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THE IMPACT OF VASCULAR STRUCTURE AND FUNCTION ON TUMOR GROWTH, RETINOPATHY, AND ANTIANGIOGENIC THERAPY

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Med kärlek till mor, far och bror

ABSTRACT

Tumors produce multiple angiogenic factors to stimulate neovascularization that is essential for tumor growth, invasion and metastasis. Despite the known angiogenic functions of each individual factor, the interplay between these angiogenic factors in the tumor local environment and in modulation of the host functions remain poorly understood. This thesis addresses these important and complex issues using various in vitro and in vivo experimental models. The findings from these studies provide mechanistic insights on the of complex interplay between various angiogenic factors and on the systemic impact of these tumor-derived factors on the host. In paper I, we showed one of the first examples of two unrelated angiogenic factors, i.e., FGF-2 and PDGF-BB that reciprocally modulate tumor angiogenesis and metastasis. We found that FGF-2 markedly upregulates the expression levels of PDGFR α and β in endothelial cells, leading to the hypersensitive response of these cells toward PDGF-BB stimulation. Reciprocally, PDGF-BB is also able to upregulate the expression level of FGFR-1 in perivascular cells. The angiogenic synergism and vascular remodeling by these two factors markedly accelerates tumor growth rates, leading to an invasive phenotype in a mouse xenograft tumor model. In paper II, we describe a negative interplay between two angiogenic factors that significantly modulate the number and structure of tumor blood vessels. PlGF could negatively modulate VEGF functions by the formation of heterodimers during their synthesis in the same cell. We show that the PlGF-VEGF heterodimers are less potent than VEGF homodimers and thus significantly reduce the angiogenic activity of VEGF. Surprisingly, we found that modulation of the VEGF-induced angiogenic activity by PlGF markedly normalize tumor vessels. PlGF-induced vascular normalization may imply increased therapeutic efficacy of antiangiogenic drugs. In paper III, we showed that tumor-derived VEGF and PlGF can enter into the circulation and systemically impair vascular functions of other tissue and organs. In this paper, we proposed a new mechanism of cancer-associated retinopathy caused by tumor-derived angiogenic factors. Unlike vasculatures distributed in other tissues, the retinal vasculature predominantly expresses VEGFR1, but lacks a detectable level of VEGFR2. Tumor-derived VEGF and PlGF significantly ablate vascular pericytes, leading to increased leakiness of the retinal vasculature. These findings suggest that targeting tumor-derived VEGF, PlGF or other related angiogenic factors might provide a new therapeutic strategy for the treatment of cancer-associated retinopathy, which otherwise remains untreatable. In paper IV, we discovered a new mechanism underlying the combination therapy between antiangiogenic drugs and chemotherapeutics. Based on preclinical findings, we propose that decreased chemotoxicity by antiangiogenic drugs might significantly contribute to survival advantages of patient receiving a combination of these two classes of drugs. We have also rationalized the optimal delivery of these two drugs by using a sequential procedure rather than a simultaneous schedule as currently recommended in clinical settings. Taken together, this thesis work reveals several novel mechanisms of tumor angiogenesis and provides novel information of antiangiogenic therapy, which can be further optimized based on the work reported in this thesis. This thesis work represents translational and mechanistic studies which can lead to clinically meaningful implications and our eventual goal is to improve survival and the quality of life for millions of cancer patients.

LIST OF PUBLICATIONS

- I. Nissen LJ, Cao R, **Hedlund EM**, Wang Z, Zhao X, Wetterskog D, Funa K, Bråkenhielm E, Cao Y.
Angiogenic factors FGF2 and PDGF-BB synergistically promote murine tumor neovascularization and metastasis.
J Clin Invest (2007) 117:2766-77
- II. **Hedlund EM**, Hosaka K, Zhong Z, Cao R, Cao Y.
Malignant cell-derived PlGF promotes normalization and remodeling of the tumor vasculature.
Proc Natl Acad Sci U S A (2009) 106:17505-10
- III. Cao R, Xue Y, **Hedlund EM**, Zhong Z, Tritsarlis K, Tondelli B, Lucchini F, Zhu Z, Dissing S, Cao Y.
VEGFR1-mediated pericyte ablation links VEGF and PlGF to cancer-associated retinopathy.
Proc Natl Acad Sci U S A (2010) 107:856-61
- IV. Zhang D, **Hedlund EM**, Lim S, Chen F, Zhang Y, Sun B, Cao Y.
Antiangiogenic agents significantly improve survivals of tumor-bearing mice by increasing tolerance to chemotherapy-induced toxicity.
Proc Natl Acad Sci U S A (2011) 108:4117-22

