymphoma
PC	Pericyte
PDGF	Platelet-derived growth factor
PHD	Prolyl hydroxylase
PlGF	Placenta growth factor
RTK	Receptor tyrosine kinase
TGF	Transforming growth factor
TIR	Toll/IL-1R
TNF	Tumor necrosis factor
VSMC	Vascular smooth muscle cell
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 CANCER

The World Health Organization reported that 8.2 million deaths due to cancer occurred worldwide during 2012. These numbers are projected to increase by 75% over the coming two decades[1]. According to recent estimations, about one third (1/3) of the Swedish population will suffer from cancer[2].

Cancer is a complex disease that is not restricted to the simple outgrowth of a malignant tumor at a single location in the body. Rather, the disease is affecting the host systemically and most commonly, tumor cells are spreading throughout the body, forming metastases[3]. The consequences of metastatic growth, ultimately, are the major causes of death for cancer patients[4]. To reduce mortality, additional efforts are necessary to understand the processes by which tumors are spreading, which constitutes one major hurdle in the treatment of cancer patients[5]. This thesis work aimed to reveal mechanisms microenvironmental regulation of tumor metastasis.

1.2 HALLMARKS OF CANCER

The formation of a tumor is an intricate process with a few shared common features, known as the hallmarks of cancer. The six hallmarks were first described by Hanahan and Weinberg in 2000, and later revisited in 2011 when four additional features were included[6]. Three of the hallmarks are specifically addressed in this current thesis; 1) induction of vessel growth by angiogenesis, 2) generation of a tumor-promoting microenvironment, and 3) metastasis (Figure 1). These hallmarks will be discussed in detail throughout the thesis.

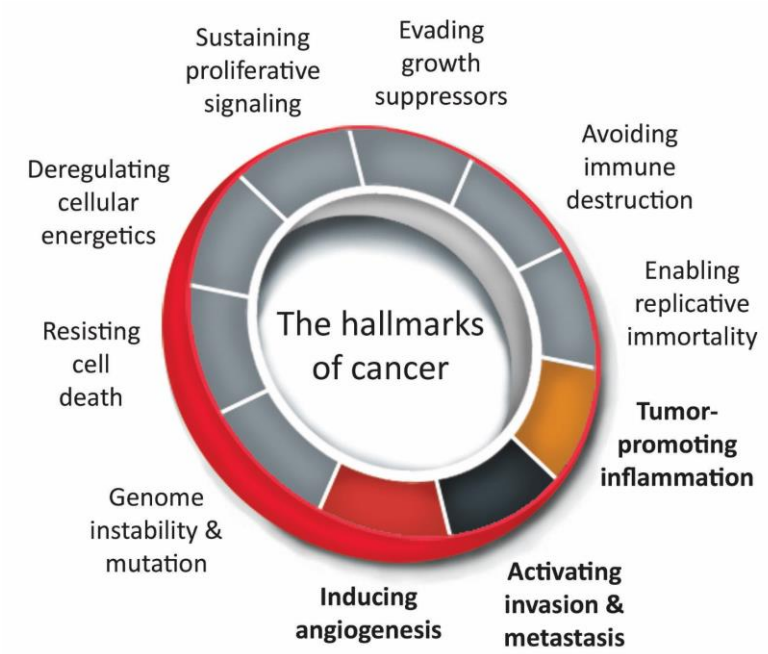

Figure 1. *The hallmarks of cancer.*

1.3 ANGIOGENESIS

There are multiple steps that are crucial during the formation of a solid tumor. Understanding how angiogenesis and modulation of blood vessels promotes tumor metastasis was of central focus of the current study. Angiogenesis is a process based on a sprouting principle where committed endothelial cells (ECs) from a pre-existing vessel are instructed to form new vessels[7]. The concept of tumor angiogenesis was initiated by Dr. Judah Folkman in 1971. He hypothesized that tumor masses would require additional blood supply to sustain their growth beyond 2 mm^3, and that tumors are able to initiate vessel growth[8]. Tumors that fail to induce such blood vessel growth will regress or remain at a dormant stage. The field of anti-angiogenic targeting therapy was initiated based on this theory, and will be discussed further in sections 1.8.2 and 1.9.

As the tumor mass continues to grow, it transitions from a stage of being avascular (lacking blood vessels), to being able to induce the first sprout, subsequently experiencing enhanced growth with continuous increase in the intratumoral vascularity[9] (Figure 2).

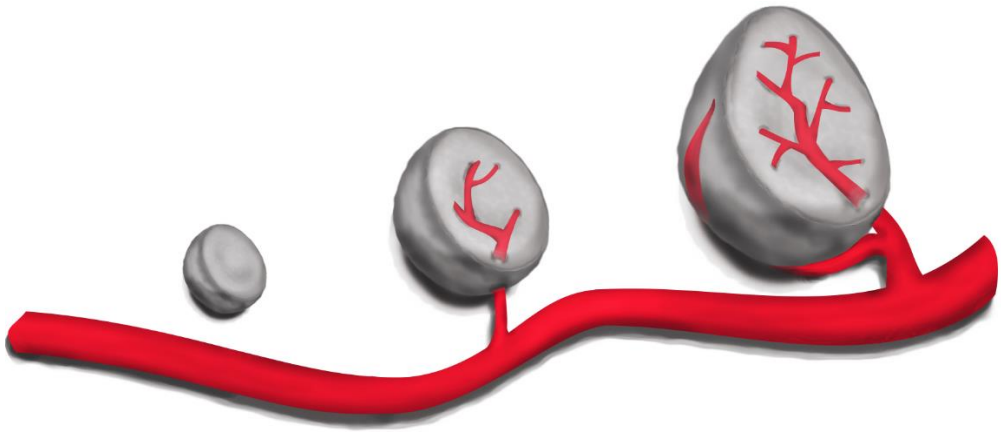

Figure 2. *Stepwise growth of new blood vessels into the growing tumor mass by angiogenesis.*

Coordinated events are required to permit sprouting of a quiescent vessel. The majority of mature vessel consists of multiple layers surrounding the inner EC layer, including mural cells and a basement membrane[10]. The basement membrane is a unique part of the extracellular matrix (ECM), mainly composed of laminin and collagen, and provides stability for the vessel, as well as playing a crucial role for EC survival[11, 12]. When angiogenesis is initiated, this fibrous structure needs to be degraded by ECM enzymes; the proteases[13]. Upon receiving the appropriate signals, one selected cell from each branch-point, the tip EC, will extend filopodia, start migrating towards the angiogenic stimuli and lead the way for the sprout. Following this tip cell are cells known as stalk cells that mainly proliferate and produce ECM to form and stabilize the new vessel[14].

Angiogenesis is, however, not restricted to tumor tissues. On the contrary, the mode of generating new vessels by sprouting angiogenesis is essential for various physiological processes, such as in developmental tissues in the embryo, during wound healing, and for the reproductive system[7, 15, 16]. If the angiogenic process is not occurring within certain limitations, it can lead to the development of diseases or even be lethal. In addition to cancer, dysregulated blood vessel growth is involved in the pathology of atherosclerosis, obesity, and retinopathy[9, 17, 18]. The blood system is vital as its main functions are to supply oxygen and nutrients, as well as removing metabolic waste products from tissues. Although the basis of angiogenesis during physiology and pathology is very similar and involves many of the same steps, the vascular outcome is, importantly, quite different in a tumor compared to healthy tissues. The capillaries that are formed during the tightly regulated angiogenic process occurring in healthy tissues are organized and smooth[19]. They are usually well-balanced, commonly characterized by high

perfusion and low permeability owing to high coverage with mural cells, such as pericytes (PCs) and vascular smooth muscle cells (VSMCs), as well as an intact basement membrane[12, 20]. Although there are exceptions and some healthy vessels are relatively leaky. In the tumor, the angiogenic process lacks tight regulation and thereby generating a rather immature vascular network consisting of disorganized, leaky, and, in many cases, dysfunctional vessels[21, 22]. The perivascular coverage of these vessels is often poor[23, 24]. Consequently, the structure facilitates intra- and extravasation of cells to and from the tissue, hence, increasing the possibility of metastatic seeding via the vascular system[25, 26]. The vascular endothelial growth factor (VEGF)-A signaling is the most potent inducer of angiogenesis and will be discussed in detail in section 1.6.1.1.

1.4 VASCULAR REMODELING

The vascular characteristics are not only depending on the active process of angiogenesis. Vascular remodeling plays an important role in determining the structure and quality of vessels within the tissue, both during physiological processes and under pathological conditions[27-29]. This is important for both the blood and lymphatic systems. The newly formed blood vessel is dependent on extracellular signals and support from mural cells for its survival. This provides an opportunity for regulating the vascularity by modulating, for example, the perivascular coverage. Several factors have been reported to have vascular remodeling properties[30, 31]. One of the most potent family of proteins that has been widely studied in this context, is the angiopoietin (Ang) family consisting of four identified members, the Ang-1-4[32], where Ang-1 and -2 have received most attention so far. By interacting with their receptor tyrosine kinases (RTKs), namely Tie-1 and Tie-2, Ang-1 and -2 have been shown to have opposing functions. Ang-1 plays a role in vessel stability and maturation by providing adhesion and survival signals. On the contrary, Ang-2 is promoting vascular de-stabilization and regression by ablating the perivascular coverage, thereby removing the support-system[33]. Ultimately, the balance between Ang-1 and Ang-2 determines vascular stability and provides an opportunity to fine-tune the vascular remodeling according to the need of the tissue.

In addition, as discussed later in sections 1.6.1.2 and 1.6.2.1, as well as in Papers I and III, some other common growth factors expressed in tumors, such as VEGF-B and platelet-derived growth factor (PDGF)-BB, are also exerting their functions by vascular remodeling.

1.5 LYMPHANGIOGENESIS

Alongside the development of a blood vasculature system, lymphatic vessels are simultaneously formed in a process known as lymphangiogenesis[34-36]. In contrast to the blood system, postnatal lymphangiogenesis does not occur under physiological circumstances, however, there can be extensive remodeling[37]. The lymphatic vascular network is a very important system with various functions, including maintenance of tissue homeostasis by regulating fluids and macromolecules, and is also involved in the immune system by providing means of transportation of immune cells from tissues to lymph nodes where antigen presentation is occurring[35]. Moreover, while the structure of a blood vessel is complex, consisting of several layers and secondary cells, the majority of lymphatic vessels are a simple assembly of lymphatic ECs (LECs). Although, there are support cells surrounding the large lymph vessels too. Unlike the blood system, except the major collective lymphatic vessels, the lymphatic system does not need additional support as it has a relatively passive and low pressure mode of transportation, relying on contractions of skeletal muscles for its circulation of lymphatic fluid[38]. Importantly, the LECs are forming valves within the vessels to ensure a unidirectional flow of fluids[39].

The most common symptom of a malfunctioning lymphatic system is lymphedema, which can be the result of insufficient drainage of fluids from tissues leading to swelling. This can either be caused by lymphatic hypoplasia due to an inherited genetic mutation, by surgical intervention, or as a consequence of infections[40]. In the tumor tissue, the lymphatic system is a common route of tumor dissemination[41]. The intravascular environment in a lymph vessel is less stressful compared to blood vessels, leading to increased chance of tumor cell survival and successful distal seeding[42]. As discussed later in section 1.6.1.3 and Paper IV, the main pro-lymphangiogenic signaling pathway is VEGF-C/VEGFR3.

1.6 TUMOR-RELATED GROWTH FACTORS

Many proteins found in tumors are classified as growth factors. Signaling by these factors is instrumental for basically all steps in tumor progression. The high frequency of activating-mutations in growth factor-related genes, e.g. receptors and downstream components, commonly found in tumors, is really emphasizing the importance this type of signaling. Some proteins are regulating multiple pro-tumorigenic steps, such as epidermal growth factor (EGF) which is involved in early clonal expansion, angiogenesis and invasion[43], whereas other factors, such as VEGF-A mainly regulate angiogenesis. In addition to VEGF-A, several other factors have been found to function as positive or negative regulators of angiogenesis[44]. Under

physiological circumstances, the balance is in favor of inhibition, whereas in a pathological setting, for instance in a tumor, this balance is skewed. An 'angiogenic switch' is required to initiate the angiogenic process[45].

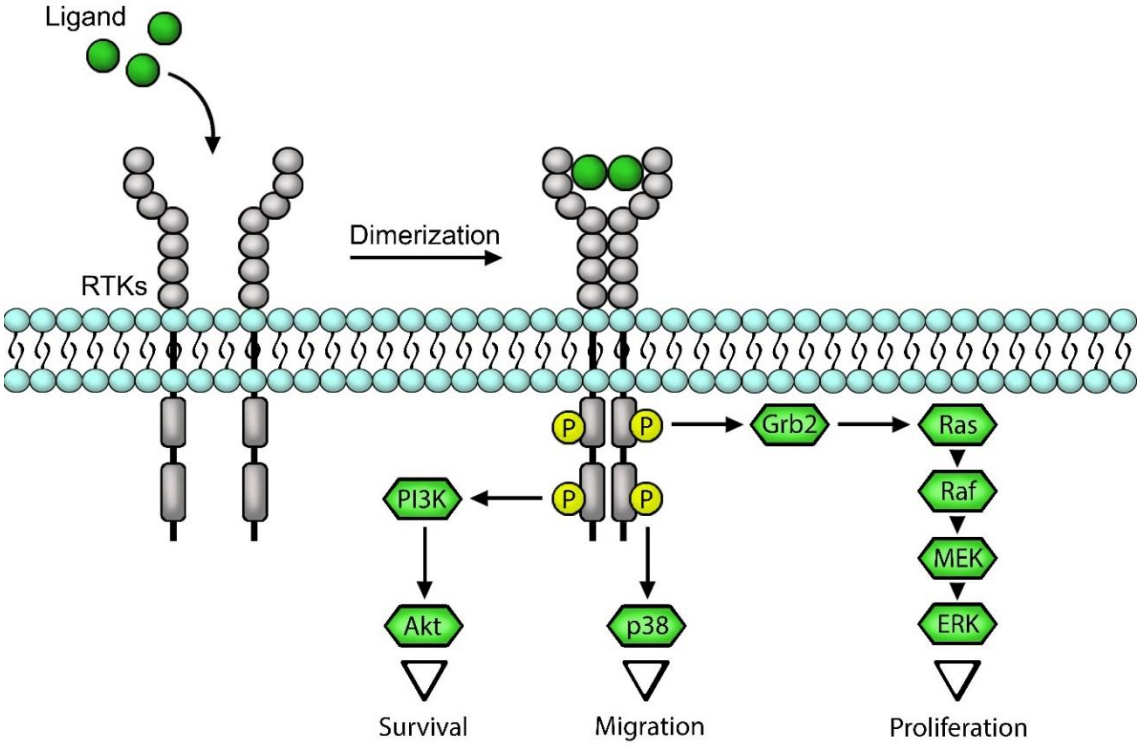

Figure 3. *Classical RTK signaling pathways.*

The pro-angiogenic growth factors most commonly active in the tumor are ligands that signal through RTKs (Figure 3). These receptor proteins are, in general, membrane-bound with a single transmembrane domain spanning the plasma membrane. The extracellular domain is responsible for ligand recognition and binding, which can occur as monomer or dimer (for simplicity, only ligand dimers are illustrated in Figure 3). Upon ligation, the receptor proteins will dimerize to form hetero or homodimers. Receptordimerization subsequently leads to activation of the intracellular tyrosine kinase domains, which will become phosphorylated and activate classical downstream adaptor proteins[46-48]. Various signaling events are initiated depending on the type of extracellular signal received. Biological outcomes involving proliferation are generally conveyed through Ras-Raf-MEK-ERK pathway. PI3K-Akt activation is related to survival, whereas p38 is responsible for migratory behavior[46, 49-52].

The three most common and relevant protein families will be introduced in the following sections, including: the VEGF-family, PDGF-family, and fibroblast growth factor (FGF)-family.

1.6.1 Vascular endothelial growth factor (VEGF)-family

The VEGF family consists of five structurally similar members, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). To exert their functions, these proteins are relying on binding to specific RTKs. There are three different VEGF receptors; R1, R2, and R3. VEGFR1, which can also be spliced to generate a soluble, non-membrane bound form, is able to bind VEGF-A, VEGF-B, and PlGF[53-55]. Signaling through this receptor plays a role in vascular modulation. Noticeably, the binding affinity of VEGF-A to VEGFR1 is higher compared to VEGFR2, whereas the signaling capacity through VEGFR1 is much lower compared to VEGFR2. VEGFR1 is therefore also thought to be important in sequestering VEGF-A, hence, acting as a negative regulator of angiogenesis[56-58]. VEGFR2 on the other hand is responsible for the most potent pro-angiogenic signal by binding VEGF-A, but VEGF-C and VEGF-D are also capable of interacting with this receptor. The last member, VEGFR3, is ligated by VEGF-C and VEGF-D[59]. Activation of this receptor is important for lymphangiogenesis and essential for tip cell formation during angiogenesis. In addition to the importance for physiological processes, this family of proteins has been shown to play a role in other diseases than cancer, such as neurodegenerative disorders, age-related macular degeneration, and rheumatoid arthritis[9, 60].

1.6.1.1 VEGF-A

VEGF-A is the most potent pro-angiogenic factor playing instrumental roles under physiological and pathological conditions. VEGF-A mainly signals through binding to VEGFR2. Global knock-out of either VEGF-A or VEGFR2 is embryonically lethal[61-63], emphasizing the crucial role of this pathway. Expression of VEGF-A has been found to be induced by a wide range of stimuli. In addition to hypoxia-dependent transcription and mRNA stabilization, factors such as PDGF, FGF, and EGF are able to effectively regulate VEGF-A expression[64, 65]. As VEGF-A is mainly a mitogen, its primary function in ECs is to promote proliferation, but is also involved in migration and survival, hence is important for the overall angiogenic process[66, 67]. One key feature of VEGF-A is its ability to increase vascular permeability, which plays a critical role in acute inflammation as well as for angiogenesis[68].

Noticeably, both the receptor and ligand are expressed by different cell types[69], however, an interesting study demonstrated that VEGFR2 is largely restricted to the vascular ECs in human tumors[70]. VEGF-A is commonly expressed at elevated levels in both pre-clinical tumor models[71, 72] and in human tumors[73, 74]. In the clinical setting, studies have indicated a strong correlation between high levels of VEGF-A and poor prognosis in many cancer types, including prostate cancer[75], breast cancer[76], and gastric cancer[77]. Anti-VEGF therapy will be discussed in sections 1.8.2 and 1.9.