RELATED PUBLICATIONS

- V. Xue Y, Cao R, Nilsson D, Chen S, Westergren R, **Hedlund EM**, Martijn C, Rondahl L, Krauli P, Walum E, Enerbäck S, Cao Y. FOXC2 controls Ang-2 expression and modulates angiogenesis, vascular patterning, remodeling, and functions in adipose tissue. *Proc Natl Acad Sci U S A* (2008) 105:10167-72.
- VI. Cao Y, Cao R, **Hedlund EM**. Regulation of tumor angiogenesis and metastasis by FGF and PDGF signaling pathways. *J Mol Med* (2008) 86(7):785-9. Review.
- VII. Dahl Ejby Jensen L, Cao R, **Hedlund EM**, Söll I, Lundberg JO, Hauptmann G, Steffensen JF, Cao Y. Nitric oxide permits hypoxia-induced lymphatic perfusion by controlling arterial-lymphatic conduits in zebrafish and glass catfish. *Proc Natl Acad Sci U S A* (2009) 106:18408-13.
- VIII. Rouhi P, Lee SL, Cao Z, **Hedlund EM**, Jensen LD, Cao Y. Pathological angiogenesis facilitates tumor cell dissemination and metastasis. *Cell Cycle* (2010) 9:913-7. Review.
- IX. Cao R, Lim S, Ji H, Zhang Y, Yang Y, Honek J, **Hedlund EM**, Cao Y. Mouse corneal lymphangiogenesis model. *Nat Protoc* (2011) 6:817-26.
- X. Wang Z*, Xue Y*, Jensen LD, Lim Sharon, Ye X, **Hedlund EM**, Wu Y, Zhu Z, Cao R and Cao Y. PDGF-B modulates hematopoiesis and tumor angiogenesis by switching on hypoxia-independent erythropoietin production in stromal cells. Submitted manuscript, in revision (*Co-first author)
- XI. **Hedlund EM**, Yang X, Yang Y, Hosaka H, Cao Y. Tumor-derived PlGF potentiates therapeutic efficiency of antiangiogenic drugs targeting the VEGFR-2 pathway. Manuscript

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LIST OF ABBREVIATIONS

BM	Bone marrow
CAR	Cancer-associated retinopathy
CML	Chronic myelogenous leukemia
CSF-1R	Colony-stimulating factor-1 receptor
CTX	Cyclophosphamide
Dll4	Delta like ligand 4
DR	Diabetic retinopathy
EC	Endothelial cells
EMT	Epithelial-mesenchymal transition
ECM	Extracellular matrix
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor Receptor
FDA	Food and Drug Administration
G-CSF	Granulocyte colony-stimulating factor
HGB	Hemoglobin
HSPG	Heparan sulfate proteoglycans
HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor-1
IP3	Inositol trisphosphate
IFN	Interferon
IFP	Interstitial fluid pressure
K14	Keratin-14
KO	Knockout
LLC	Lewis Lung Carcinoma
LRD	Lysine-fixable rhodamine labeled dextran
MMP	Metalloproteinase inhibitors
NRP	Neuropilin
OV	Orf virus
PLC γ	Phospholipase C γ
PI3K	Phosphoinositide-3 kinase
PEDF	Pigment epithelium-derived factor growth factor
PIGF	Placental growth factor
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PKC	Protein kinase C
RTK	Receptor tyrosine kinase
RBC	Red Blood Cell
ROP	Retinopathy of prematurity
SMA	Smooth muscle actin
SMC	Smooth muscle cells
sVEGFR	Soluble Vascular Endothelial Growth Factor Receptor
TGF	Transforming growth factor
Tg	Transgenic
TNF	Tumor necrosis factor
TKIs	Tyrosine kinase inhibitors
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VPF	Vascular permeability factor
WBC	White Blood Cells