1.6.1.2 VEGF-B

The functions of VEGF-B are not well-studied compared with VEGF-A, and the biological roles of its signaling are still controversial. Global deletion of VEGF-B in mice seems to only produce minor defects in the heart, such as an atrial conduction abnormality [78, 79]. This data somewhat diminishes the possibility of VEGF-B playing any critical roles during development. VEGF-B interacts with VEGFR1 to transduce its signals, and interestingly, knock-out of VEGFR1 is embryonically lethal[80]. In contrast, when only the intracellular tyrosine-kinase domain, responsible for signal transduction, is deleted (VEGFR1-TK), mice are viable, further complicating the potential role of VEGFR1-binding ligands[81]. It should be pointed out that in this model, the receptor is still present and able to bind VEGF-A, VEGF-B and PlGF. The regulation of VEGF-B expression remains to be elucidated. Common growth factors, cytokines and hormones do not seem to be involved, and in contrast to VEGF-A, VEGF-B is not induced by hypoxia[82, 83]. As an endothelial mitogen, VEGF-B is only displaying modest angiogenic capacity[84]. It is debated whether VEGF-B is to be considered pro- or anti-angiogenic[85-87]. Studies have shown that the angiogenic effects of VEGF-B could be a result of modulating the VEGF-A signaling[88]. By activation of VEGFR1, VEGF-B seems to be involved in vascular remodeling as its signaling leads to expression of plasminogen activator inhibitor-1 and urokinase, both of which are important for protecting the ECM from degradation[89, 90]. Despite an unclear picture of the biological contributions of VEGF-B, one study reported the expression of VEGF-B in a wide range of human tumors[91], and high expression levels are correlated with metastasis in colorectal cancer[92], and breast cancer patients[93].

1.6.1.3 VEGF-C

VEGF-C exerts its biological functions mainly through VEGFR3 activation. This member of the VEGF family plays an instrumental role in the lymphangiogenic process, but has also been

shown to be involved in blood vessel formation[94, 95]. Experimental data emphasizes the critical role of this signaling pathway as it was shown that embryos do not survive until birth when VEGF-C is globally deleted; this was due to the lack of lymphatic vessels[96]. Interestingly, VEGFR3 deficient mice die earlier compared to VEGF-C null mice, and in contrast, these mice display severe defects in the blood vasculature[97]. Mechanistically, VEGF-C is a potent driver of lymphangiogenesis by promoting lymphatic ECs proliferation, migration and survival[84, 98]. Production of VEGF-C can be upregulated by various growth factors, including PDGF and EGF[82], or pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α[99]. There is conflicting evidence on whether or not hypoxia is involved[82, 100-102]. One recent publication identified hypoxia as an inducer of VEGF-C expression; however, this regulation was hypoxia inducible factor (HIF)-1α-independent[103]. In line with its lymphangiogenic properties, pre-clinical studies have confirmed the ability to promote intra-tumoral lymphatic growth. For example, in a human breast cancer xenograft model, where tumor cells were propagated to overexpress VEGF-C, a significant increase in lymphangiogenesis was observed. This was, moreover, correlated with lymph node and lung metastases[104]. VEGF-C has also been identified to play a role in human cancer patients. In one extensive study, it was reported that VEGF-C mRNA levels could be detected in roughly 50% of cases, including breast cancer and melanoma[91]. Other studies have detected VEGF-C in tumors such as colorectal cancer[105, 106], gastric cancer[107], and esophageal cancer[108]. Additionally, a link between VEGF-C expression and lymph node metastasis was reported for breast cancer[109], gastric cancer[110], and esophageal cancer[111, 112]. In all the above cases, VEGF-C was also correlated to poor patient prognosis.

1.6.2 Platelet-derived growth factor (PDGF)-family

The four ligands belonging to the PDGF family mostly form homodimers (AA, BB, CC, and DD); however, heterodimerization can also occur resulting in AB dimers. Two different RTK protein chains, α and β, respectively, are forming homo- or heterodimers to function as receptors. Receptor dimers consisting of αα are able to bind AA, BB, AB, and CC ligands, whereas receptor dimers of ββ are binding BB and DD ligands. Various experimental data shows that all ligand combinations, except AA, can interact with αβ receptor heterodimers[113]. Expression of these ligands and receptors can be found in a broad spectrum of cell types, including VSMCs, PCs, ECs, and fibroblasts[114]. The PDGF family is generally important for transducing mitogenic as well as chemotactic signals[115, 116], thereby playing a role in development, wound healing, and tumor angiogenesis, including both hematogenous and lymphatic vessel growth[113, 117]. Evidence for implication of abnormal PDGF signaling has been

found in various pathological conditions and processes, including cancer, diabetic retinopathy[118], and atherosclerosis[119, 120].

1.6.2.1 PDGF-B

PDGF-B is the major factor responsible for the recruitment of PCs and VSMCs to vessels to provide necessary support and, hence, is essential for the formation of mature blood vessels[121, 122]. Signaling from PDGF-B by activation of its PDGFR-β receptor on PCs and VSMCs is essential for proliferation, migration and survival[123, 124]. The mechanisms of PDGF-B-induced angiogenesis are still debated. Experimental data support the finding that PDGF-B directly promotes sprouting of ECs *in vitro*[123, 125], however, to date, it has not yet been confirmed *in vivo*[117]. The indispensable role of PDGF-B signaling in development was realized when global knock-out models were generated. Both PDGF-B and PDGFR-β null mice die perinatally due to severe hemorrhages, confirming the involvement of PDGF signaling for proper vascular development[126, 127]. In fact, the vascular endothelium, including in tumors, is a key source of PDGF-B[128]. However, importantly, tumor cells also express PDGF-B[129]. There are multiple factors with potential to regulate PDGF-B expression, including TNF-α[130] and transforming growth factor (TGF)-β[131]. Hypoxia has also been confirmed to have an important regulatory function by stimulating PDGF-B expression[132, 133]. In the tumor setting, PDGF-B can act either directly on tumor cells, or indirectly be promoting tumor progression by its angiogenic effects[134, 135]. In line with the pro-tumorigenic effects of PDGF-B, significant correlations between its expression and poor prognosis have been demonstrated in lung cancer[136, 137]. Interestingly, PDGF-B expression has been linked to lymph node metastasis in gastric cancer[138]. Potential of PDGF-targeting therapy will be discussed in sections 1.8.3 and 1.9.

1.6.3 Fibroblast growth factor (FGF)-family

At the time of writing, 22 different proteins have been identified and classified as members of the FGF family[139]. Their biological effects are in general mediated by four different RTKs: FGFR1, FGFR2, FGFR3, and FGFR4. Interestingly, four members, the FGF-11, -12, -13, -14, are not signaling through these surface receptors, but rather have intracellular functions[140]. Furthermore, most FGFs are secreted from cells, however, certain members, including FGF-2, lack a secretory signaling peptide[141]. Release of FGF-2 is believed to occur through an endoplasmic-reticulum-independent mechanism (exocytosis), or from damaged cells[142]. The signaling axis of FGFs and their receptors are involved in multiple biological processes,

including differentiation, proliferation, migration, and survival[143, 144]. In a broad sense, they are instrumental for the overall healthy organ development, but particularly in skeletal and vascular development[145, 146]. Global deletion models in mice reveal dramatically different outcomes dependent of which FGF-member that is removed. Some are viable with varying degrees of phenotypes, whereas others are embryonically lethal[147]. FGFs have also been implicated in development of diseases such as cancer[148], Parkinson's disease[149], Alzheimer's disease[150], and skeletal diseases[151].

1.6.3.1 FGF-2

FGF-2 has not only been identified to be a potent regulator of angiogenesis[152], but also plays a role in lymphangiogenesis[153, 154]. FGF-2 induced angiogenesis has been reported to occur through both VEGF-A dependent and independent mechanisms[155]. Interestingly, FGF-2 signaling leads to VEGF-A upregulation in ECs[156-158]. Mechanistically, FGF-2 is a pleiotropic factor acting on various cell types, such as ECs, PCs and VSMCs[159], to regulate multiple processes including proliferation, migration, survival, and differentiation[152, 160, 161]. FGF-2 signaling in VSMCs modulates contractility of the vessels and maintains the vascular tone. Consequently, FGF-2 is essential for regulation of blood pressure, inflammation, wound healing, metabolism, and bone formation[161-163]. Despite being involved in vital processes, mice with global deletion of FGF-2 are still viable and fertile. These mice only display moderate phenotypes, including reduced blood pressure, thrombocytosis, delayed wound healing, and neuronal defects[161, 163]. FGF-2 expression is induced in response to hypoxia[164], but can also be regulated by factors such as PDGF-B[165], EGF[166], and TGF-β[167]. FGF-2 is known to contribute to tumorigenesis and its expression has been detected both in tumor cells and stromal cells[168, 169]. Furthermore, elevated levels of FGF-2 were detected in a wide range of cancer types[170]. Nevertheless, the clinical impact of FGF-2 expression is not clear. There are reports indicating significant correlation with poor prognosis in head and neck squamous cell carcinoma[171], and oral squamous cell carcinoma patients[172]. In a study on breast cancer, the findings were opposite where patients with high levels of FGF-2 showed improved survival[173]. Moreover, survival in lung cancer patients appears to be dependent on the cellular source of FGF-2 as it was demonstrated that high tumor cell expression of FGF-2 correlated with poor prognosis, whereas opposite correlation was found if the FGF-2 was expressed in the stromal compartment[174]. Taken together, additional studies are required before conclusions of the tumor-contributing properties of FGF-2 can be drawn.

1.7 CYTOKINES

Cytokines are small specialized secreted proteins classified according to their immune response-related effects. In general, cytokines are defined as being pleiotropic, i.e. the effect of one cytokine can be different depending on the target cell type. In addition, there is a redundancy in the system, meaning that different cytokines have overlapping functions[175]. There are several subgroups of cytokines, the; interleukins (ILs), interferons (IFNs), colony stimulating factors (CSFs), TGFs, TNFs, and chemokines[176]. Chemokines are characterized by having an effect on cell migration, as well as adhesion and activation[177]. Furthermore, cytokines can be grouped according to their pro- or anti-inflammatory effect; however, that can be context dependent as well. Four different classes of receptor proteins have been identified to be involved in cytokine signaling. These receptors are divided into type I cytokine receptors, type II cytokine receptors, TNF receptor family, and IL-1 receptor family[178]. Downstream signaling pathways of cytokine receptors include most of the common adaptor proteins such as ERK, Akt, p38, but frequently also NF-κB and JAK/STAT components for various biological outcomes[179-181]. The category of cytokines is comprised of a broad spectrum of proteins, and as one might expect, the processes where these are involved are equally diverse. Implication of cytokine signaling have be noted in the regulation of, for example, inflammation, infection, injury, body temperature, and psychological state including mood and appetite[182-185].

1.7.1 Interleukin (IL)-1 family

The IL-1 superfamily of cytokines contains five identified members; IL-1, IL-18, IL-33, IL-36, and IL-37. The receptors for the IL-1 ligands are structurally similar and rely on similar principles for activation[186]. The intracellular domain, responsible for initiation of downstream signaling, is composed of a special Toll/IL-1R (TIR) domain[187]. In contrast to RTKs, most of these receptors do not form dimers among themselves, but rather depend on dimerization with an IL-1R accessory protein (IL-1RAcP) which also contains an intracellular TIR domain. Upon ligand binding, the receptor will interact with the IL-1RAcP leading to TIR domain dimerization, allowing for downstream signaling events to occur. Usually the intracellular signaling is conveyed through ERK, p38, or NF-κB leading to various biological outcomes, such as proliferation, migration, and production of cytokines, chemokines and tissue remodeling effector proteins[176, 188, 189] (Figure 4). In addition to the involvement of biological processes previously described for cytokines in general, ILs can be drivers of pathological conditions, such as cancer[190]. Specifically, during diseases where chronic inflammation is an

underlying cause, including atherosclerosis[191], Alzheimer's disease[192], and rheumatoid arthritis[193], ILs commonly play an important role.

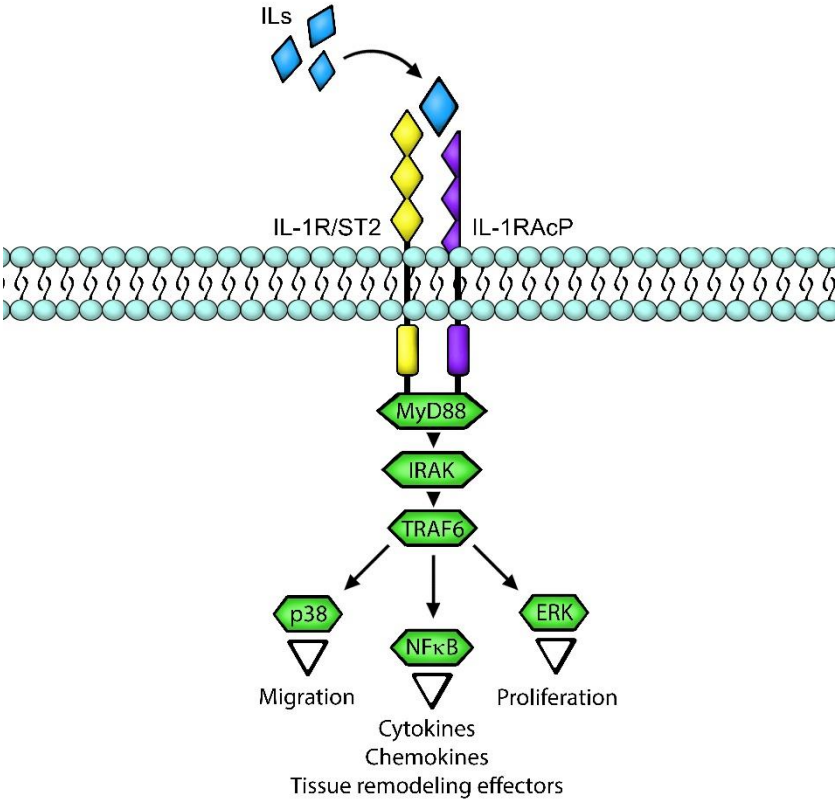

Figure 4. *Interleukin signaling pathways.*

1.7.1.1 IL-33

IL-33 has been widely studied in various immunological settings as it was identified to be a potent regulator of the innate immunity as well as the adaptive T-cell response[194, 195]. Studies in both IL-33 and ST2 (IL-1RL1)-deficient mice revealed no obvious abnormal phenotypes under pathogen-free conditions and mice were fertile and developed normally. The mice do, however, have reduced immune reactivity and impaired production of certain cytokines[194, 196, 197]. IL-33 is constitutively expressed in many different tissues[198, 199], but typically is restricted to epithelial cells, fibroblasts and smooth muscle cells[200]. In humans, expression was first described in ECs[201, 202], yet, such expression appears to be lacking in mice[199, 203]. Interestingly, in contrast to most other cytokines, IL-33 seems to be exclusively located in the nucleus[195, 198], implying that IL-33 can act as a nuclear factor[204, 205]. Importantly, in line with the nuclear localization pattern and its constitutive expression, IL-33 has been recognized as an alarming cytokine signaling upon tissue damage or as a result of necrotic cell death[205, 206]. Cellular stress

or injury would, hence, lead to the extracellular release of IL-33, functioning as an alarm signal and trigger an immune response. This process does not occur during apoptosis as active caspases would cleave IL-33 to become inactive[207]. Recent findings have, however, reported that living cells also actively can secrete IL-33[208, 209]. Extracellular biological activity is mediated by binding to the ST2 receptor present on many immune cells, including macrophages[210, 211], mast cells[212], and CD4+ T-cells[213]. IL-33 has been demonstrated to be involved in cellular differentiation, activation, adhesion, proliferation, and cytokine production in its target cells[214]. Pro-inflammatory cytokines, such as TNF-α and IFN-γ, in turn, potently regulate IL-33 expression[212]. Implication of IL-33 in pathology includes cancer, asthma and rheumatoid arthritis[215, 216]. Regarding the role of IL-33 signaling in cancer, results from pre-clinical models are pointing towards different directions. There are several tumor studies performed in mice that indicate a pro-tumorigenic effect of IL-33 on tumor progression, invasion and metastasis in breast cancer[217], ovarian cancer[218], gastric cancer[219], and colorectal cancer[220, 221]. In contrast, opposing results were recently published by others using mouse models of breast cancer and melanoma[222], as well as colon cancer[223]. In one of these studies, the anti-tumorigenic effects were probably due to an increased number of cytotoxic T-cells and natural killer cells[222]. In cancer patients, the findings are reflecting the pro-tumorigenic effects of IL-33 and strong associations between high IL-33 expression and poor prognosis have been reported in ovarian cancer[218], glioma[224], and squamous cell carcinoma of the tongue[225].

1.8 THE METASTATIC CASCADE AND INVOLVEMENT OF THE TUMOR MICROENVIRONMENT

The involvement of tumor microenvironment in initiation, progression and spreading of tumors has been well recognized during the past years and was introduced as an important key compartment in the updated version of hallmarks of cancer in 2011[6]. It is known that an extensive dynamic communication occurs within tumors; between tumor cells, vascular cells, perivascular cells, and fibroblasts, as well as immune cells. In fact, the tumor cells are not only able to escape the regulatory systems, but also induce an active signaling to produce a favorable environment. Under those signals, tumor cells educate the body's own cells to use their functions for tumor-promoting advantages[226]. This interplay is of crucial importance for providing immune escape, treatment resistance mechanisms, and facilitating tumor spreading[227]. Microenvironmental opportunities for cancer therapy will be discussed in the following sections.

1.8.1 The metastatic process

Despite being an inefficient process where tumor cells either do not survive in the circulation or fail to establish a metastatic colony[228], metastatic spread is very common and in fact responsible for around 90% of cancer-related deaths[229]. These numbers justify the prominent role of acquiring an invasive phenotype as one of the hallmarks of cancer. There are several important and challenging steps that occur during the process of metastatic dissemination and eventually lead to successful colonization[230] (summarized in Figure 5).

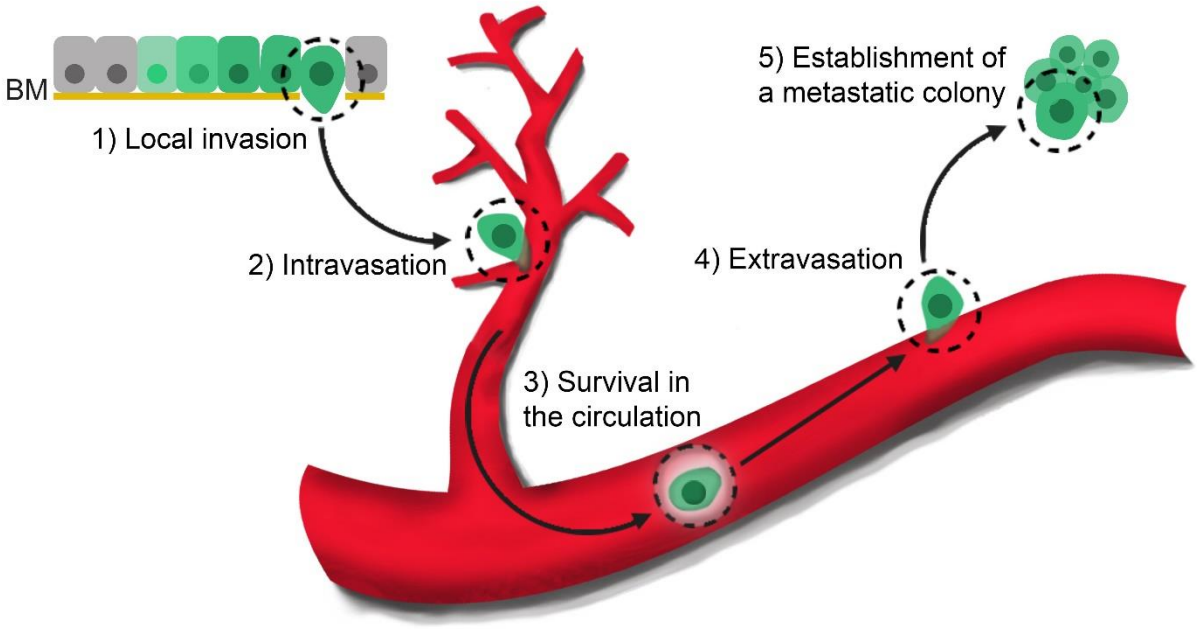

Figure 5. *A simplified overview of the metastatic process.*

In brief, cellular changes occur within the primary tumor to generate tumor cells with more invasive characteristics that are able to break loose, invade locally, and migrate towards the blood and lymphatic vessels. The cells then need to intravasate into the vascular system. As previously discussed, the environment in the circulation, especially the blood system, is mechanically harsh but there are also plenty of immune cells posing a threat to the tumor cells. The tumor cells struggle to survive such stressful conditions. Tumor cells are usually arrested in capillaries at the site of a distant organ, commonly lungs and liver. Here, the tumor cells must extravasate in order to form metastatic colonies. Subsequent metastatic growth is dependent on several aspects. In fact, if the environment is unfavorable, tumor cells have been found to remain in a dormant stage for years before regrowth is initiated and metastases can be clinically detectable[231].

The complex process of metastatic dissemination can be influenced at many levels and by many different factors. As in most steps in tumor progression, the microenvironment has been found to play instrumental roles in the metastatic process[232]. This can be exemplified by hypoxia, which is commonly found in the growing tumor and is known to be one of the major inducers of metastasis-related genes[233, 234]. Under normoxic conditions, the HIF-1α, a transcription factor, is targeted for degradation. This process is dependent on hydroxylation of the HIF-1α protein by a group of enzymes known as prolyl hydroxylases (PHDs). When there is a lack of oxygen, these PDHs are not active and hence, HIF-1α can be accumulated and translocated to the nucleus where it will regulate transcription of its target genes[235]. Importantly, hypoxia affects all cells in the tumor tissue. Again, there is an effect on the stromal compartment by factors produced under hypoxic conditions to recruit cells and engage them in the metastatic process to facilitate dissemination. For example, certain immune cells, including macrophages (Mφ) and neutrophils that can be recruited to boost tumor cell proliferation and promote invasion by different mechanisms[236, 237]. Clinically, it has been reported that expression of HIF-1 and HIF-2 correlates to poor prognosis in various tumor types, for instance in late stage breast cancer[238], colorectal cancer[239], and metastatic renal cancer[240]. Collectively, these findings further confirm the role of direct and indirect regulation by hypoxia in the tumor progression and metastasis.

1.8.1.1 Local invasion

Prior to understanding the process of local invasion, it is central to understand the fundamental structures and cell behaviors of tissues. Importantly, as tumor cells in the primary tumor are frequently (approx. 90%) of epithelial origin[241], the cells are well organized and have a specific polarization with apical (luminal), lateral, and basal sides. On the basal side of the cell layer, cells are anchored to the basement membrane via, for example, integrins. On the lateral sides between epithelial cells, there are several different categories of junctions, such as tight junctions, adherens junctions, and desmosomes that provide strong cell-cell adhesions[242]. All these aforementioned features are limiting cell migration. During tumor progression, tumor cells are gaining various advantages by gene mutations or regulation of gene expression. In context of primary tumor invasion, the cells need to detach from neighboring cells. A cell can accomplish that by different means. One such process known to occur; is epithelial-to-mesenchymal transition (EMT)[243]. Probably the most well-studied factor in the context of EMT is TGF-β[244]. Signaling through the TGF-β type I and II receptors, activates Smad2/3 downstream effector proteins and eventually initiates several important signaling events. In the

frame of reference of EMT, TGF-β signaling will, for example, lead to the loss of E-cadherin, the receptor that forms adherens junctions. As a consequence of this event, β-catenin, a transcription factor that is associated to E-cadherin, will be released and relocate to the nucleus where it can upregulate various genes involved in proliferation, survival, and differentiation. Collectively, the process of EMT leads to increased migratory capacity and reduced cell-cell junctions. Together, these downstream events of TGF-β signaling are sufficient to induce the EMT program and allow cells with an acquired mesenchymal phenotype to detach[245, 246].

1.8.1.2 Intravasation

Following acquisition of migratory and invasive phenotypes, tumor cells are facing another rate-limiting step, the intravasation process. Entry into the blood system seems to require more advanced steps compared to entry into the lymphatic system, for reasons discussed in sections 1.3 and 1.5. The approaches by which tumor cells are crossing the endothelial barrier can be different. Two modes of intravasation by transmigration; paracellular (between ECs) and transcellular (through ECs), have been proposed to be utilized by tumor cells[247]. Paracellular transmigration is believed to be the most common mechanism *in vivo*. The molecular details underlying this process are not fully understood, however, it has been shown that matrix metalloproteinases (MMPs) are involved. One report identified a regulation of endothelial permeability by MMP-1 via activation of the protease-activated receptor-1 expressed on ECs, leading to increased tumor cell intravasation[248]. Importantly, for tumor cells to gain access to the circulation, the ECM and basement membrane act as a protective barrier that needs to be degraded. Various MMPs and other proteases, which can be both tumor cell-derived or produced by non-malignant cells such as Mφ, are responsible for degrading different components of these structures[249]. Blocking of, for example, MMP-9 in pre-clinical models has been shown to lead to significantly impaired metastatic capacity[250, 251].

1.8.1.3 Extravasation

Tumor cells that are able to survive in the blood circulatory system have a new challenging step to complete, the process of extravasation, before they have gained access to the distal organ. In regards to the immune system, extravasation is the manner by which leukocytes enter tissues. Tumor cells are believed to utilize the same steps of extravasation, i.e. rolling, adhesion, and transmigration[252]. The underlying mechanisms are not yet well characterized in the case of tumors. There are studies indicating a role for integrins as the means by which tumor cells are

capable to attach to the endothelium and subsequently transmigrate[253]. Specifically, integrin β1 has been identified as a mediator of extravasation[254] and has been shown to be associated with poor prognosis in various type of cancer types, including lung cancer[255], breast cancer[256], and metastatic melanoma[257]. Interestingly, in a pre-clinical model, tumor cells were recently found to promote their extravasation by inducing necroptosis (a form of programmed cell-death) in ECs to gain access to lung tissues. This process was shown to be mediated by death-receptor 6 activation[258]. In addition, transmigration can be facilitated by enhancing vascular permeability, which can be accomplished by secretion of factors such as VEGF-A[259], TGF-β[260], or angiopoietin-like-4[261]. After successfully completing this difficult step-wise process, the tumor cells have the opportunity to form an established metastatic colony.

Non-malignant cells have been identified to be critical in the overall metastatic process. As the central focus of the current study, was to investigate the mechanisms of tumor microenvironment in promoting metastasis, the roles of ECs, PCs, and Mφ will be discussed in the following sections in relation to metastasis and potential targeted therapy. Although the contributions of tumor-associated fibroblasts are being well recognized in tumor development and progression, the topic is outside to scope of our findings, and hence will not be discussed in detail.

1.8.2 Endothelial cells (ECs)

Blood vessels are a crucial part of the architecture within a tumor and, as detailed earlier, provides a common route for dissemination. As the main role of the blood system is to deliver oxygen, the vessels are in that sense also regulating tissue hypoxia, which is known to affect various aspects of tumor progression, as previously discussed in section 1.8.1. Even in a healthy tissue, the ECs are important for maintaining tissue homeostasis and regulating tissue inflammation by, for example, controlling the transmigration of immune cells[262]. In addition to the previously mentioned, obvious vessel-related functions, the ECs have been found to make further contributions. In fact, recent findings indicate a link to direct tumor-promoting effects on proliferation and invasion exerted by EC-derived factors[263]. Also, tumor cell-EC communication has been reported to render ECs resistant to a number of chemotherapeutic agents by acquiring expression of anti-apoptotic proteins. As a result, the tumor vasculature can remain even after treatment and continue to support surviving tumor cells, again contributing to resistance[264].

When looking at the blood vasculature as a target for treatment, the initial strategy and idea was to starve the tumor. Killing of ECs would lead to reduced tumor vascularity, consequently less oxygen and nutrients will reach the tissue and increase the chance of apoptosis and tumor regression[265]. Early experimental data was in support of this hypothesis and blockade of VEGF-A signaling in pre-clinical models proved to be successful in a range of different tumor models[266]. These findings led to the development of a first anti-VEGF-A neutralizing antibody, Bevacizumab (Genentech)[267]. Clinically, the results have been less beneficial and researcher are still attempting to understand the mechanisms. However, Bevacizumab is currently approved by the Food and Drug Administration (FDA) for treatment of various tumor types, in general, at an advanced metastatic or recurrent stage. Importantly, the approval is based on combination treatment with chemotherapeutics. Despite a promising initial hypothesis, emerging evidence is, however, suggesting that induction of hypoxic conditions might not be that beneficial. Reports are instead proposing a strategy leading to improved blood perfusion and alleviated hypoxia. This would be preferred, especially in combinational settings, since it has been shown to increase delivery of a secondary drug, commonly a chemotherapeutic agent[265]. Additional efforts are ongoing. The second and third generations of anti-angiogenic agents, such as the tyrosine kinase inhibitors sorafenib (Bayer) and sunitinib (Pfizer), are now available for clinical use. The key question is how to select the right treatment for individual patients.

1.8.3 Pericytes (PCs)

The tumor-regulating functions of PCs are still being debated. As PCs are covering the outside of the capillary blood vessels to provide support, they also constitute an effective barrier to prevent intravasation of cells and hence are, in that context, believed to limit the metastatic potential of a tumor[268]. One report indicated a strong correlation between low perivascular coverage and poor prognosis in clinical samples from breast cancer patients[269]. It is, however, important to realize that the tumor vasculature is highly heterogeneous with different levels of coverage in different areas, which may affect treatment outcome[270]. We and others have shown how PCs can actively participate to promote metastasis by different mechanisms. On one hand, PCs have been found to undergo transition into tumor-associated fibroblasts and contribute to metastasis[271]. Additionally, we identified PCs as a source of IL-33, which was reported to promote metastasis by modulating the immune cell compartment[221, 272]. The therapeutic setting can be exemplified by imatinib (Novartis), a small molecule, which is approved as first-line treatment of KIT+ gastrointestinal stromal tumors[273]. Imatinib has several targets, including

PDGFRs, and is reported to modulate the vascular coverage by ablating PCs[274]. This consequently renders ECs more susceptible for combination treatment with, for example, anti-VEGF-targeting agents. Such combinations have shown promising results in various pre-clinical models[275, 276]. However, one recent clinical study on combination therapy in melanoma failed to observe any clinical benefits[277].

Available literature is not only focused on if there is a therapeutic effect of targeting PCs in tumors, or not. But also, caution is to be taken, as it has been reported that targeting PCs can also increase the risk for metastasis. Interestingly, targeting PCs at early stages in tumors or when the level of PDGF-BB is high seem to reduce tumor progression and inhibit dissemination. In contrast, in late stage tumors or if levels of PDGF-BB are low, metastasis was promoted in settings where PCs were targeted[278, 279]. In light of these reports, it is noteworthy to mention that, in the clinical setting, cancer is usually detected at a later stage, possibly reducing the potential success of this approach. In contrast to this strategy, some results are suggesting that attempts should be made to stabilize the EC-PC interactions to increase the PC coverage, which could be accomplished by antagonizing the Ang-2 signaling[280]. Trebananib (Amgen), a peptibody blocking both Ang-1 and -2 interaction with Tie-2, was reported to increase progression-free survival in recurrent ovarian cancer patients when combined with paclitaxel compared to paclitaxel alone[281]. A more specific antibody, Nesvacumab (Regeneron), which is only neutralizing Ang-2, is showing promising anti-tumorigenic effects in early clinical trials[282].

1.8.4 Macrophages (Mφ)

The components of the immune system protect the body against danger and potential diseases. Tumor cells are, however, to a large extent capable of evading the immune surveillance and persist[283]. As is true for other cellular compartments, tumor cells are able to modulate the phenotypes of immune cells. Immune cells are intrinsically dynamic and their functions and polarization can be changed according to the environmental need[284]. One population of immune cells found in abundance in tumor tissues is Mφ. Macrophages can be tissue-resident or recruited from the periphery and they play an important role in host defense both by direct phagocytosis of microbes and activation of T- and B-lymphocytes[285, 286]. Depending on the circumstances, Mφ can become “classically activated” to generate M1 type, which is pro-inflammatory, or “alternatively activated” to become anti-inflammatory M2 type[287]. In the context of tumor studies, the M1 subtype has been identified to be anti-tumorigenic by e.g. stimulating the immune response. In contrast, the M2 subtype has been found to act in favor of

the tumor by engaging in various processes. For example, these cells are producing anti-inflammatory cytokines to dampen the immune response[288]. In addition, they are an important source of matrix degrading proteins that facilitates tumor cell invasion, but also contribute to resistance by producing pro-angiogenic factors[289]. Moreover, Mφ have been shown to play an even more active role in the metastatic process by guiding and helping tumor cells to intravasate[290, 291]. At least in part, this Mφ-tumor cell communication was shown to involve CSF-1R[292, 293]. Collectively, there is clear evidence for Mφ being an important contributor to tumor progression.

Recently, growing interest in the population of Mφ as an attractive target for therapy has led to development of CSF-1R neutralizing antibodies and small molecules. The mechanisms behind the potential success of such approach are still under investigation, some reports indicate a process of re-programming upon treatment[294], whereas others point towards induction of cell death[295]. Of note, a recent publication described an increase in spontaneous metastatic potential when neutralizing CSF-1R in a breast cancer model, indicating that caution might be needed[296]. Additional studies are required before final conclusions can be drawn on the future of CSF-1R targeting agents. Interestingly, a different Mφ-targeted approach, taking advantage of the fundamental characteristics of this cellular population of being an effective contributor to cell and pathogen destruction, is based on CD40 activation. CD40 is expressed on many cell types and was found to activate Mφ leading to IFN-γ-mediated tumor cell killing[297]. Based on that notion, agonistic CD40 antibodies have been developed. One such antibody, CP-870,893 (Pfizer), is currently in clinical trials[298, 299] after displaying promising anti-tumorigenic activity in mouse models[300].

1.9 TREATMENT OF CANCER PATIENTS

From the evolvement of modern cancer surgery in the 19th century, radiotherapy in early 20th century, and chemotherapy in the middle of the same decade, today's cancer therapy has come a long way. The realization that tumors are able to regrow or become resistant when applying these therapeutic interventions[301, 302], contributed to continuous effort being put on finding new strategies for cancer treatment. Along with great progress in tumor detection using computed tomography and introduction of detection and screening processes, several different types of treatments were developed, including improved chemotherapeutics, hormonal therapy for breast and prostate cancer patients, vaccination for colon and cervical cancer, and new radiotherapy techniques. Tumors, however, often respond poorly to many of these available drugs[303].

In recent years, interesting progress in the field of targeted therapies has improved the opportunities for individual treatment and increased overall survival in many types of cancer. Although the already established tamoxifen treatment is regarded as a targeted therapy by its binding to the estrogen receptor and blocking its interaction with estrogen, the first antibody-based drug designed for a molecular target in cancer, rituximab (IDEC Pharmaceuticals), received a lot of attention and initiated the field of targeted therapy. In 1997 it became the first drug ever of its kind to get approval by the FDA, the indication was treatment of Non-Hodgkin's lymphoma (NHL). Rituximab is a monoclonal antibody targeting the CD20 antigen expressed on B-cells, which is the most common malignant cell type in NHL[304]. Following this approval, additional tumor cell-targeting antibodies were approved, such as trastuzumab (target; HER2, Genentech) in breast cancer, and cetuximab (target; EGFR, ImClone Systems) in colon cancer. Not only antibody-based approaches have been developed, small molecules are an attractive alternative due to their chemical stability, potency, and less immune-reactive properties along with being more cost-effective[305]. FDA-approved small molecules includes imatinib (target; Bcr-Abl, c-Kit and PDGFRs, Novartis) in chronic myeloid leukemia (CML), and gefitinib (target; EGFR, AstraZeneca) in lung cancer.

All these aforementioned therapies are based on targeting tumor cells, and are commonly facing the problem of resistance in the clinical setting. As discussed in previous sections, the different compartments of the tumor microenvironment have been identified to influence all the steps of tumor progression, including metastasis and resistance[306-308]. Despite the layer of complexity of microenvironmental interactions, it provides an attractive opportunity for therapeutic interventions. In fact, looking at clinical trials and already FDA-approved drugs, there are targets for different compartments in the tumor microenvironment. One of those attractive targets are the immune cells with the overall aim to amplify the anti-tumorigenic effects of the immune system. Such approaches are employed by drugs targeting proteins expressed on T-cells (CTLA-4 and PD-1), or macrophages (CD40 and CSF-1R). Some examples are: ipilimumab (target; CTLA-4, Bristol-Myers Squibb), nivolumab (target; PD-1, Bristol-Myers Squibb), CP-870,893 (target; CD40, Pfizer), and AMG-820 (target; CSF-1R, Amgen). Furthermore, several drugs have been generated to modulate vascular and perivascular cells by targeting VEGFRs, PDGFRs, or the Ang system. Some examples are: bevacizumab (target; VEGF-A, Genentech/Roche), aflibercept (target: VEGF-A, VEGF-B, and PlGF, Regeneron), sunitinib (target; VEGFRs and PDGFRs, Flt3, and CSF-1R, Pfizer), imatinib (target; Bcr-Abl, c-Kit and PDGFRs, Novartis), sorafenib (target; VEGFRs, PDGFRs, Raf, and Kit, Bayer), pazopanib (target; VEGFRs, PDGFRs, Kit, GlaxoSmith Kline), AMG-386 (target; Ang-2, Amgen), trebananib (target; Ang-1 and Ang-2, Amgen), and nesvacumab

(target; Ang-2, Regeneron). These drugs have generated promising pre-clinical data and showed potential in clinical trials in combination with other anti-cancer therapies. However, the anti-angiogenic drugs, so far, to reach the clinic have only limited efficacy[309]. Pre-clinical data from a study using VEGFR inhibitor in a mouse pancreatic tumor model, demonstrated that anti-angiogenic targeting in late-stage tumors, a point where patients usually come to the clinic, had limited effect compared to early treatment. It was concluded that VEGFR blockade was inefficient in regressing tumors with pre-established vascular network. Furthermore, this study indicated that combination therapy where VEGFR and PDGFR are targeted concomitantly, yielded superior anti-tumorigenic properties, even in late-stage tumors[275].

As is true for most treatment options today, drug resistance is one of the major hurdles. In the frame of references of anti-angiogenic therapy, there are many hypotheses to how tumors are becoming resistant. Again, the microenvironment seems to play an important role in the contribution to resistance. Upon anti-angiogenic therapy, some tumor vessels will regress, leading to a hypoxic environment. On one hand, hypoxic tumor cells are resistant to chemotherapy and radiotherapy[310, 311], and there is a selection of cells that do survive, for example those insensitive to apoptosis and thereby not responsive to drugs with apoptosis-dependent mechanisms[311]. On the other hand, as mentioned in an earlier section, hypoxia is regulating a wide range of processes by controlling gene expression in basically all cells within the tumor. Many of those processes are involved in escape from cell death. These issues are challenging, but there is intriguing research ongoing in the field of targeting hypoxia in tumors to improve the efficacy of secondary drugs[312].