1 INTRODUCTION

1.1 ANGIOGENESIS, A HISTORICAL VIEW

In the early 1970s Dr Judah Folkman (Harvard Medical School, USA) proposed that a solid tumor of 2-3 mm in size is in need of neovascularization, to transform from a dormant to growing tumor [1]. Today it is known that maximum size for dormant tumors is even decreased to 1 mm. This process of neovascularization, or rather angiogenesis, was not new knowledge, but it was not until now that the importance of tumor angiogenesis was highlighted. Already in the late 17th century a Scottish surgeon, Dr John Hunter, and scientists studied the anatomy of the vasculature and revealed voluminous several facts, including that the maternal and fetal blood is separated, the anatomy of the lymphatic system which was a puzzle at the time, and he described for the first time angiogenesis, revealed after reindeers were exposed to cold [2]. Although it was not until 1935 the term angiogenesis was used in the description of neovascularization in the placenta by Dr Arthur T Hertig [3]. Angiogenesis originates from the Greek word *angêion* meaning vase and *genesis* meaning birth.

1.2 THE PROCESS OF ANGIOGENESIS

Angiogenesis is a process where new vessels sprout from an existing vessel. The stimulated endothelial cells (ECs) produce proteases to degrade the nearby basement membrane. The ECs will then start to proliferate, differentiate and migrate into the tissue, forming a new vessel. Growth factors, produced by the thin layer of ECs, will be released and attract mural cells; smooth muscle cells (SMCs) and pericytes, to line the vessels for stability and support [4].

More specifically, one of the ECs expressing Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) will differentiate into a so-called tip cell with extensive filopodia protrusions in the presence of a chemottractant such as the angiogenic factor; Vascular Endothelial Growth Factor-A (VEGF-A). This molecule can be either expressed by, for example tumor cells, or be upregulated also in non-malignant cells due to a hypoxic environment. The tip cell will be the leading cell of the sprout of the vessel, and the tip cell will migrate in the direction of the attractants. The tip cell will not form a lumen, to shape a new vessel, but this role is rather performed by the cells trailing along, called stalk cells. These stalk ECs, which do not form the filopodias, proliferate and forming a stalk containing a lumen, connected to the pre-existing vessel and thereby extending the blood circulation. The stalk EC phenotype is induced by activation of the Notch1 receptor by Delta like ligand 4 (Dll4) and expressed on neighboring tip cell, which leads to a down regulation of VEGFR2 in the stalk cells [5, 6]. The stalk cells attract mural cells; small capillaries will be covered by pericytes and larger vessels will be covered by SMCs. The pericytes will suppress migration and proliferation of the ECs, and support the stability of the vessels in a VEGF-A-dependent manner [6, 7]. Furthermore, vascular ablation of pericytes will lead to decreased stability and increased vessel permeability or leakage.

Another well characterized angiogenic factor involved in the vascular development is Platelet-Derived Growth Factor-BB (PDGF-BB) and its receptor β (PDGFR β) [8]. PDGF-BB is known to be a SMC migratory factor [9], but the importance of this factor in EC migration is more speculative as most mature ECs in healthy tissues seems not to express the receptor [8].

1.3 VASCULAR PERMEABILITY

Depending on who reads the words vascular permeability, it will have different meanings. For the physiologist the interest of permeability is generally of the passive barrier constituted by the ECs, as a membrane-like filter, where small molecules can pass through freely, e.g. O_2 and CO_2 and where a limited number of plasma proteins pass through large pores. The physiologist often uses calculations of different perfusion parameters to evaluate the perfusion value. The vascular biologist on the other hand, preferably studies the vessel permeability in the term of the properties of the vessels. These properties include the blood flow or the visible extravasation. For example, the mice get an injection of a dye, and the tissue of interest is analyzed; using the dye as a perfusion value.