Taken together, drugs targeting the microenvironment can be successful when applied in appropriate settings. It is crucial to gain further understanding of the intratumoral communication that has been proven to limit the therapeutic effects, and to apply the right combinations of drugs, in the right settings to achieve improved treatment of cancer patients.

2 AIMS

The overall aim of this thesis was to study the influence of tumor microenvironment in the process of metastasis and to investigate current cancer therapy.

More specifically, the aims were:

I. To explore the role of PDGF-BB in the tumor microenvironment

II. To investigate the mechanisms behind PDGF-targeting therapy in cancer

III. To study the influence of VEGF-B in context of tumor metastasis

IV. To investigate lymphangiogenesis and potential interplay of two common lymphangiogenic factors; VEGF-C and FGF-2

3 RESULTS

The constituent papers in this thesis work describe different mechanisms employed by various cellular compartments in regulating the metastatic process. We report how modulation of angiogenesis (paper I, and III) and lymphangiogenesis (paper IV) influence the metastatic potential of tumors. In addition, we identify mechanisms underlying the pro-metastatic contributions of PCs (papers I, and II) and Mφ (papers II, and III) in the tumor microenvironment.

3.1 PAPER I

PDGF-BB expression serves as a biomarker for anti-PDGF therapy by influencing the vascular remodeling and having an impact on tumor metastasis

PDGF-BB is a known mitogen that contributes to tumor growth, and plays an instrumental role in PCs recruitment to vessels. Despite several studies in pre-clinical and clinical settings, the role and mechanisms of PDGF-BB-modulated tumor vasculature remains largely unclear. Understanding the role of PDGF-BB in cancer is motivated by the frequent expression of PDGF-BB in tumors, which has been noted in various types of tumors[313]. The fact that anti-PDGF drugs are currently a part of clinical practice for targeting of PDGF signaling in tumors, justifies further investigations on the underlying mechanisms to guide appropriate use of anti-PDGF targeting. We and others previously identified how PDGF signaling is essential for vascular remodeling, however, overexpression of PDGF-BB has been found to increase metastasis in pre-clinical models. In this study, we wanted to understand the mechanisms of PDGF-BB effects on the vascular phenotype and impact on metastasis in tumors, as well as clarify the impact of anti-PDGF targeting.

To address the role of PDGF-BB in tumors, we employed human tumor xenografts in mice. A human epidermoid cancer cell line (A431) was selected for its high expression, and a neuroblastoma cell line (IMR32) displayed relatively low PDGF-BB expression. Vascular analysis of these tumors, revealed significant differences in the perivascular coverage. Interestingly, despite the role of recruiting PCs to the vasculature, the high PDGF-BB expressing A431 tumors showed less coverage and reduced total number of PCs. To validate that PDGF-BB was playing a role in the vascular phenotype, we propagated murine tumor models to overexpress PDGF-BB at different levels. In these models, we were able to reproduce the findings observed in the human tumor models. We could also confirm that vascular disorganization was increased as the level of PDGF-BB increased.

To elucidate the effects of anti-PDGF drugs in settings where expression of PDGF-BB is different, we treated the human and mouse tumors with a neutralizing PDGFR-β antibody or imatinib. From these experiments, it was evident that the tumors responded unexpectedly different. In line with the suggested mechanism of such drugs, the vascular coverage was significantly decreased in tumors with low PDGF-BB levels. In contrast, to our surprise, blockade in tumors with high expression of PDGF-BB, displayed improved vascular coverage. Further analyses of the vascular qualities by injection of low molecular weight dextran (70kDa) indicated that the high vascular leakage observed in PDGF-BB high tumors, was significantly reduced upon anti-PDGF treatment. Here, again, opposing results were detected in tumors with low PDGF-BB expression.

As the degree of vascular coverage and permeability are possibly related to the feasibility of intravasation, we wanted to study the metastatic outcome. Detection of circulating tumor cells (CTCs) after treatment with imatinib, showed that dissemination was inhibited in A431 tumors, hence when PDGF-BB is high. Inversely, IMR32 tumors had a trend of increased CTCs. These findings where reproduced in the murine tumor model, where CTCs, in addition, were also found to be correlated to pulmonary metastasis.

To further understand how PDGF-BB is regulating PCs in the tumor, we conducted several *in vitro* studies using isolated PCs. Interestingly, persistent stimulation with high doses of PDGF-BB leads to downregulation of surface-available PDGFR-β by promoting internalization. In line with these *in vitro* studies, levels of PDGFR-β in tumor tissues exposed to high PDGF-BB expression were downregulated. By performing a gene array of PDGF-BB stimulated PCs and comparing to non-stimulated control PCs, we identified a wide range of genes with differential expression. We chose to focus on genes involved in EC-PC interaction, and identified integrin α1 as being the most downregulated adhesion molecule. To generate a functional adhesion molecule, integrin α1 combines with integrin β1, both of which were confirmed by qPCR to be downregulated upon PDGF-BB stimulation. These results indicate that loss of α1β1-binding is a consequences of prolonged PDGF-BB stimulation and PDGFR-β downregulation. Subsequent experiments with treatment of tumors with α1β1 neutralizing antibodies could successfully reproduce the loss of PCs observed in PDGF-BB high tumors. Collectively, this data demonstrates that high doses of tumor cell-derived PDGF-BB, at least in part, leads to reduced vascular coverage by downregulation of integrin α1β1-dependent EC-PC interaction. This destabilization does not occur during angiogenesis when ECs are the main source, probably due to the relatively low production of PDGF-BB.

In summary, we report that PDGF-BB expression levels strongly determine the treatment outcome when using anti-PDGF drugs, and that treatment may potentially promote metastasis. Furthermore, we identify the underlying mechanisms of PDGF-BB-induced vascular remodeling in tumors. In addition, we found that PDGF-BB may serve as a biomarker for selection of patients for anti-PDGF therapy.

3.2 PAPER II

PDGF-BB is inducing an IL-33-dependent communication between perivascular stromal cells and macrophages leading to a metastasis-promoting microenvironment

When analyzing tumor tissues from PDGF-BB overexpressing tumors, we noted that the population of Mφ where drastically increased in numbers. Interestingly, Mφ are not directly responsive to PDGF-BB stimulation as they lack PDGFR expression, indicating a potential role for an indirect mechanism. In this study, we set out to investigate how PDGF-BB is able to trigger an increase in Mφ. Additionally, as Mφ have been demonstrated to be important in tumor cell dissemination, we wanted to explore any potential impact on metastasis in our tumor settings.

For initial studies, we chose to use the previously identified high PDGF-BB expressing human tumor cell line, A431. As a control, shRNA was used to downregulate the PDGF-BB levels. By performing immunohistochemical staining and FACS analyses of such tumors, we could conclude that Mφ were effectively reduced when PDGF-BB was downregulated. To confirm the role of PDGF-BB we used two established murine tumor cell lines with stable PDGF-BB overexpression. Here, we detected significant increases in PDGF-BB high tumors compared to vector-only transfected tumors, further confirming the contributions of PDGF-BB. In an effort to point-out which receptor that was responsible, we treated tumors with imatinib (blocks both PDGFR-α and -β), a specific anti-PDGFR-α, or an anti-PDGFR-β neutralizing antibody. Imatinib and PDGFR-β blockades were able to inhibit PDGF-BB-induced Mφ infiltration, whereas PDGFR-α failed to do so. Co-localization experiments demonstrated that Mφ in the murine tumor model lacks PDGFR expression. As it is known that the main cellular target of PDGF-BB is PCs, we focused on this cell type.

The effects of PDGF-BB on PCs, in addition to the main role of recruitment onto vessels, is not known. By performing a gene array of PDGF-BB stimulated PCs and comparing to non-stimulated control PCs, we were able to identify potential mediators of PDGF-BB-induced Mφ infiltration. Among all genes, IL-33 was the most upregulated and known to regulate

various immune cells and, hence, was selected for subsequent studies. Analyses of IL-33 protein and mRNA levels in PDGF-BB tumors validated PDGF-BB-driven increase in IL-33 expression. Specifically, detailed analysis identified PDGFR-β-positive cells, including PCs, to be the main source of IL-33. Previously, PDGFR-β was identified *in vivo* for Mφ infiltration. To verify if PCs are producing IL-33 in response to PDGFR-β activation, we performed blockade experiment *in vitro* and could conclude the involvement of PDGFR-β, whereas PDGFR-α not seemed to be involved. The underlying molecular mechanisms of PDGF-BB-induced upregulation of IL-33 was previously unidentified. *In vitro* experiments and array analysis of PDGF-BB-stimulated PCs revealed that Sox7, a transcription factor, is mediating the transcriptional regulation of IL-33 by binding to SRY-boxes in the promoter region.

So far, IL-33 has been identified as a potential link between PDGF-BB and Mφ infiltration. To investigate the effect of IL-33 on Mφ, we performed *in vitro* studies using a murine Mφ-like cell line. As IL-33 is known to signal through its receptor ST2, we first confirmed that our cell line expressed this receptor by FACS and RT-PCR. IL-33 potently induced activation of ERK, p38, and NFκB pathways in these cells. Upon IL-33 stimulation, increased migratory capacity along with activated morphological changes could be detected. Together with induction of several M2-related genes, including CD206, these results are confirming the ability of IL-33 to activate and polarize Mφ.

In tumor settings, the role of IL-33 remains largely unknown, but has been well studied for its role in modulating the immune system. Here, we used a murine pancreatic tumor cell line, Panc02, selected because of its high expression of IL-33 in tumor tissue. In addition, we generated murine tumor cells with stable expression of IL-33. In this gain-of-function tumor model, we detected an increase in Mφ infiltration. Notably, no significant effect on tumor growth was observed, probably indicating a direct tumor-promoting effect of PDGF-BB in those tumors, independent of IL-33. IL-33 effects on the Mφ population was further confirmed in loss-of-function models by implanting Panc02 tumors in mice lacking IL-33 or ST2 gene expression, or treating with soluble ST2 protein (acting as a decoy receptor). In these models, Mφ infiltration was significantly impaired. Similar results could be reproduced by using PDGF-BB expressing tumors in the same models, further strengthening the link between PDGF-BB and IL-33.

The field of tumor immunology is boosting, especially tumor-associated Mφ are popular to study in tumors. Mφ are plastic and depending on the environment, different subtypes can be induced, and we wanted to investigate the effects of IL-33. By performing array, qPCR, and

FACS analyses, along with immunohistochemical staining of independent markers, we could conclude that IL-33 is a potent inducer of Mφ polarization towards M2, which has been shown to be the tumor-promoting subtype.

Furthermore, as we previously found that PDGF-BB expressing tumor show increased metastatic capacities, we wanted to explore the IL-33 potential in relation to metastasis. Using subcutaneous and liver implantation models, it became evident that IL-33 is driving tumor cell dissemination, concluded from CTC detection, as well as metastatic growth as we detected increased pulmonary metastasis. Depletion of Mφ with clodronate liposome treatment, significantly impaired the metastatic potential of IL-33-driven tumors, including the gain-of-function overexpressing tumors, as well as Panc02 tumors.

In summary, we provide detailed information of PDGF-BB-induced metastasis in tumors by a previously unknown mechanism. We identify the common inflammatory cytokine, IL-33, as being responsible for promoting the Mφ infiltration seen in PDGF-BB expressing tumors. Furthermore, we confirm that this population of Mφ are the critical mediators of metastasis. In light of these findings, new strategies, including IL-33 blockade and Mφ targeting, for therapeutic interventions can be developed to prevent tumor metastasis.

3.3 PAPER III

Identification of VEGF-B as a marker of poor prognosis by modulating the tumor microenvironment and promoting metastasis

Tumor angiogenesis and vascular remodeling are important processes for tumor development and progression[314]. There are many potent regulators of the vasculature found to be expressed in tumors. Here, we focused on one of the VEGF-family members, VEGF-B. Whereas VEGF-A-driven angiogenesis has been widely studied, the VEGFR1-only binding ligands, especially VEGF-B, remains less characterized. Early reports on the pro-angiogenic activity of VEGF-B has not been able to be reproduced, and its dependence on VEGF-A is debated. This promoted us to engage in a thorough study to investigate the role of VEGF-B in a tumor setting.

As VEGF-B expression has been detected in various human tumors, we selected 28 different human tumor cell line to screen for VEGF-B expression. From this experiment, we identified two human melanoma cell lines with differential expression, the MDA-MB-435 was among the highest, and UACC-62 expressed relatively low amounts. To generate appropriate

controls, we used specific shRNA to downregulate VEGF-B expression in MDA-MB-435, while we propagated UACC-62 to overexpress VEGF-B.

Loss-of-function experiments were performed by implantation of the two MDA-MB-435 cell lines in mice. Notably, loss of VEGF-B expression had no impact on primary tumor growth. Examination of the vascular phenotype in these tumors, revealed a significant increase in vessel density and disorganization in the shRNA-tumors compared to endogenously high VEGF-B expressing tumors. In correlation to these findings, the vasculature displayed improved perfusion and decreased permeability. As a consequence, tumors showed reduced level of tissue hypoxia. Interestingly, we also detected a decrease in the number of tumor-associated Mφ, as determined by CD206 (M2 marker) and F4/80 (pan-marker) co-staining, in VEGF-B-downregulated tumors. Collectively, these results indicate that VEGF-B is able to significantly modulate the tumor microenvironment, function as a negative regulator of angiogenesis, and have pronounced effect on the vasculature.

Next, we studied the effect on VEGF-B in an independent, gain-of-function model, where we implanted control and VEGF-B overexpressing UACC-62 cells in mice. Introduction of VEGF-B expression in this tumor model, in contrast to the loss-of-function model, led to reduced tumor growth. Furthermore, VEGF-B tumors displayed decreased vascular density with fewer branch-points. Despite this normalized appearance, pericytes coverage was poor, and endothelial cell junctions, as determined by VE-cadherin staining, were impaired. Consequently, the vasculature was leaky and poorly perfused, showing increase level of hypoxia. In addition, VEGF-B significantly increased the number of tumor-associated Mφ. For confirmation purposes, we generated a murine tumor cells to stably express VEGF-B using fibrosarcoma cell line, T241, with no significant endogenous VEGF-B expression. This model was able to reproduce the findings detected in the human tumor models. These results suggest that VEGF-B is inhibiting tumor growth and angiogenesis, along with displaying vascular remodeling properties rendering tumors hypoxic, and contributing to increased numbers of Mφ infiltration.

The vascular phenotypes observed under the influence of VEGF-B signaling, together with the increase in hypoxia and expansion of tumor-associated Mφ, which both have been linked to tumor cell dissemination, promoted us to investigate the metastatic potential in our available models. In the first model, blood was collected from tumor-bearing mice and analyzed for CTCs. For MDA-MD-435 tumors where VEGF-B had been downregulated, we saw a significant reduction in CTCs compared to controls. In contrast, the overexpression of VEGF-B in UACC-62 and fibrosarcoma tumors contributed to increased number of CTCs.

These indications of tumor cell dissemination were also correlated to the degree of pulmonary metastasis in all three models, where knock-down reduced the metastatic incidence, and overexpression led to increased metastasis.

The influence of VEGF-B on the vasculature has previously been indicated to occur through a VEGF-A-dependent mechanism. Here, we took advantage of a murine tumor cell line, 528ras, that lacks VEGF-A expression (originating from a VEGF-A null mouse). These cells were propagated to express VEGF-B and subsequently implanted into mice. Although many parameters were similar as detected in the previous VEGF-A positive tumors, there were important differences. Unexpectedly, the vasculature displayed a disorganized phenotype in VEGF-B expressing tumors, which is in contrast to previous models, suggesting that the effects of VEGF-B on vascular remodeling occurs in collaboration with VEGF-A. Although the perfusion and hypoxia did not differ between the two groups, permeability was increased and vascular coverage was reduced. The population of tumor-associated Mφ, however, was still increased. Importantly, independent of VEGF-A, VEGF-B promoted tumor cell dissemination as we detected increased CTCs and pulmonary metastasis. This effect of VEGF-B was confirmed in a model where we treated T241 tumors with anti-VEGF-A neutralizing antibody. Here, again, VEGF-B still promoted metastasis.

VEGF-B is known to interact with VEGFR1 to convey its biological signals. So we took an approach where T241 tumors were implanted in VEGFR1-TK knockout mice. Tumors generated in these mice were to a large extent similar to tumors grown in wild-type mice, where VEGF-B altered the tumor microenvironment, including Mφ, and promoted metastasis. These findings indicate that VEGF-B in the tumor setting, is promoting metastasis via, at least in part, a VEGFR1-independent mechanism.

Finally, to validate if there was any clinical relevance of our findings, we analyzed the correlation of VEGF-B expression and survival. Patients in high expression groups of melanoma and lung cancer were found to have significantly poorer survival rates.

In summary, we identify VEGF-B as a promoter of metastasis by modulating the tumor microenvironment, including altering the vascular phenotype and increasing the population of tumor-associated Mφ. We further demonstrate that pro-metastatic effects of VEGF-B is independent of VEGF-A and VEGFR1 activation. Clinically, patients with high levels of VEGF-B had decreased survival, hence, suggesting that VEGF-B is a marker of poor prognosis.

3.4 PAPER IV

VEGFR3 mediates FGF-2 induced lymphangiogenesis and metastasis

Tumor cell dissemination into the lymphatic system is commonly detected in cancer patients. VEGF-C is the main pro-lymphangiogenic factor, but members of other growth factor families, including FGF-2, have demonstrated lymphangiogenic properties. Several factors are simultaneously co-expressed in the tumor microenvironment, but little is generally known about any potential interplay. Results from one previous study indicated that there might be a link between VEGF-C and FGF-2 signaling, as FGF-2-induced lymphangiogenesis could be blocked by VEGFR3 inhibition[153]. In this study, we aimed to further investigate the underlying mechanisms of FGF-2-induced lymphangiogenesis and potential involvement of VEGF-C/VEGFR3 signaling.

For initial studies, we chose to use an established mouse cornea assay model to investigate the angiogenic effects of VEGF-C and FGF-2. The cornea is providing an advantageous system to study angiogenesis *in vivo* by its nature of being an avascular tissue[315]. Here, the factors were implanted as pellets in the avascular cornea and allow for studies of blood and lymph vessel growth. We confirmed the individual pro-angiogenic and lymphangiogenic properties of VEGF-C and FGF-2. To our surprise, co-implantation of both factors resulted in synergistic effect on both angiogenesis and lymphangiogenesis.

Next, we performed *in vitro* studies to gain further understanding of the synergism on a cellular level. For these experiments, we used primary mouse and human LECs. Both VEGF-C and FGF-2 induced proliferation in mLECs and hLECs, demonstrating that both factors are directly effecting these cells. This effect was significantly potentiated upon co-stimulation. Further experiments demonstrated that mainly FGFR1 was expressed, whereas FGFR2-4 were barely detectable, indicating that the FGF-2-FGFR1 axis was responsible for FGF-2 induced effects. Interestingly, qPCR analyses demonstrated that FGFR1 levels were increased by both VEGF-C and FGF-2 stimulation. Similarly, both factors also upregulated VEGFR3 expression. These results suggest a reciprocal amplification of the receptors induced by the ligands.