VEGF-A is the most well known permeability factor, and can cause immediate but limiting hyper-permeability of healthy vessels, and also chronic hyperpermeability in disease, such as rheumatoid arthritis, wound healings, or in tumors [10]. VEGF-A can cause EC fenestrations, pore-like openings, though the Calcium activation [11]. These EC fenestrae allow rapid molecular exchange between the tissue and the blood circulation, and the fenestrations are present of the sinusoidal vessels in lymphoid tissue, endocrine organs, hematopoietic organs, and the placenta villi. Recently it has been shown that in the PDGF-mediated angiogenesis, VEGF-A is causing pericyte loss and thereby immature vessels. VEGF-A activates VEGFR2, which will inhibit PDGFR β by forming a complex with it [12]. For more description of the VEGF-A expression in fenestrated tissue, see the section of VEGF.

Why vascular permeability is so interesting in tumors, is not just leakiness of the vessel. But rather, it is possible for the tumor cells to enter the blood vessel, follow the blood stream and stop by a location, where it possibility starts to proliferate and

cause a new secondary tumor, a metastasis, which has high possibility of being lethal for the patients [13] (figure 1). This has been seen in patients. In a study of 92 surgically removed tumors of colorectal cancer, the higher tumor vessels density and lesser SMCs coverage were strongly correlated with metastasis and poorer prognosis [14].

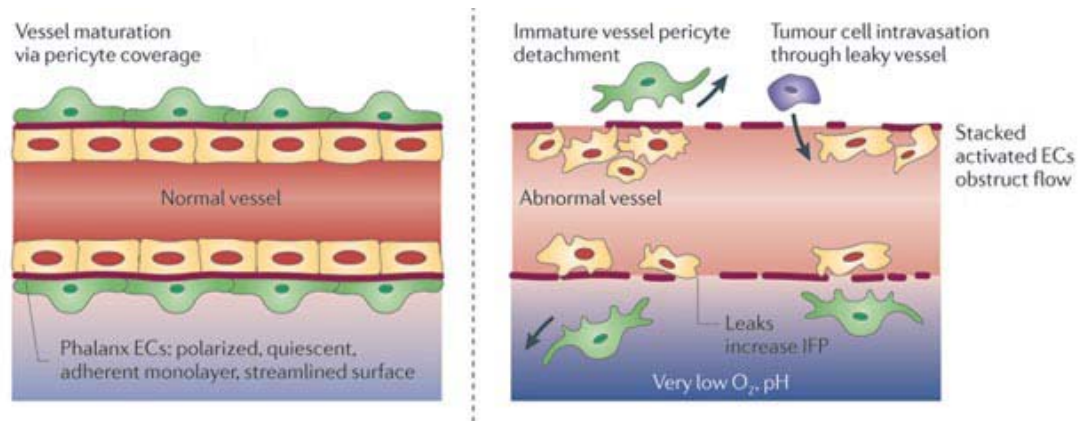


Figure 1. Healthy and tumor vessels (modified from [15]).

1.4 TUMOR ANGIOGENESIS AND METASTASIS

Tumor angiogenesis is referred to when the tumor releases a multitude of growth factors, ECM breaking signals, cytokines, and etc, which stimulate the growth of blood vessels into the tumor. The nearby tissues or more specifically the nearby vascular cells, will respond to some or several of these factors. If the tumor appears in the area of a nearby vessel, this increases the risk of progressive tumor growth due to tumor angiogenesis. Without the blood vessel the solid tumor will most likely stay dormant [1]. The vessels of the healthy tissue stay quiescent due to the dominance of angiogenic inhibitors over the stimulators. In the tumor tissue, this is usually not the case, due to the overproduction of angiogenic factors. The tumor grows due to the increased blood supply, although the tumor vasculature becomes impaired and thereby hypoxic. The hypoxia causes an up-regulation of VEGF-A by the hypoxia