To study the involvement of VEGFR3 and FGFR1 in mediating biological processes upon stimulation, we used specific receptor neutralizing antibodies. FGF-2-induced proliferation and migration of mLEC was completely blocked by the addition of an anti-FGFR1 antibody, but no significant inhibitory effects upon VEGFR3-blockade. Opposite findings were observed with VEGF-C stimulation as only VEGFR3 neutralizing antibody had inhibitory

effects. Collectively, *in vitro* experiments indicate that these ligands are exerting the lymphangiogenic activities via their own receptor-specific axis.

Moving back to the cornea assay, to verify the role of FGFR1 in transducing FGF-2 activities *in vivo*, we co-implanted VEGF-C and FGF-2 and treated with the anti-FGFR1 neutralizing antibody. FGFR1-blockade significantly inhibited angiogenesis and lymphangiogenesis. Furthermore, we applied VEGFR3-blockade and could observe complete inhibition of VEGF-C-induced lymphangiogenesis. Unexpectedly, VEGFR3-blockade also significantly inhibited FGF-2-induced lymphangiogenesis. To understand how VEGFR3 is involved in FGF-2 promoted lymphangiogenesis, we took a closer look at the tip cells, as it was reported that tip cell formation is crucial for the angiogenic process. Individually, both VEGF-C and FGF-2 could induce tip cells in the growing lymph vessels. Co-implantation significantly contributed to an additive effect. Surprisingly, VEGFR3-blockade completely suppressed lymphatic tip cell formation in all conditions (FGF-2/VEGF-C/VEGF-C+FGF-2), but no inhibition was detected for blood vessel growth. These results are suggesting that FGF-2-promoted lymphangiogenesis is dependent on VEGFR3 for tip cell formation. In addition, we demonstrated that VEGF-C stimulates angiogenesis through activating VEGFR2, rather than VEGFR3.

Based on these initial findings, we wanted to investigate the effects of VEGF-C and FGF-2 in the tumor setting to study lymphangiogenesis and metastasis. For this purpose, we propagated a murine fibrosarcoma cell line, T241, to overexpress (and secrete) FGF-2 or VEGF-C. Implantation of these cells into mice showed that both VEGF-C and FGF-2 individually are promoting tumor growth, angiogenesis, and intratumoral lymphangiogenesis. Interestingly, all these processes were significantly potentiated beyond the additive effects when implanting a mixture of VEGF-C and FGF-2 expressing tumor cells, demonstrating collaborative effect of the two factors. Finally, as our previous findings clearly showed that both blood and lymph vessels were promoted in these models, we sought to examine the metastatic capacity. Individually, mice with VEGF-C expressing tumors displayed more metastatic burden in lungs and lymph nodes compared to FGF-2 implanted tumors. Notably, co-implantation of VEGF-C and FGF-2 expressing tumor cells drastically increased the number of visible metastatic nodules present in the lungs, and all mice in this group developed lymph node metastasis.

In summary, there is a clear microenvironmental interaction between different factors that regulate various processes in the tumor. Here, we revealed the underlying mechanisms of FGF-2-induced lymphangiogenesis by showing how VEGFR3-blockade completely

inhibited lymphangiogenic sprouting upon FGF-2 stimulation *in vivo* in both cornea and tumor models. We showed that tip cell formation is dependent on VEGFR3 activation. Furthermore, we identified how VEGF-C and FGF-2 are reciprocally amplifying each other's signaling to synergistically promote blood and lymph vessel growth, leading to metastasis. Collectively, our results demonstrate the importance of microenvironmental communication between growth factors in tumors. Therefore, combination therapy should be designed to interfere with such interplay.

4 DISCUSSION AND CONCLUSIONS

Metastatic disease is responsible for the majority of cancer-related deaths and the underlying mechanisms for tumor cell dissemination remains largely unknown. Increasing evidence is indicating how non-malignant cells of the host are contributing to a pro-metastatic phenotype[227]. Continuous efforts are being put on understanding the communication between compartments in the tumor microenvironment. The tumor microenvironment is highly complex, consisting of various cellular types, including ECs, PCs, VSMCs, fibroblasts, and immune cells. These cells are responsible for growth of blood and lymph vessels, generating connective tissues, and inflammation. The microenvironment provides, as previously mentioned, an opportunity for therapeutic intervention. By increasing the knowledge behind microenvironmental communication, we can begin to understand how to optimally block tumor-promoting processes.

Therapeutic targeting aiming to alter the density and structures of blood vessels to become less accessible to tumor cell intravasation would decrease metastasis and increase patient survival. It has proven not to be that straight-forward, as we provide several examples of in our current work, and additional efforts are needed. Can use vascular targeting and remodeling to reduce vascular density and improve the quality of the vessels that do remain? So far, the clinical experience of anti-angiogenic targeting has reduced the interest of such approaches. If anti-angiogenic and angiogenesis-modulating therapies are applied in the right setting, tumor would struggle to survive. The questions are *when* and *how* to treat. Anti-angiogenic therapy can be applied by different strategies. Either directly targeting the ECs by modulating the VEGFs/VEGFRs signaling axis, or by indirect targeting of the vascular support cells. Anti-PDGF targeting is one example of the latter. Clinically, several drugs are available that prevent activation of PDGFRs, including imatinib, sunitinib, and sorafenib. Except for imatinib in treatment of leukemia (blocking Bcr-Abl in CML), there is no molecular indication for their use in specific patients, pointing-out the need for new understanding of the underlying mechanisms. We sought to investigate the underlying principles for PDGF-BB signaling in tumors. In paper I, we report how one growth factor, PDGF-BB, produce opposing outcomes depending on its expression level, especially following therapeutic intervention using imatinib. On one hand, PDGF-BB is frequently expressed in tumors and its biological effects are mediated by directly acting on PDGFR-β expressing cells, such as PCs. Interestingly, in contrast to the role in recruitment of PCs to vessels, we found that high levels of PDGF-BB decreased vascular coverage and increased vessel disorganization. We identify a functional link

relating PDGF-BB signaling and the impairment of integrin interaction between ECs and PCs, leading to detachment of PCs. Consequently, the metastatic potential is increased in these tumors. These results are indicating that PDGF-BB signaling should be targeted in cancer patients. Our findings, however, demonstrated that the issue is more complex. In tumors where PDGF-BB is relatively low, anti-PDGF targeting is causing metastasis rather than having an anti-tumorigenic effect. Similar mechanisms could be true for other factors as well, indicating the importance of understanding the context-dependent outcomes before applying any type of therapy. Collectively, we point-out the need of a clear indication for therapy. In the clinical setting, it can be difficult to justify the use of a specific therapeutic agent. Many patients are found to be non-responsive to the treatment and there is a need for improvements in selection of patients. Reliable biomarkers that can predict treatment outcome and select patients that will benefit from a certain treatment. Here, we identify PDGF-BB as a potential biomarker for anti-PDGF targeting therapy.

In tumors, there are both direct and indirect mechanisms for regulation of various processes. For example, blood vessels are carriers of oxygen, hence, direct targeting of blood vessels, will indirectly lead to increased hypoxia, which is responsible for many tumor promoting processes. Hypoxia is involved in the regulation of PDGF-BB expression[132, 133]. The contributions of PDGF-BB to the tumor tissue, in addition to the extensively studied effects on PCs, are not clear. Growth factors, such as PDGF-BB, are not only potently regulating the functions of cellular populations through their specific receptor signaling axis, but indirect mechanisms are equally important. It will be essential to understand the secondary impacts of signaling by one factor, in addition to the direct effects in targeted cells. This could provide novel strategies for therapy. We exemplify such complex interaction using PDGF-BB as we previously noticed that PDGF-BB signaling in tumors is contributing to increased numbers of Mφ despite the lack of PDGFR expression by this cell type. In paper II, we report a previously unknown mechanism of PDGF-BB-induced Mφ infiltration. By activation of PDGFR-β-positive cells, PDGF-BB is stimulating the production of IL-33. In turn, IL-33 is regulating different populations of cells that express the ST2 receptor, including Mφ. Interestingly, not only numbers of Mφ were increased, but we also detected a clear polarization towards M2 phenotype. Many publications have indicated that M2 Mφ have tumor promoting functions and are important in the metastatic process. In our models, we confirmed the contributions of tumor-associated Mφ to metastasis, demonstrating that the metastatic potential of PDGF-BB is mediated through Mφ via IL-33 signaling. The question that remains to be addressed is *how* Mφ are facilitating metastasis. Are there unidentified mechanisms in addition to the ones that have been reported? Even without further understanding, the population of Mφ, on its own, is emerging as an important target for

prevention of metastasis. Here we identify and highlight a link between the growth factor signaling and cytokine systems, indicating the importance of microenvironmental interplay. Essentially, our findings could provide a new therapeutic opportunity, especially in inflammatory-driven tumors. Whereas anti-PDGF targeting is already established, targeting of Mφ is currently being developed by different strategies and it will be interesting to follow clinical trials using such therapies. We show how IL-33 drives the increase of Mφ and is responsible for polarization towards M2, hence, targeting of IL-33 would offer an upstream approach to prevent tumor-associated Mφ. Blocking IL-33 signaling would be feasible by neutralizing antibodies or soluble receptor protein. In fact, recently it was announced that AnaptysBio is entering an IL-33 neutralizing antibody (ANB020) in phase I clinical trials aiming for patients with asthma, atopic dermatitis and peanut allergy (Clinical Trial ID: NCT02920021). An application in cancer would be an interesting addition and, based on our results, potentially useful for preventing metastasis.

The blood vascular system is a common route of dissemination and angiogenesis is a pre-requisite for growth of a solid tumor. In addition to diverse vascular phenotypes being generated during the sprouting of vessels under influence of different factors, where VEGF-A is known to generate a disorganized tumor vascular network, there is an important contribution of vascular remodeling[314]. Much remains to be learnt about the regulation of this process and its involvement in tumor progression. In paper III, we demonstrate how VEGF-B promotes metastasis by modulating the tumor vasculature and inflammation. Importantly, gross examination of the vascular structures generated under the influence of VEGF-B signaling, appeared to be normalized. When analyzing the vessels, we could identify a poor vascular coverage and impaired EC junctions, leading to a leaky and tumor cell-accessible vessel structure. This finding pointed-out that vascular density and degree of disorganization, in terms of branch-points, cannot reliably be used for metastatic prediction. In combination with an altered tumor microenvironment, including vascular remodeling, increased numbers of Mφ and increased hypoxia, despite simultaneously retarding tumor growth, VEGF-B potently promoted metastasis. Collectively, VEGF-B exemplifies the difficulty of tumor biology. Tumors are inhibited in their growth, which is a good clinical sign, and vascular density is reduced, but tumors are still metastatic. Further efforts are needed to understand how these processes are being uncoupled. Of importance for targeting of VEGF-B signaling in tumors, even with being identified as only binding to VEGFR1, in this work, we demonstrated that VEGF-B-induced metastasis is VEGFR1-independent. These results are suggesting that intracellular targeting of VEGFR1 by small molecules, would not effectively block the pro-metastatic properties of VEGF-B. Additional studies are needed to identify a potential receptor

mediating these VEGFR1-independent processes of VEGF-B. Such findings would discover a novel target for prevention of metastasis.

One additional important compartment of the tumor microenvironment and a common route of dissemination, is the lymphatic system. Cancer patients often develop lymph node metastasis[316] and will, in for example breast cancer, frequently undergo surgery to remove sentinel lymph nodes along with the primary tumor. The main system for regulation of lymphangiogenesis is VEGF-C/VEGFR3 signaling, although additional factors also have pro-lymphangiogenic properties. Further complicating the matter, is the fact that many factors are simultaneously expressed in the tumor tissue. The possible relationship between signaling pathways in regards to angiogenesis and dissemination is unclear, but potentially important for resistance and treatment outcome. Clinically, this issue is not addressed. In paper IV, we identify a synergistic interplay of two growth factors, VEGF-C and FGF-2, in promoting metastasis by collaboratively inducing blood and lymph vessel growth. The most important conclusion from our experiments is the demonstration how VEGFR3-blockade is able to completely prevent FGF-2-induced lymphangiogenesis. Importantly, blood vessel growth promoted by FGF-2 is VEGFR3-independent and instead meditated by FGFR1 activation. These findings are showing that lymphangiogenesis is triggered in a sequential process and that VEGFR3 is essential for the initial step, the tip cell formation. This would suggest that VEGFR3-targeting, irrespectively of the driving force, will be important in tumors with high potential to induce lymphangiogenesis with subsequent lymphatic dissemination. Importantly, our findings are showing that FGF-2-induced blood vessel growth is independent of VEGFR3-activation. Collectively, it is becoming evident that combinational targeting will be necessary to prevent interplays such as these.

In this thesis work, we identify new concepts in tumor biology by revealing previously unknown mechanisms:

- High levels of tumor-derived PDGF-BB lead to reduced vascular coverage and facilitated tumor dissemination. We suggest to use PDGF-BB as a biomarker for anti-PDGF therapy
- IL-33 as a novel potential therapeutic target for inflammation-induced metastasis
- VEGF-B as a promoter of metastasis through a VEGFR1-independent mechanism
- VEGFR3-activity as being required for lymphatic tip cell formation, even under non-VEGFR3-ligand stimulation

In conclusion, we identify how tumors are employing different mechanisms for supporting the metastatic process by modulating different aspects of the tumor microenvironment. There is no effective treatment currently available for patients with metastatic disease. By characterizing the common factors expressed in tumor tissues and revealing the underlying mechanisms of the involvement of the tumor microenvironment, we can define new targets and strategies for therapy. Prevention of tumor cell dissemination will be critical to reduce mortality.

5 ACKNOWLEDGEMENTS

The work described in this thesis was accomplished at the Department of Microbiology, Tumor- and Cell Biology (MTC), Karolinska Institutet. I would like to express my sincere gratitude to everyone who helped me and created a wonderful environment during these years.

Professor Yihai Cao, my supervisor – When I first contacted you during my Bachelor's program to ask if I could do a project work in your group, I remember being amazed that you called me a few days after I sent my email and apologized for not returning to me earlier. This shows what kind of person and leader you are, always treating everyone the same, no matter a student or a fellow professor. Thank you for giving me the opportunity and believing in me throughout these years, this is something I appreciate and highly value! You taught me a lot about research and life in general. Thank you for providing an environment where not only research is in focus, but also organizing group events to refresh and strengthen the relations between lab members. I am very grateful that you took me in as a doctoral candidate and financed me during these years.

Dr. Lasse Jensen, my co-supervisor – Denmark and Sweden are not always the best of friends, but that's just part of the relationship. As one of the most senior members of the group and now leading your own group in Linköping, you are an inspiration. I admire your love for research and the hard work you put in to push your career forward. You have always been helpful and I value your scientific input. Your bank of knowledge is very impressive! Thank you for being my co-supervisor during my Ph.D. studies.

Dr. Sonia Lain, my mentor – Thank you Sonia for agreeing to be my mentor and for inviting me to discuss the progress of my research. Your friendly presence in the corridor has been appreciated! Thank you also for bringing in a lot of international students to your group for us in the corridor to get to know.

Dr. Kayoko Hosaka – How could I ever thank you enough! You kindly took me as your student right from the start and you showed me around. You taught me everything from cell culture work, to how the lab was operating, and how to perform animal experiments. I am impressed by the skills you possess and the amount of time and effort you put into your research. As a friend, you are so kind and helpful, likes to share your thoughts but also and active listener. You are always in the lab, from early morning to late evening. In addition to all the help you given me and your kind collaborations, I enjoyed the time we spent outside the lab, going to the gym, hiking, and kayaking. Thank you for inviting me to your apartment for

dinner gatherings, I'm happy that you managed to get it! I wish you the best of luck in your career. In the future, you can show me Japan, and the lovely Mt Fuji!

Dr. Yunlong Yang – Thank you for a very productive collaboration and kind help with ours and my projects. Thank you for all the support and motivation! You are always happy to discuss, help – you are a great teacher. I have learnt a lot from you and at the same time we had a lot of fun!

Dr. Jennifer Honek – My awesome friend and former colleague! You are truly inspirational and I am so happy that I got to know you. Having you in the lab meant a lot to me, as do our friendship outside work. You showed me the way to succeed as a Ph.D. candidate - your dissertation was amazing. Now I hope I can follow your example! Thank you for everything you've done for and with me in and outside of work. Also, I appreciate you taking the time to proofread my thesis. Send my regards to scratchy!

Dr. Pegah Rouhi – You and your family are amazing! I highly value all the laughter, all the discussions, dinner at your place, and everything else. I miss you in the lab, things are not the same without you. Believe it or not, but I have learnt so much from you in life. I admire your spirit and attitude. Thank you for being you, and for being my friend!

Carina Fischer – We have become very good friends! It's been a pleasure to have you in the lab and to hang out with you and **Tony** outside of the lab. Thank you for introducing me to climbing, although I'm not reaching your level yet! Please keep up the good work and soon it will be your turn to defend. Thank you also for the great input and help with my writing!

Dr. Sharon Lim and **Dr. Junwei Zhang** – Thank you both so much for making a great atmosphere in the office and for putting up with all my talking and poor jokes! You have been very helpful both in research and in life to have you as my friends and colleagues. You are both extremely productive and good at what you do. And thanks your trying to teach me some Chinese. Xie xie ni! Tack för allt och för all hjälp med avhandlingen!

What would I have done without all the kind friends and colleagues from Japan! - **Dr. Takahiro Seki**, **Dr. Hideki Iwamoto**, **Dr. Masaki Nakamura**, and **Dr. Abe Mitsuhiko**. You all have contributed to my smile every day, although I know that you don't always understand my complicated jokes. All of you are great people, very helpful and knowledgeable, and good friends. Thank you for proofreading my thesis. If Japan is anything like you guys, it must be an amazing place!

I would like to thank all present and former lab members, visiting researcher and students that made the lab a joyful, happy, inspiring and productive place to work and to share memorable events with. Specifically, I want to thank **Dr. Renhai Cao** and **Li Chen** for always being helpful and kind. Thank you **Dr. Xiaojuan Yang** and **Dr. Ji Hong** for great collaborations and for helping me during my studies.

I would like to extend a big thank you to all the helpful people in the MTC service group: **Torbjörn**, **Magnus**, **David**, and **Per**. Also there are several people in the study-department of the administration that made my life easier: the previous study administrator **Anita**, the current study administrator **Åsa**, and our Director of third-level studies **Velmurugesan Arulampalam**. Further, thank you **Kristina** in the economy-department, I really appreciate the kind help and interesting conversations. I also want to thank all the other people in the administration for your helpful assistance. Importantly, I want to thank all the amazing people in the MTC animal house, especially **Elin**, **Ida**, **Torunn**, and **Kenth**, and our veterinarian **Anna** for all your contributions, kind assistance, and nice conversations!

Marcus Ladds – Thank you for all the conversations, laughter, kind help, and for constantly changing back the smiley on your white board. The hobo will not be forgotten, and soon enough, Dr. Hobo!

Habib Sakil – Thank you for sharing my frustrations and for motivating me (sometimes with chocolate!). I really appreciate our friendship, and I wish you the best of luck with your PhD studies!

I want to thank all the awesome people previously and currently at MTC, including but not limited to: **Mariam**, **Jacob**, **Nyosha**, **Wisam**, **Marina, Marijke**, **Matteo**, **Ana, Vanessa**, **Silke**, **Candido**, **Alonso**, **Hamid,** and **Vishal**.

During my short stay at CCK, I met many wonderful people, the **CCK-people**, you know who you are! A special thanks to **Professor Arne Östman** for providing me with the opportunity to join your group for an exciting project work! I learnt a lot and I had the best time, getting to know great people. Thank you all the members of Arne's group during my time there! Especially, I want to thank **Dr. Elin Sjöberg –** Such an amazing person and it was great to attend your dissertation!

Fanny (Pang) Krantz – Underbara Pingu, kära djurgårdare! We have known each other for many years now! You are a wonderful person and a great friend. I'm so glad for everything we have been experienced so far, any more adventures to come. You and your **Stefan** (in his own

way, haha) are some of the best, and I could not be happier for the new addition to your family! I wish you were here, but I will do my best to join you in the US instead.

Peter Saliba Gustafsson – Our biomedical studies seem so far away, time really flies, but we have some great memories! I am happy to have you and your wife(!) **Erika** as friends. Even though we, most of the times, only managed to meet up for lunch, it's a nice break from work and I always enjoy it. Also enjoy sharing our experiences in the lab, although they are sometimes too frustrating. Thank you for all these years, and I look forward to your upcoming defense as well!

Dr. Cecilia Nordfors – You were the first among us to get the title, and how impressive of you! It is always a pleasure to meet you and be inspired by your personality and happy attitude, you are a good friend. Thank you for motivating me!

All the kind people I got to know during my climbing sessions: **Daniela**, **Mirela**, **Jilin**, **Per**, **Gaëlle**, and **João**.

Tack alla gamla klasskompisar och vänner från Hälsingland, speciellt **Sven** och **Mårten**, för att ni konstant frågat om jag hittat ett botemedel än och därmed motiverat mig till att fortsätta sikta framåt! Även tack för att bidra till att få tänka på annat och ibland inte tänka alls – vilket vi vet hur det brukar sluta.

Camilla Pettersson – Bästa Camilla! Du är så underbar och härlig, inspirerande och motiverande. Du kan ju typ allt, och blir hela tiden imponerad av dig. Du får mig alltid på bra humör. Tack för att du finns, din vänskap betyder väldigt mycket!

Till sist, vill jag tacka min familj för allt ni har gjort och för att jag fått chansen att utforma min framtid precis som jag önskat och att ni alltid stöttat mig! Hoppas att ni ska vara stolta över den resa jag hittills gjort, och kommer fortsätta göra. **Mamma**, du har varit helt otrolig som funnits där som mitt största stöd och supporter. **Pappa**, alla systrar: **Frida**, **Malin**, **Marie**, **Carina** med respektive familjer, tack för att ni funnits där, visat intresse och hjälpt till. Ett stort tack till älskade **mormor och morfar**, och **farmor** för att ni trott på mig. Älskar er alla!

6 REFERENCES

1. World Health Organization. World Cancer Report. (2014).
2. Socialstyrelsen. Cancer i siffror 2013. (2013).
3. Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell* **127,** 679-695 (2006).
4. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. *Nature medicine* **12,** 895-904 (2006).
5. Hunt KK, Vorburger SA. Tech.Sight. Gene therapy. Hurdles and hopes for cancer treatment. *Science* **297,** 415-416 (2002).
6. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* **144,** 646-674 (2011).
7. Carmeliet P. Angiogenesis in health and disease. *Nature medicine* **9,** 653-660 (2003).
8. Folkman J. Tumor angiogenesis: therapeutic implications. *The New England journal of medicine* **285,** 1182-1186 (1971).
9. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* **407,** 249-257 (2000).
10. Tennant M, McGeachie JK. Blood vessel structure and function: a brief update on recent advances. *The Australian and New Zealand journal of surgery* **60,** 747-753 (1990).
11. Paulsson M. Basement membrane proteins: structure, assembly, and cellular interactions. *Critical reviews in biochemistry and molecular biology* **27,** 93-127 (1992).
12. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nature reviews Cancer* **3,** 422-433 (2003).
13. Chang C, Werb Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. *Trends in cell biology* **11,** S37-43 (2001).
14. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of cell biology* **161,** 1163-1177 (2003).
15. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. *The journal of investigative dermatology Symposium proceedings / the Society for Investigative Dermatology, Inc [and] European Society for Dermatological Research* **5,** 40-46 (2000).
16. Breier G. Angiogenesis in embryonic development--a review. *Placenta* **21 Suppl A,** S11-15 (2000).
17. Chung AS, Ferrara N. Developmental and pathological angiogenesis. *Annual review of cell and developmental biology* **27,** 563-584 (2011).
18. Cao Y. Angiogenesis in malignancy. *Seminars in cancer biology* **19,** 277-278 (2009).
19. Papetti M, Herman IM. Mechanisms of normal and tumor-derived angiogenesis. *American journal of physiology Cell physiology* **282,** C947-970 (2002).
20. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro-oncology* **7,** 452-464 (2005).
21. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nature medicine* **17,** 1359-1370 (2011).
22. Ribatti D, Nico B, Crivellato E. The role of pericytes in angiogenesis. *The International journal of developmental biology* **55,** 261-268 (2011).
23. Jain RK, Booth MF. What brings pericytes to tumor vessels? *The Journal of clinical investigation* **112,** 1134-1136 (2003).
24. McDonald DM, Baluk P. Imaging of angiogenesis in inflamed airways and tumors: newly formed blood vessels are not alike and may be wildly abnormal: Parker B. Francis lecture. *Chest* **128,** 602S-608S (2005).

25. Zetter BR. Angiogenesis and tumor metastasis. *Annual review of medicine* **49,** 407-424 (1998).
26. Kirsch M, Schackert G, Black PM. Metastasis and angiogenesis. *Cancer treatment and research* **117,** 285-304 (2004).
27. Stenmark KR, Davie N, Frid M, Gerasimovskaya E, Das M. Role of the adventitia in pulmonary vascular remodeling. *Physiology* **21,** 134-145 (2006).
28. Osol G, Mandala M. Maternal uterine vascular remodeling during pregnancy. *Physiology* **24,** 58-71 (2009).
29. Dzau VJ, Gibbons GH. Vascular remodeling: mechanisms and implications. *Journal of cardiovascular pharmacology* **21 Suppl 1,** S1-5 (1993).
30. Tabruyn SP, Colton K, Morisada T, Fuxe J, Wiegand SJ, Thurston G, et al. Angiopoietin-2-driven vascular remodeling in airway inflammation. *The American journal of pathology* **177,** 3233-3243 (2010).
31. Glade Bender J, Cooney EM, Kandel JJ, Yamashiro DJ. Vascular remodeling and clinical resistance to antiangiogenic cancer therapy. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* **7,** 289-300 (2004).
32. Tsigkos S, Koutsilieris M, Papapetropoulos A. Angiopoietins in angiogenesis and beyond. *Expert opinion on investigational drugs* **12,** 933-941 (2003).
33. Felcht M, Luck R, Schering A, Seidel P, Srivastava K, Hu J, et al. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. *The Journal of clinical investigation* **122,** 1991-2005 (2012).
34. Wilting J, Tomarev SI, Christ B, Schweigerer L. Lymphangioblasts in embryonic lymphangiogenesis. *Lymphatic research and biology* **1,** 33-40 (2003).
35. Scavelli C, Weber E, Agliano M, Cirulli T, Nico B, Vacca A, et al. Lymphatics at the crossroads of angiogenesis and lymphangiogenesis. *Journal of anatomy* **204,** 433-449 (2004).
36. Kitajewski J, Shawber CJ, Simons M. Lymphatics in health and disease: a new thematic series in vascular cell. *Vascular cell* **5,** 14 (2013).
37. Karpanen T, Wirzenius M, Makinen T, Veikkola T, Haisma HJ, Achen MG, et al. Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. *The American journal of pathology* **169,** 708-718 (2006).
38. von der Weid PY, Zawieja DC. Lymphatic smooth muscle: the motor unit of lymph drainage. *The international journal of biochemistry & cell biology* **36,** 1147-1153 (2004).
39. Rovenska E, Rovensky J. Lymphatic vessels: structure and function. *The Israel Medical Association journal : IMAJ* **13,** 762-768 (2011).
40. Brouillard P, Boon L, Vikkula M. Genetics of lymphatic anomalies. *The Journal of clinical investigation* **124,** 898-904 (2014).
41. Stacker SA, Williams SP, Karnezis T, Shayan R, Fox SB, Achen MG. Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nature reviews Cancer* **14,** 159-172 (2014).
42. Christiansen A, Detmar M. Lymphangiogenesis and cancer. *Genes & cancer* **2,** 1146-1158 (2011).
43. Witsch E, Sela M, Yarden Y. Roles for growth factors in cancer progression. *Physiology* **25,** 85-101 (2010).
44. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in cancer. *Vascular health and risk management* **2,** 213-219 (2006).
45. Folkman J. Role of angiogenesis in tumor growth and metastasis. *Seminars in oncology* **29,** 15-18 (2002).
46. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* **141,** 1117-1134 (2010).

47. Hubbard SR, Miller WT. Receptor tyrosine kinases: mechanisms of activation and signaling. *Current opinion in cell biology* **19,** 117-123 (2007).
48. He L, Hristova K. Physical-chemical principles underlying RTK activation, and their implications for human disease. *Biochimica et biophysica acta* **1818,** 995-1005 (2012).
49. Sangwan V, Park M. Receptor tyrosine kinases: role in cancer progression. *Current oncology* **13,** 191-193 (2006).
50. Regad T. Targeting RTK Signaling Pathways in Cancer. *Cancers* **7,** 1758-1784 (2015)
51. Katz M, Amit I, Yarden Y. Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochimica et biophysica acta* **1773,** 1161-1176 (2007).
52. Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nature reviews Cancer* **4,** 361-370 (2004).
53. Lange C, Storkebaum E, de Almodovar CR, Dewerchin M, Carmeliet P. Vascular endothelial growth factor: a neurovascular target in neurological diseases. *Nature reviews Neurology* **12,** 439-454 (2016).
54. Otrock ZK, Makarem JA, Shamseddine AI. Vascular endothelial growth factor family of ligands and receptors: review. *Blood cells, molecules & diseases* **38,** 258-268 (2007)
55. Shibuya M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. *Genes & cancer* **2,** 1097-1105 (2011).
56. Cao Y. Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Science signaling* **2,** re1 (2009).
57. Meyer RD, Mohammadi M, Rahimi N. A single amino acid substitution in the activation loop defines the decoy characteristic of VEGFR-1/FLT-1. *The Journal of biological chemistry* **281,** 867-875 (2006).
58. Shibuya M. Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis. *Angiogenesis* **9,** 225-230; discussion 231 (2006).
59. Takahashi S. Vascular endothelial growth factor (VEGF), VEGF receptors and their inhibitors for antiangiogenic tumor therapy. *Biological & pharmaceutical bulletin* **34,** 1785-1788 (2011).
60. Holmes DI, Zachary I. The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. *Genome biology* **6,** 209 (2005).
61. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435-439 (1996).
62. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380,** 439-442 (1996).
63. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376,** 62-66 (1995).
64. Ferrara N. Vascular endothelial growth factor as a target for anticancer therapy. *The oncologist* **9 Suppl 1,** 2-10 (2004).
65. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **13,** 9-22 (1999).
66. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69 Suppl 3,** 4-10 (2005).
67. Fischer C, Mazzone M, Jonckx B, Carmeliet P. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nature reviews Cancer* **8,** 942-956 (2008).
68. Sirois MG, Edelman ER. VEGF effect on vascular permeability is mediated by synthesis of platelet-activating factor. *The American journal of physiology* **272,** H2746-2756 (1997).

69. Goel HL, Mercurio AM. VEGF targets the tumour cell. *Nature reviews Cancer* **13,** 871-882 (2013).
70. Smith NR, Baker D, James NH, Ratcliffe K, Jenkins M, Ashton SE, et al. Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16,** 3548-3561 (2010).
71. Dabrosin C, Margetts PJ, Gauldie J. Estradiol increases extracellular levels of vascular endothelial growth factor in vivo in murine mammary cancer. *International journal of cancer* **107,** 535-540 (2003).
72. Manenti L, Riccardi E, Marchini S, Naumova E, Floriani I, Garofalo A, et al. Circulating plasma vascular endothelial growth factor in mice bearing human ovarian carcinoma xenograft correlates with tumor progression and response to therapy. *Molecular cancer therapeutics* **4,** 715-725 (2005).
73. Ferrer FA, Miller LJ, Andrawis RI, Kurtzman SH, Albertsen PC, Laudone VP, et al. Vascular endothelial growth factor (VEGF) expression in human prostate cancer: in situ and in vitro expression of VEGF by human prostate cancer cells. *The Journal of urology* **157,** 2329-2333 (1997).
74. Terris B, Scoazec JY, Rubbia L, Bregeaud L, Pepper MS, Ruszniewski P, et al. Expression of vascular endothelial growth factor in digestive neuroendocrine tumours. *Histopathology* **32,** 133-138 (1998).
75. Zhan P, Ji YN, Yu LK. VEGF is associated with the poor survival of patients with prostate cancer: a meta-analysis. *Translational andrology and urology* **2,** 99-105 (2013).
76. Gasparini G. Prognostic value of vascular endothelial growth factor in breast cancer. *The oncologist* **5 Suppl 1,** 37-44 (2000).
77. Ozdemir F, Akdogan R, Aydin F, Reis A, Kavgaci H, Gul S, et al. The effects of VEGF and VEGFR-2 on survival in patients with gastric cancer. *Journal of experimental & clinical cancer research : CR* **25,** 83-88 (2006).
78. Bellomo D, Headrick JP, Silins GU, Paterson CA, Thomas PS, Gartside M, et al. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circulation research* **86,** E29-35 (2000).
79. Aase K, von Euler G, Li X, Ponten A, Thoren P, Cao R, et al. Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect. *Circulation* **104,** 358-364 (2001).
80. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376,** 66-70 (1995).
81. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 9349-9354 (1998).
82. Enholm B, Paavonen K, Ristimaki A, Kumar V, Gunji Y, Klefstrom J, et al. Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene* **14,** 2475-2483 (1997).
83. Li X, Eriksson U. Novel VEGF family members: VEGF-B, VEGF-C and VEGF-D. *The international journal of biochemistry & cell biology* **33,** 421-426 (2001).
84. Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacological reviews* **56,** 549-580 (2004).
85. Albrecht I, Kopfstein L, Strittmatter K, Schomber T, Falkevall A, Hagberg CE, et al. Suppressive effects of vascular endothelial growth factor-B on tumor growth in a mouse model of pancreatic neuroendocrine tumorigenesis. *PloS one* **5,** e14109 (2010).

86. Silvestre JS, Tamarat R, Ebrahimian TG, Le-Roux A, Clergue M, Emmanuel F, et al. Vascular endothelial growth factor-B promotes in vivo angiogenesis. *Circulation research* **93,** 114-123 (2003).
87. Li X, Kumar A, Zhang F, Lee C, Tang Z. Complicated life, complicated VEGF-B. *Trends in molecular medicine* **18,** 119-127 (2012).
88. Sands M, Howell K, Costello CM, McLoughlin P. Placenta growth factor and vascular endothelial growth factor B expression in the hypoxic lung. *Respiratory research* **12,** 17 (2011).
89. Olofsson B, Korpelainen E, Pepper MS, Mandriota SJ, Aase K, Kumar V, et al. Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 11709-11714 (1998).
90. Andre T, Kotelevets L, Vaillant JC, Coudray AM, Weber L, Prevot S, et al. Vegf, Vegf-B, Vegf-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. *International journal of cancer* **86,** 174-181 (2000).
91. Salven P, Lymboussaki A, Heikkila P, Jaaskela-Saari H, Enholm B, Aase K, et al. Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. *The American journal of pathology* **153,** 103-108 (1998).
92. Jayasinghe C, Simiantonaki N, Kirkpatrick CJ. VEGF-B expression in colorectal carcinomas and its relevance for tumor progression. *Histology and histopathology* **28,** 647-653 (2013).
93. Gunningham SP, Currie MJ, Han C, Robinson BA, Scott PA, Harris AL, et al. VEGF-B expression in human primary breast cancers is associated with lymph node metastasis but not angiogenesis. *The Journal of pathology* **193,** 325-332 (2001).
94. Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V, et al. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development* **122,** 3829-3837 (1996).
95. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, et al. Vascular endothelial growth factor C induces angiogenesis in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 14389-14394 (1998).
96. Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nature immunology* **5,** 74-80 (2004).
97. Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, et al. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* **282,** 946-949 (1998).
98. Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proceedings of the National Academy of Sciences of the United States of America* **95,** 548-553 (1998).
99. Cha HS, Bae EK, Koh JH, Chai JY, Jeon CH, Ahn KS, et al. Tumor necrosis factor-alpha induces vascular endothelial growth factor-C expression in rheumatoid synoviocytes. *The Journal of rheumatology* **34,** 16-19 (2007).
100. Kim BS, Chen J, Weinstein T, Noiri E, Goligorsky MS. VEGF expression in hypoxia and hyperglycemia: reciprocal effect on branching angiogenesis in epithelial-endothelial co-cultures. *Journal of the American Society of Nephrology : JASN* **13,** 2027-2036 (2002).
101. Ristimaki A, Narko K, Enholm B, Joukov V, Alitalo K. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *The Journal of biological chemistry* **273,** 8413-8418 (1998).