inducible factor-1 (HIF-1) [16]. This leads to further angiogenesis and the VEGF-A induced vessels are severely disorganized, tortured, full of vascular plaques, irregular diameters, and excessive branches. The blood flow is irregular with high interstitial fluid pressure (IFP), the vessels are highly permeable, and are causing hypertension [15]. These vessels generally lack mural cell coverage, which are important for the stability. Through these vessels it is possible for small sub-populations of tumor cells to 'escape'. They can migrate through the blood vasculature and colonize a new tissue, and cause a metastasis. The ECs lining the tumor vasculature have an irregular shape (not seen in healthy tissue), are disorganized, and sometimes the EC lie on top of each other, causing large holes in the vessels [17]. Highly aggressive tumor cells can also form a tubular structure, without the presence of ECs connecting to the blood vessels. This is called vascular-mimicry [18]. Thereby the tumor has found another way to enter the blood system and spread. Another way for the tumor to metastasize is through the so called epithelial-mesenchymal transition (EMT). The tumor cells in the invasive frontline of the progressing and growing tumor convert into a mesenchymal phenotype. This switches the cells to a more migratory phenotype and enables them to enter the blood stream and metastasize in other places. Once settled in a different place they convert back to epithelial state of the primary tumors [19].

1.4.1 Vessel normalization

Cancer treatments can generally be divided into two groups, as discussed in more detail later in the therapy section. First and pre-dominantly are the chemotherapeutic drugs which kill the tumor cells. The second group is targeting the tumor vasculature. Administration of anti-angiogenic drugs will disrupt the tumor vasculature and thereby the nutrient and oxygen supply [15].

Yet, there is an alternative theory. To normalize the tumor vasculature by anti-angiogenic drugs, through inhibition of the pro-angiogenic signals. This causes vessels to become functional. The phenotype of a normalized tumor vasculature consists of straight vessels, of a narrow diameter, which are relatively long, and therefore look similar to those seen in healthy (normal) tissue. As described above, the IFP is high in the tumor and this is preventing the therapeutic drugs to enter the tumor. Thereby the drugs are primarily circulating in the non-tumor vascular system causing side-effects, without causing any damage of the actual tumor. By normalizing the tumor vasculature, the IFP will decrease, allowing efficiently drug delivery to the tumor, thereby maximizing the therapeutic efficiency of these agents the tumor tissue. Another reason to normalize the tumor vasculature would be to prevent or decrease the metastatic feature to the tumor, via the blood circulation [13, 15].

1.4.2 Tumor microenvironment

Already mentioned is the tumor, and in this thesis I will exclusively refer to tumors of the solid kind. In solid tumors the tumor microenvironment consists of the tumor cells, which is a cell that has undergone a genetic or epigenetic change. This change has led to the malfunctioning of the control machinery such that the cell which should undergo apoptosis will not, but rather continue to live and make daughter cells, which in their turn might even develop more modification. These modifications, which are referred to as the *Hallmarks of cancer* includes the cells ability to avoid anti-growth signals and apoptosis, self-sufficient in growth signals, prolonged life by limitless replication, sustained angiogenesis and ability to invade other tissues and metastasize [20]. The tumor contains more than just tumor cells. A great variety of cells, called the stroma; including stromal cells, inflammatory cells, endothelial cells, smooth muscle cells (SMC) and pericytes. The stromal cells comprise fibroblasts and

macrophages, whereas the inflammatory cells include an even greater amount of cellular components, such as macrophages, dendritic cells, natural killer cells, and lymphocytes. [ref]

The tumor microenvironment produces a multitude of the soluble components. These are of course of high importance, since these are the ones which influence the surrounding nearby cells, but also can be transported through the vessels out to the tissues and organs all-around the body. These soluble components, which are produced by both the tumor cells but also the stroma, can in fact cause mild but also severe systemic syndromes. [ref]

What makes up these soluble components? This varies from tumor to tumor, dependent on which original cell caused the tumor, but to generalize the answer; the soluble components are cytokines, chemokines, stromal factors, inflammatory factors, and angiogenic factors.