102. Simiantonaki N, Jayasinghe C, Michel-Schmidt R, Peters K, Hermanns MI, Kirkpatrick CJ. Hypoxia-induced epithelial VEGF-C/VEGFR-3 upregulation in carcinoma cell lines. *International journal of oncology* **32,** 585-592 (2008).
103. Morfoisse F, Kuchnio A, Frainay C, Gomez-Brouchet A, Delisle MB, Marzi S, et al. Hypoxia induces VEGF-C expression in metastatic tumor cells via a HIF-1alpha-independent translation-mediated mechanism. *Cell reports* **6,** 155-167 (2014).
104. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature medicine* **7,** 192-198 (2001).
105. George ML, Tutton MG, Janssen F, Arnaout A, Abulafi AM, Eccles SA, et al. VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. *Neoplasia* **3,** 420-427 (2001).
106. Zhang C, Hao L, Wang L, Xiao Y, Ge H, Zhu Z, et al. Elevated IGFIR expression regulating VEGF and VEGF-C predicts lymph node metastasis in human colorectal cancer. *BMC cancer* **10,** 184 (2010).
107. Yonemura Y, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, et al. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **5,** 1823-1829 (1999).
108. Mobius C, Freire J, Becker I, Feith M, Brucher BL, Hennig M, et al. VEGF-C expression in squamous cell carcinoma and adenocarcinoma of the esophagus. *World journal of surgery* **31,** 1768-1772; discussion 1773-1764 (2007).
109. Liang B, Li Y. Prognostic Significance of VEGF-C Expression in Patients with Breast Cancer: A Meta-Analysis. *Iranian journal of public health* **43,** 128-135 (2014).
110. Yonemura Y, Endo Y, Tabata K, Kawamura T, Yun HY, Bandou E, et al. Role of VEGF-C and VEGF-D in lymphangiogenesis in gastric cancer. *International journal of clinical oncology* **10,** 318-327 (2005).
111. Tanaka T, Ishiguro H, Kuwabara Y, Kimura M, Mitsui A, Katada T, et al. Vascular endothelial growth factor C (VEGF-C) in esophageal cancer correlates with lymph node metastasis and poor patient prognosis. *Journal of experimental & clinical cancer research : CR* **29,** 83 (2010).
112. Xia H, Shen J, Chen S, Huang H, Xu Y, Ma H. Overexpression of VEGF-C correlates with a poor prognosis in esophageal cancer patients. *Cancer biomarkers : section A of Disease markers* **17,** 165-170 (2016).
113. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes & development* **22,** 1276-1312 (2008).
114. Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiological reviews* **79,** 1283-1316 (1999).
115. Davis JB, Stroobant P. Platelet-derived growth factors and fibroblast growth factors are mitogens for rat Schwann cells. *The Journal of cell biology* **110,** 1353-1360 (1990).
116. Westermark B, Siegbahn A, Heldin CH, Claesson-Welsh L. B-type receptor for platelet-derived growth factor mediates a chemotactic response by means of ligand-induced activation of the receptor protein-tyrosine kinase. *Proceedings of the National Academy of Sciences of the United States of America* **87,** 128-132 (1990).
117. Hoch RV, Soriano P. Roles of PDGF in animal development. *Development* **130,** 4769-4784 (2003).
118. Freyberger H, Brocker M, Yakut H, Hammer J, Effert R, Schifferdecker E, et al. Increased levels of platelet-derived growth factor in vitreous fluid of patients with proliferative diabetic retinopathy. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association* **108,** 106-109 (2000).

119. Rubin K, Tingstrom A, Hansson GK, Larsson E, Ronnstrand L, Klareskog L, et al. Induction of B-type receptors for platelet-derived growth factor in vascular inflammation: possible implications for development of vascular proliferative lesions. *Lancet* **1,** 1353-1356 (1988).
120. Wilcox JN, Smith KM, Williams LT, Schwartz SM, Gordon D. Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by in situ hybridization. *The Journal of clinical investigation* **82,** 1134-1143 (1988).
121. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* **125,** 1591-1598 (1998).
122. Lindahl P, Johansson BR, Leveen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* **277,** 242-245 (1997).
123. Battegay EJ, Rupp J, Iruela-Arispe L, Sage EH, Pech M. PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. *The Journal of cell biology* **125,** 917-928 (1994).
124. Hellstrom M, Kalen M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* **126,** 3047-3055 (1999).
125. Cao R, Brakenhielm E, Li X, Pietras K, Widenfalk J, Ostman A, et al. Angiogenesis stimulated by PDGF-CC, a novel member in the PDGF family, involves activation of PDGFR-alphaalpha and -alphabeta receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **16,** 1575-1583 (2002).
126. Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes & development* **8,** 1875-1887 (1994).
127. Soriano P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes & development* **8,** 1888-1896 (1994).
128. Abramsson A, Lindblom P, Betsholtz C. Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *The Journal of clinical investigation* **112,** 1142-1151 (2003).
129. Coltrera MD, Wang J, Porter PL, Gown AM. Expression of platelet-derived growth factor B-chain and the platelet-derived growth factor receptor beta subunit in human breast tissue and breast carcinoma. *Cancer research* **55,** 2703-2708 (1995).
130. Silberstein FC, De Simone R, Levi G, Aloisi F. Cytokine-regulated expression of platelet-derived growth factor gene and protein in cultured human astrocytes. *Journal of neurochemistry* **66,** 1409-1417 (1996).
131. Rydziel S, Canalis E. Expression and growth factor regulation of platelet-derived growth factor B transcripts in primary osteoblast cell cultures. *Endocrinology* **137,** 4115-4119 (1996).
132. Schito L, Rey S, Tafani M, Zhang H, Wong CC, Russo A, et al. Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* **109,** E2707-2716 (2012).
133. Yoshida D, Kim K, Noha M, Teramoto A. Hypoxia inducible factor 1-alpha regulates of platelet derived growth factor-B in human glioblastoma cells. *Journal of neuro-oncology* **76,** 13-21 (2006).
134. Board R, Jayson GC. Platelet-derived growth factor receptor (PDGFR): a target for anticancer therapeutics. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* **8,** 75-83 (2005).
135. Wu E, Palmer N, Tian Z, Moseman AP, Galdzicki M, Wang X, et al. Comprehensive dissection of PDGF-PDGFR signaling pathways in PDGFR genetically defined cells. *PloS one* **3,** e3794 (2008).

136. Donnem T, Al-Saad S, Al-Shibli K, Andersen S, Busund LT, Bremnes RM. Prognostic impact of platelet-derived growth factors in non-small cell lung cancer tumor and stromal cells. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* **3,** 963-970 (2008).
137. Raica M, Cimpean AM. Platelet-Derived Growth Factor (PDGF)/PDGF Receptors (PDGFR) Axis as Target for Antitumor and Antiangiogenic Therapy. *Pharmaceuticals* **3,** 572-599 (2010).
138. Kodama M, Kitadai Y, Sumida T, Ohnishi M, Ohara E, Tanaka M, et al. Expression of platelet-derived growth factor (PDGF)-B and PDGF-receptor beta is associated with lymphatic metastasis in human gastric carcinoma. *Cancer science* **101,** 1984-1989 (2010).
139. Krejci P, Prochazkova J, Bryja V, Kozubik A, Wilcox WR. Molecular pathology of the fibroblast growth factor family. *Human mutation* **30,** 1245-1255 (2009).
140. Goldfarb M. Fibroblast growth factor homologous factors: evolution, structure, and function. *Cytokine & growth factor reviews* **16,** 215-220 (2005).
141. Muller HM, Steringer JP, Wegehingel S, Bleicken S, Munster M, Dimou E, et al. Formation of disulfide bridges drives oligomerization, membrane pore formation, and translocation of fibroblast growth factor 2 to cell surfaces. *The Journal of biological chemistry* **290,** 8925-8937 (2015).
142. Mignatti P, Morimoto T, Rifkin DB. Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *Journal of cellular physiology* **151,** 81-93 (1992).
143. Shaoul E, Reich-Slotky R, Berman B, Ron D. Fibroblast growth factor receptors display both common and distinct signaling pathways. *Oncogene* **10,** 1553-1561 (1995).
144. Thisse B, Thisse C. Functions and regulations of fibroblast growth factor signaling during embryonic development. *Developmental biology* **287,** 390-402 (2005).
145. Auguste P, Javerzat S, Bikfalvi A. Regulation of vascular development by fibroblast growth factors. *Cell and tissue research* **314,** 157-166 (2003).
146. Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. *Nature reviews Cancer* **10,** 116-129 (2010).
147. Ornitz DM, Itoh N. Fibroblast growth factors. *Genome biology* **2,** REVIEWS3005 (2001)
148. Abdel-Rahman WM, Kalinina J, Shoman S, Eissa S, Ollikainen M, Elomaa O, et al. Somatic FGF9 mutations in colorectal and endometrial carcinomas associated with membranous beta-catenin. *Human mutation* **29,** 390-397 (2008).
149. van der Walt JM, Noureddine MA, Kittappa R, Hauser MA, Scott WK, McKay R, et al. Fibroblast growth factor 20 polymorphisms and haplotypes strongly influence risk of Parkinson disease. *American journal of human genetics* **74,** 1121-1127 (2004).
150. Stopa EG, Gonzalez AM, Chorsky R, Corona RJ, Alvarez J, Bird ED, et al. Basic fibroblast growth factor in Alzheimer's disease. *Biochemical and biophysical research communications* **171,** 690-696 (1990).
151. Ornitz DM, Marie PJ. Fibroblast growth factor signaling in skeletal development and disease. *Genes & development* **29,** 1463-1486 (2015).
152. Kottakis F, Polytarchou C, Foltopoulou P, Sanidas I, Kampranis SC, Tsichlis PN. FGF-2 regulates cell proliferation, migration, and angiogenesis through an NDY1/KDM2B-miR-101-EZH2 pathway. *Molecular cell* **43,** 285-298 (2011).
153. Kubo H, Cao R, Brakenhielm E, Makinen T, Cao Y, Alitalo K. Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 8868-8873 (2002).

154. Pepper MS, Wasi S, Ferrara N, Orci L, Montesano R. In vitro angiogenic and proteolytic properties of bovine lymphatic endothelial cells. *Experimental cell research* **210,** 298-305 (1994).
155. Alessi P, Leali D, Camozzi M, Cantelmo A, Albini A, Presta M. Anti-FGF2 approaches as a strategy to compensate resistance to anti-VEGF therapy: long-pentraxin 3 as a novel antiangiogenic FGF2-antagonist. *European cytokine network* **20,** 225-234 (2009).
156. Andres G, Leali D, Mitola S, Coltrini D, Camozzi M, Corsini M, et al. A pro-inflammatory signature mediates FGF2-induced angiogenesis. *Journal of cellular and molecular medicine* **13,** 2083-2108 (2009).
157. Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, et al. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *The Journal of cell biology* **141,** 1659-1673 (1998).
158. Stavri GT, Zachary IC, Baskerville PA, Martin JF, Erusalimsky JD. Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. *Circulation* **92,** 11-14 (1995).
159. Parsons-Wingerter P, Elliott KE, Clark JI, Farr AG. Fibroblast growth factor-2 selectively stimulates angiogenesis of small vessels in arterial tree. *Arteriosclerosis, thrombosis, and vascular biology* **20,** 1250-1256 (2000).
160. Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. *Nature reviews Drug discovery* **8,** 235-253 (2009).
161. Montero A, Okada Y, Tomita M, Ito M, Tsurukami H, Nakamura T, et al. Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. *The Journal of clinical investigation* **105,** 1085-1093 (2000).
162. Fei Y, Xiao L, Doetschman T, Coffin DJ, Hurley MM. Fibroblast growth factor 2 stimulation of osteoblast differentiation and bone formation is mediated by modulation of the Wnt signaling pathway. *The Journal of biological chemistry* **286,** 40575-40583 (2011).
163. Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoying JB, Haudenschild CC, et al. Fibroblast growth factor 2 control of vascular tone. *Nature medicine* **4,** 201-207 (1998).
164. Conte C, Riant E, Toutain C, Pujol F, Arnal JF, Lenfant F, et al. FGF2 translationally induced by hypoxia is involved in negative and positive feedback loops with HIF-1alpha. *PloS one* **3,** e3078 (2008).
165. Pintucci G, Yu PJ, Saponara F, Kadian-Dodov DL, Galloway AC, Mignatti P. PDGF-BB induces vascular smooth muscle cell expression of high molecular weight FGF-2, which accumulates in the nucleus. *Journal of cellular biochemistry* **95,** 1292-1300 (2005).
166. Moffett J, Kratz E, Myers J, Stachowiak EK, Florkiewicz RZ, Stachowiak MK. Transcriptional regulation of fibroblast growth factor-2 expression in human astrocytes: implications for cell plasticity. *Molecular biology of the cell* **9,** 2269-2285 (1998).
167. Song QH, Klepeis VE, Nugent MA, Trinkaus-Randall V. TGF-beta1 regulates TGF-beta1 and FGF-2 mRNA expression during fibroblast wound healing. *Molecular pathology : MP* **55,** 164-176 (2002).
168. Cao Y, Cao R, Hedlund EM. R Regulation of tumor angiogenesis and metastasis by FGF and PDGF signaling pathways. *Journal of molecular medicine* **86,** 785-789 (2008).
169. Dvorak P, Dvorakova D, Hampl A. Fibroblast growth factor signaling in embryonic and cancer stem cells. *FEBS letters* **580,** 2869-2874 (2006).

170. Nguyen M, Watanabe H, Budson AE, Richie JP, Hayes DF, Folkman J. Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers. *Journal of the National Cancer Institute* **86,** 356-361 (1994).
171. Rades D, Seibold ND, Gebhard MP, Noack F, Bruchhage KL, Schild SE. Fibroblast growth factor 2 is of prognostic value for patients with locally advanced squamous cell carcinoma of the head and neck. *Strahlentherapie und Onkologie : Organ der Deutschen Rontgengesellschaft [et al]* **190,** 68-74 (2014).
172. Hase T, Kawashiri S, Tanaka A, Nozaki S, Noguchi N, Kato K, et al. Correlation of basic fibroblast growth factor expression with the invasion and the prognosis of oral squamous cell carcinoma. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* **35,** 136-139 (2006).
173. Yiangou C, Gomm JJ, Coope RC, Law M, Luqmani YA, Shousha S, et al. Fibroblast growth factor 2 in breast cancer: occurrence and prognostic significance. *British journal of cancer* **75,** 28-33 (1997).
174. Donnem T, Al-Shibli K, Al-Saad S, Busund LT, Bremnes RM. Prognostic impact of fibroblast growth factor 2 in non-small cell lung cancer: coexpression with VEGFR-3 and PDGF-B predicts poor survival. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* **4,** 578-585 (2009).
175. Ozaki K, Leonard WJ. Cytokine and cytokine receptor pleiotropy and redundancy. *The Journal of biological chemistry* **277,** 29355-29358 (2002).
176. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et biophysica acta* **1843,** 2563-2582 (2014).
177. Lira SA, Furtado GC. The biology of chemokines and their receptors. *Immunologic research* **54,** 111-120 (2012).
178. Kelso A. Cytokines: principles and prospects. *Immunology and cell biology* **76,** 300-317 (1998).
179. Krementsov DN, Thornton TM, Teuscher C, Rincon M. The emerging role of p38 mitogen-activated protein kinase in multiple sclerosis and its models. *Molecular and cellular biology* **33,** 3728-3734 (2013).
180. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor perspectives in biology* **1,** a001651 (2009).
181. Leonard WJ, Lin JX. Cytokine receptor signaling pathways. *The Journal of allergy and clinical immunology* **105,** 877-888 (2000).
182. Dantzer R, Wollman EE, Vitkovic L, Yirmiya R. Cytokines, stress, and depression. Conclusions and perspectives. *Advances in experimental medicine and biology* **461,** 317-329 (1999).
183. Kent S, Bluthe RM, Dantzer R, Hardwick AJ, Kelley KW, Rothwell NJ, et al. Different receptor mechanisms mediate the pyrogenic and behavioral effects of interleukin 1. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 9117-9120 (1992).
184. Krueger JM, Toth LA. Cytokines as regulators of sleep. *Annals of the New York Academy of Sciences* **739,** 299-310 (1994).
185. Maier SF, Watkins LR. Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychological review* **105,** 83-107 (1998).
186. O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Science's STKE : signal transduction knowledge environment* **2000,** re1 (2000).
187. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* **117,** 3720-3732 (2011).

188. Krumm B, Xiang Y, Deng J. Structural biology of the IL-1 superfamily: key cytokines in the regulation of immune and inflammatory responses. *Protein science : a publication of the Protein Society* **23,** 526-538 (2014).
189. Weber A, Wasiliew P, Kracht M. Interleukin-1 (IL-1) pathway. *Science signaling* **3,** cm1 (2010).
190. Lewis AM, Varghese S, Xu H, Alexander HR. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. *Journal of translational medicine* **4,** 48 (2006).
191. von der Thusen JH, Kuiper J, van Berkel TJ, Biessen EA. Interleukins in atherosclerosis: molecular pathways and therapeutic potential. *Pharmacological reviews* **55,** 133-166 (2003).
192. Weisman D, Hakimian E, Ho GJ. Interleukins, inflammation, and mechanisms of Alzheimer's disease. *Vitamins and hormones* **74,** 505-530 (2006).
193. Burger D, Dayer JM, Palmer G, Gabay C. Is IL-1 a good therapeutic target in the treatment of arthritis? *Best practice & research Clinical rheumatology* **20,** 879-896 (2006).
194. Oboki K, Ohno T, Kajiwara N, Arae K, Morita H, Ishii A, et al. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 18581-18586 (2010).
195. Peine M, Marek RM, Lohning M. IL-33 in T Cell Differentiation, Function, and Immune Homeostasis. *Trends in immunology* **37,** 321-333 (2016).
196. Hoshino K, Kashiwamura S, Kuribayashi K, Kodama T, Tsujimura T, Nakanishi K, et al. The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. *The Journal of experimental medicine* **190,** 1541-1548 (1999).
197. Townsend MJ, Fallon PG, Matthews DJ, Jolin HE, McKenzie AN. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *The Journal of experimental medicine* **191,** 1069-1076 (2000).
198. Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PloS one* **3,** e3331 (2008).
199. Pichery M, Mirey E, Mercier P, Lefrancais E, Dujardin A, Ortega N, et al. Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: in situ analysis using a novel Il-33-LacZ gene trap reporter strain. *Journal of immunology* **188,** 3488-3495 (2012).
200. Martin NT, Martin MU. Interleukin 33 is a guardian of barriers and a local alarmin. *Nature immunology* **17,** 122-131 (2016).
201. Baekkevold ES, Roussigne M, Yamanaka T, Johansen FE, Jahnsen FL, Amalric F, et al. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *The American journal of pathology* **163,** 69-79 (2003).
202. Onda H, Kasuya H, Takakura K, Hori T, Imaizumi T, Takeuchi T, et al. Identification of genes differentially expressed in canine vasospastic cerebral arteries after subarachnoid hemorrhage. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **19,** 1279-1288 (1999).
203. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23,** 479-490 (2005).
204. Ali S, Mohs A, Thomas M, Klare J, Ross R, Schmitz ML, et al. The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *Journal of immunology* **187,** 1609-1616 (2011).

205. Cayrol C, Girard JP. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Current opinion in immunology* **31,** 31-37 (2014).
206. Miller AM. Role of IL-33 in inflammation and disease. *Journal of inflammation* **8,** 22 (2011).
207. Luthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, et al. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* **31,** 84-98 (2009).
208. Kakkar R, Hei H, Dobner S, Lee RT. Interleukin 33 as a mechanically responsive cytokine secreted by living cells. *The Journal of biological chemistry* **287,** 6941-6948 (2012).
209. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *Journal of immunology* **186,** 4375-4387 (2011).
210. Hazlett LD, McClellan SA, Barrett RP, Huang X, Zhang Y, Wu M, et al. IL-33 shifts macrophage polarization, promoting resistance against Pseudomonas aeruginosa keratitis. *Investigative ophthalmology & visual science* **51,** 1524-1532 (2010).
211. Kearley J, Silver JS, Sanden C, Liu Z, Berlin AA, White N, et al. Cigarette smoke silences innate lymphoid cell function and facilitates an exacerbated type I interleukin-33-dependent response to infection. *Immunity* **42,** 566-579 (2015).
212. Meephansan J, Tsuda H, Komine M, Tominaga S, Ohtsuki M. Regulation of IL-33 expression by IFN-gamma and tumor necrosis factor-alpha in normal human epidermal keratinocytes. *The Journal of investigative dermatology* **132,** 2593-2600 (2012).
213. Lohning M, Stroehmann A, Coyle AJ, Grogan JL, Lin S, Gutierrez-Ramos JC, et al. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 6930-6935 (1998).
214. Lu J, Kang J, Zhang C, Zhang X. The role of IL-33/ST2L signals in the immune cells. *Immunology letters* **164,** 11-17 (2015).
215. Palmer G, Talabot-Ayer D, Lamacchia C, Toy D, Seemayer CA, Viatte S, et al. Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis. *Arthritis and rheumatism* **60,** 738-749 (2009).
216. Palmer G, Gabay C. Interleukin-33 biology with potential insights into human diseases. *Nature reviews Rheumatology* **7,** 321-329 (2011).
217. Jovanovic IP, Pejnovic NN, Radosavljevic GD, Arsenijevic NN, Lukic ML. IL-33/ST2 axis in innate and acquired immunity to tumors. *Oncoimmunology* **1,** 229-231 (2012).
218. Tong X, Barbour M, Hou K, Gao C, Cao S, Zheng J, et al. Interleukin-33 predicts poor prognosis and promotes ovarian cancer cell growth and metastasis through regulating ERK and JNK signaling pathways. *Molecular oncology* **10,** 113-125 (2016).
219. Yu XX, Hu Z, Shen X, Dong LY, Zhou WZ, Hu WH. IL-33 Promotes Gastric Cancer Cell Invasion and Migration Via ST2-ERK1/2 Pathway. *Digestive diseases and sciences* **60,** 1265-1272 (2015).
220. Liu X, Zhu L, Lu X, Bian H, Wu X, Yang W, et al. IL-33/ST2 pathway contributes to metastasis of human colorectal cancer. *Biochemical and biophysical research communications* **453,** 486-492 (2014).
221. Zhang Y, Davis C, Shah S, Hughes D, Ryan JC, Altomare D, et al. IL-33 promotes growth and liver metastasis of colorectal cancer in mice by remodeling the tumor microenvironment and inducing angiogenesis. *Molecular carcinogenesis* (2016).
222. Gao X, Wang X, Yang Q, Zhao X, Wen W, Li G, et al. Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells. *Journal of immunology* **194,** 438-445 (2015).

223. O'Donnell C, Mahmoud A, Keane J, Murphy C, White D, Carey S, et al. An antitumorigenic role for the IL-33 receptor, ST2L, in colon cancer. *British journal of cancer* **114,** 37-43 (2016).
224. Zhang J, Wang P, Ji W, Ding Y, Lu X. Overexpression of interleukin-33 is associated with poor prognosis of patients with glioma. *The International journal of neuroscience* 1-8 (2016).
225. Ishikawa K, Yagi-Nakanishi S, Nakanishi Y, Kondo S, Tsuji A, Endo K, et al. Expression of interleukin-33 is correlated with poor prognosis of patients with squamous cell carcinoma of the tongue. *Auris, nasus, larynx* **41,** 552-557 (2014).
226. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. *Nature reviews Cancer* **4,** 839-849 (2004).
227. Chen F, Zhuang X, Lin L, Yu P, Wang Y, Shi Y, et al. New horizons in tumor microenvironment biology: challenges and opportunities. *BMC medicine* **13,** 45 (2015).
228. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *The American journal of pathology* **153,** 865-873 (1998).
229. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147,** 275-292 (2011).
230. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nature reviews Cancer* **4,** 448-456 (2004).
231. Hedley BD, Chambers AF. Tumor dormancy and metastasis. *Advances in cancer research* **102,** 67-101 (2009).
232. Mbeunkui F, Johann DJ, Jr. Cancer and the tumor microenvironment: a review of an essential relationship. *Cancer chemotherapy and pharmacology* **63,** 571-582 (2009).
233. Yamamoto Y, Ibusuki M, Okumura Y, Kawasoe T, Kai K, Iyama K, et al. Hypoxia-inducible factor 1alpha is closely linked to an aggressive phenotype in breast cancer. *Breast cancer research and treatment* **110,** 465-475 (2008).
234. Tsai YP, Wu KJ. Hypoxia-regulated target genes implicated in tumor metastasis. *Journal of biomedical science* **19,** 102 (2012).
235. Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. *Nature reviews Immunology* **9,** 609-617 (2009).
236. Egners A, Erdem M, Cramer T. The Response of Macrophages and Neutrophils to Hypoxia in the Context of Cancer and Other Inflammatory Diseases. *Mediators of inflammation* **2016,** 2053646 (2016).
237. Piccard H, Muschel RJ, Opdenakker G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. *Critical reviews in oncology/hematology* **82,** 296-309 (2012).
238. Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, et al. Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **8,** 1831-1837 (2002).
239. Baba Y, Nosho K, Shima K, Irahara N, Chan AT, Meyerhardt JA, et al. HIF1A overexpression is associated with poor prognosis in a cohort of 731 colorectal cancers. *The American journal of pathology* **176,** 2292-2301 (2010).
240. Szendroi A, Szasz AM, Kardos M, Tokes AM, Idan R, Szucs M, et al. Opposite prognostic roles of HIF1alpha and HIF2alpha expressions in bone metastatic clear cell renal cell cancer. *Oncotarget* (2016).
241. McCaffrey LM, Macara IG. Epithelial organization, cell polarity and tumorigenesis. *Trends in cell biology* **21,** 727-735 (2011).

242. Rodriguez-Boulan E, Macara IG. Organization and execution of the epithelial polarity programme. *Nature reviews Molecular cell biology* **15,** 225-242 (2014).
243. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation* **119,** 1420-1428 (2009).
244. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. *Cell research* **19,** 156-172 (2009).
245. Miyazono K. Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer. *Proceedings of the Japan Academy Series B, Physical and biological sciences* **85,** 314-323 (2009).
246. Fuxe J, Karlsson MC. TGF-beta-induced epithelial-mesenchymal transition: a link between cancer and inflammation. *Seminars in cancer biology* **22,** 455-461 (2012).
247. Reymond N, Im JH, Garg R, Vega FM, Borda d'Agua B, Riou P, et al. Cdc42 promotes transendothelial migration of cancer cells through beta1 integrin. *The Journal of cell biology* **199,** 653-668 (2012).
248. Juncker-Jensen A, Deryugina EI, Rimann I, Zajac E, Kupriyanova TA, Engelholm LH, et al. Tumor MMP-1 activates endothelial PAR1 to facilitate vascular intravasation and metastatic dissemination. *Cancer research* **73,** 4196-4211 (2013).
249. Williams CB, Yeh ES, Soloff AC. Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy. *NPJ breast cancer* **2,** (2016).
250. Shi M, Cao M, Song J, Liu Q, Li H, Meng F, et al. PinX1 inhibits the invasion and metastasis of human breast cancer via suppressing NF-kappaB/MMP-9 signaling pathway. *Molecular cancer* **14,** 66 (2015).
251. Kim J, Yu W, Kovalski K, Ossowski L. Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell* **94,** 353-362 (1998).
252. Strell C, Entschladen F. Extravasation of leukocytes in comparison to tumor cells. *Cell communication and signaling : CCS* **6,** 10 (2008).
253. Ganguly KK, Pal S, Moulik S, Chatterjee A. Integrins and metastasis. *Cell adhesion & migration* **7,** 251-261 (2013).
254. Heyder C, Gloria-Maercker E, Hatzmann W, Niggemann B, Zanker KS, Dittmar T. Role of the beta1-integrin subunit in the adhesion, extravasation and migration of T24 human bladder carcinoma cells. *Clinical & experimental metastasis* **22,** 99-106 (2005).
255. Oshita F, Kameda Y, Ikehara M, Tanaka G, Yamada K, Nomura I, et al. Increased expression of integrin beta1 is a poor prognostic factor in small-cell lung cancer. *Anticancer research* **22,** 1065-1070 (2002).
256. dos Santos PB, Zanetti JS, Ribeiro-Silva A, Beltrao EI. Beta 1 integrin predicts survival in breast cancer: a clinicopathological and immunohistochemical study. *Diagnostic pathology* **7,** 104 (2012).
257. Vihinen P, Nikkola J, Vlaykova T, Hahka-Kemppinen M, Talve L, Heino J, et al. Prognostic value of beta1 integrin expression in metastatic melanoma. *Melanoma research* **10,** 243-251 (2000).
258. Strilic B, Yang L, Albarran-Juarez J, Wachsmuth L, Han K, Muller UC, et al. Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. *Nature* **536,** 215-218 (2016).
259. Wan L, Pantel K, Kang Y. Tumor metastasis: moving new biological insights into the clinic. *Nature medicine* **19,** 1450-1464 (2013).
260. Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, et al. TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **133,** 66-77 (2008).
261. Li H, Ge C, Zhao F, Yan M, Hu C, Jia D, et al. Hypoxia-inducible factor 1 alpha-activated angiopoietin-like protein 4 contributes to tumor metastasis via vascular cell

adhesion molecule-1/integrin beta1 signaling in human hepatocellular carcinoma. *Hepatology* **54,** 910-919 (2011).
262. Muller WA. How endothelial cells regulate transmigration of leukocytes in the inflammatory response. *The American journal of pathology* **184,** 886-896 (2014).
263. Franses JW, Baker AB, Chitalia VC, Edelman ER. Stromal endothelial cells directly influence cancer progression. *Science translational medicine* **3,** 66ra65 (2011).
264. Huang L, Perrault C, Coelho-Martins J, Hu C, Dulong C, Varna M, et al. Induction of acquired drug resistance in endothelial cells and its involvement in anticancer therapy. *Journal of hematology & oncology* **6,** 49 (2013).
265. Jain RK. Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. *Cancer cell* **26,** 605-622 (2014).
266. Schlaeppi JM, Wood JM. Targeting vascular endothelial growth factor (VEGF) for anti-tumor therapy, by anti-VEGF neutralizing monoclonal antibodies or by VEGF receptor tyrosine-kinase inhibitors. *Cancer metastasis reviews* **18,** 473-481 (1999).
267. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature reviews Drug discovery* **3,** 391-400 (2004).
268. Xian X, Hakansson J, Stahlberg A, Lindblom P, Betsholtz C, Gerhardt H, et al. Pericytes limit tumor cell metastasis. *The Journal of clinical investigation* **116,** 642-651 (2006).
269. Cooke VG, LeBleu VS, Keskin D, Khan Z, O'Connell JT, Teng Y, et al. Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer cell* **21,** 66-81 (2012).
270. Smith MJ, Berger RW, Minhas K, Moorehead RA, Coomber BL. Heterogeneity of vascular and progenitor cell compartments in tumours from MMTV-PyVmT transgenic mice during mammary cancer progression. *International journal of experimental pathology* **92,** 106-116 (2011).
271. Hosaka K, Yang Y, Seki T, Fischer C, Dubey O, Fredlund E, et al. Pericyte-fibroblast transition promotes tumor growth and metastasis. *Proceedings of the National Academy of Sciences of the United States of America* **113,** E5618-5627 (2016).
272. Yang Y, Andersson P, Hosaka K, Zhang Y, Cao R, Iwamoto H, et al. The PDGF-BB-SOX7 axis-modulated IL-33 in pericytes and stromal cells promotes metastasis through tumour-associated macrophages. *Nature communications* **7,** 11385 (2016).
273. Blay JY, Shen L, Kang YK, Rutkowski P, Qin S, Nosov D, et al. Nilotinib versus imatinib as first-line therapy for patients with unresectable or metastatic gastrointestinal stromal tumours (ENESTg1): a randomised phase 3 trial. *The Lancet Oncology* **16,** 550-560 (2015).
274. Ruan J, Luo M, Wang C, Fan L, Yang SN, Cardenas M, et al. Imatinib disrupts lymphoma angiogenesis by targeting vascular pericytes. *Blood* **121,** 5192-5202 (2013).
275. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *The Journal of clinical investigation* **111,** 1287-1295 (2003).
276. Shen J, Vil MD, Zhang H, Tonra JR, Rong LL, Damoci C, et al. An antibody directed against PDGF receptor beta enhances the antitumor and the anti-angiogenic activities of an anti-VEGF receptor 2 antibody. *Biochemical and biophysical research communications* **357,** 1142-1147 (2007).
277. Flaherty KT, Hamilton BK, Rosen MA, Amaravadi RK, Schuchter LM, Gallagher M, et al. Phase I/II Trial of Imatinib and Bevacizumab in Patients With Advanced Melanoma and Other Advanced Cancers. *The oncologist* **20,** 952-959 (2015).
278. Hosaka K, Yang Y, Seki T, Nakamura M, Andersson P, Rouhi P, et al. Tumour PDGF-BB expression levels determine dual effects of anti-PDGF drugs on vascular remodelling and metastasis. *Nature communications* **4,** 2129 (2013).

279. Keskin D, Kim J, Cooke VG, Wu CC, Sugimoto H, Gu C, et al. Targeting vascular pericytes in hypoxic tumors increases lung metastasis via angiopoietin-2. *Cell reports* **10,** 1066-1081 (2015).
280. Hu B, Cheng SY. Angiopoietin-2: development of inhibitors for cancer therapy. *Current oncology reports* **11,** 111-116 (2009).
281. Monk BJ, Poveda A, Vergote I, Raspagliesi F, Fujiwara K, Bae DS, et al. Anti-angiopoietin therapy with trebananib for recurrent ovarian cancer (TRINOVA-1): a randomised, multicentre, double-blind, placebo-controlled phase 3 trial. *The Lancet Oncology* **15,** 799-808 (2014).
282. Papadopoulos KP, Kelley RK, Tolcher AW, Razak AR, Van Loon K, Patnaik A, et al. A Phase I First-in-Human Study of Nesvacumab (REGN910), a Fully Human Anti-Angiopoietin-2 (Ang2) Monoclonal Antibody, in Patients with Advanced Solid Tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* **22,** 1348-1355 (2016).
283. Vinay DS, Ryan EP, Pawelec G, Talib WH, Stagg J, Elkord E, et al. Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Seminars in cancer biology* **35 Suppl,** S185-198 (2015).
284. Vicente-Manzanares M, Sanchez-Madrid F. Cell polarization: a comparative cell biology and immunological view. *Developmental immunology* **7,** 51-65 (2000).
285. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature reviews Immunology* **14,** 392-404 (2014).
286. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Frontiers in immunology* **5,** 491 (2014).
287. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation* **122,** 787-795 (2012).
288. Yuan A, Hsiao YJ, Chen HY, Chen HW, Ho CC, Chen YY, et al. Opposite Effects of M1 and M2 Macrophage Subtypes on Lung Cancer Progression. *Scientific reports* **5,** 14273 (2015).
289. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer metastasis reviews* **25,** 315-322 (2006).
290. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, et al. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer research* **65,** 5278-5283 (2005).
291. Wang J, Cao Z, Zhang XM, Nakamura M, Sun M, Hartman J, et al. Novel mechanism of macrophage-mediated metastasis revealed in a zebrafish model of tumor development. *Cancer research* **75,** 306-315 (2015).
292. Coniglio SJ, Eugenin E, Dobrenis K, Stanley ER, West BL, Symons MH, et al. Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. *Molecular medicine* **18,** 519-527 (2012).
293. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124,** 263-266 (2006).
294. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer research* **74,** 5057-5069 (2014).
295. Ries CH, Cannarile MA, Hoves S, Benz J, Wartha K, Runza V, et al. Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy. *Cancer cell* **25,** 846-859 (2014).
296. Swierczak A, Cook AD, Lenzo JC, Restall CM, Doherty JP, Anderson RL, et al. The promotion of breast cancer metastasis caused by inhibition of CSF-1R/CSF-1 signaling

is blocked by targeting the G-CSF receptor. *Cancer immunology research* **2,** 765-776 (2014).
297. Buhtoiarov IN, Lum H, Berke G, Paulnock DM, Sondel PM, Rakhmilevich AL. CD40 ligation activates murine macrophages via an IFN-gamma-dependent mechanism resulting in tumor cell destruction in vitro. *Journal of immunology* **174,** 6013-6022 (2005).
298. Nowak AK, Cook AM, McDonnell AM, Millward MJ, Creaney J, Francis RJ, et al. A phase 1b clinical trial of the CD40-activating antibody CP-870,893 in combination with cisplatin and pemetrexed in malignant pleural mesothelioma. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **26,** 2483-2490 (2015).
299. Vonderheide RH, Flaherty KT, Khalil M, Stumacher MS, Bajor DL, Hutnick NA, et al. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **25,** 876-883 (2007).
300. Gladue RP, Paradis T, Cole SH, Donovan C, Nelson R, Alpert R, et al. The CD40 agonist antibody CP-870,893 enhances dendritic cell and B-cell activity and promotes anti-tumor efficacy in SCID-hu mice. *Cancer immunology, immunotherapy : CII* **60,** 1009-1017 (2011).
301. Ajani JA, Izzo JG, Lee JS. Chemotherapy and radiotherapy resistance: complexity, reality, and promise. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **27,** 162-163; author reply 163 (2009).
302. Willers H, Azzoli CG, Santivasi WL, Xia F. Basic mechanisms of therapeutic resistance to radiation and chemotherapy in lung cancer. *Cancer journal* **19,** 200-207 (2013).
303. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nature reviews Cancer* **13,** 714-726 (2013).
304. Plosker GL, Figgitt DP. Rituximab: a review of its use in non-Hodgkin's lymphoma and chronic lymphocytic leukaemia. *Drugs* **63,** 803-843 (2003).
305. Morrow T, Felcone LH. Defining the difference: What Makes Biologics Unique. *Biotechnology healthcare* **1,** 24-29 (2004).
306. Barker HE, Paget JT, Khan AA, Harrington KJ. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nature reviews Cancer* **15,** 409-425 (2015).
307. Albini A, Sporn MB. The tumour microenvironment as a target for chemoprevention. *Nature reviews Cancer* **7,** 139-147 (2007).
308. Sounni NE, Noel A. Targeting the tumor microenvironment for cancer therapy. *Clinical chemistry* **59,** 85-93 (2013).
309. Dey N, De P, Brian LJ. Evading anti-angiogenic therapy: resistance to anti-angiogenic therapy in solid tumors. *American journal of translational research* **7,** 1675-1698 (2015).
310. Bennewith KL, Dedhar S. Targeting hypoxic tumour cells to overcome metastasis. *BMC cancer* **11,** 504 (2011).
311. Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. *Nature reviews Cancer* **4,** 437-447 (2004).
312. Semenza GL. Targeting HIF-1 for cancer therapy. *Nature reviews Cancer* **3,** 721-732 (2003)
313. Wang J, Coltrera MD, Gown AM. Cell proliferation in human soft tissue tumors correlates with platelet-derived growth factor B chain expression: an immunohistochemical and in situ hybridization study. *Cancer research* **54,** 560-564 (1994).

314. Farnsworth RH, Lackmann M, Achen MG, Stacker SA. Vascular remodeling in cancer. *Oncogene* **33,** 3496-3505 (2014).
315. Cao R, Eriksson A, Kubo H, Alitalo K, Cao Y, Thyberg J. Comparative evaluation of FGF-2-, VEGF-A-, and VEGF-C-induced angiogenesis, lymphangiogenesis, vascular fenestrations, and permeability. *Circulation research* **94,** 664-670 (2004).
316. Rahman M, Mohammed S. Breast cancer metastasis and the lymphatic system. *Oncology letters* **10,** 1233-1239 (2015).