1.5 ANGIOGENIC FACTORS

The angiogenic factors are molecules with either of pro- or anti-angiogenic properties, see figure 2. In addition to these molecules are other factors, worth mentioning since they influence the tumor vasculature in an un-controlled way during the experimental setting, which include hypoxia and mechanical force such as blood flow. To concentrate on the vasoactive molecules, which is a continuously increases by number, these are usually divided into growth factors and inhibitory factors.

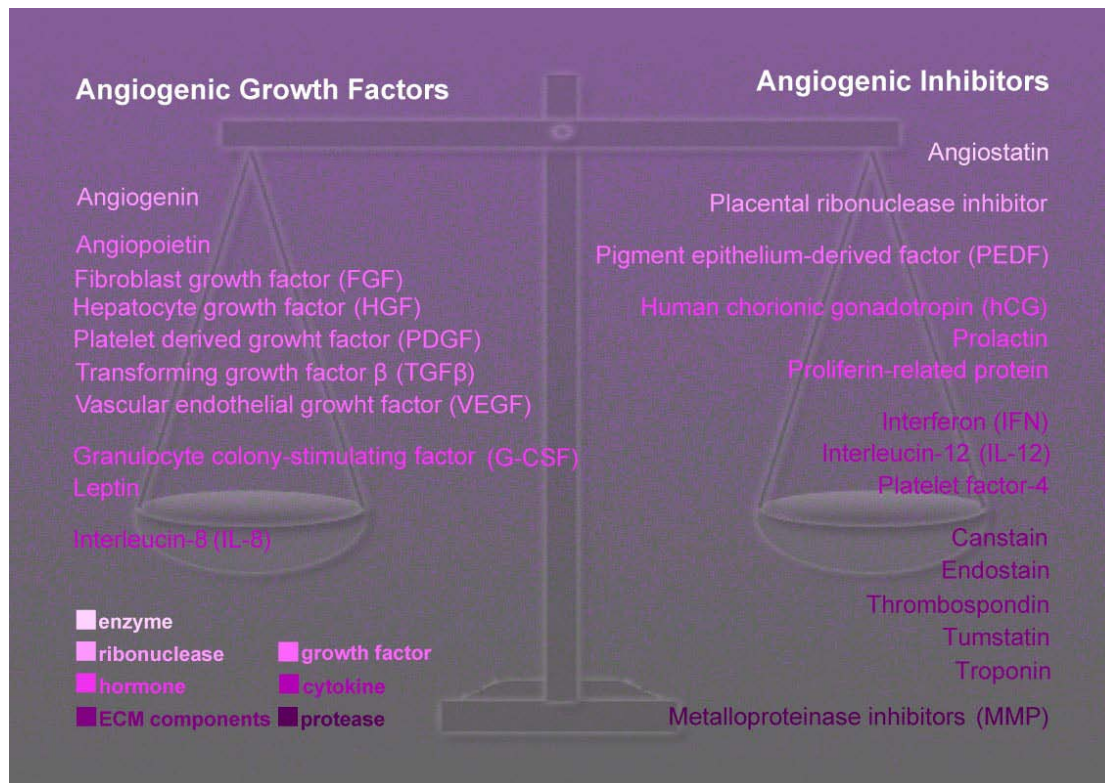


Figure 2. Angiogenic growth factors and inhibitors.

1.5.1 Growth factors

Dependent on the genetic polymorphism, many different growth factors are released by the tumor, such as angiopoietin, tumor necrosis factor (TNF), and transforming growth factor β (TGF β). The most potent and well known angiogenic growth factor produced by most tumors is the vascular endothelial growth factor (VEGF). Further, two other well known and studied, tumor produced angiogenic factors are the fibroblast growth factor (FGF) and platelet derived growth factor (PDGF).

1.5.1.1 Vascular endothelial growth factor (VEGF)

By the year 1989 a protein was found by Dr Napoleon Ferrara and coworkers and it was named VEGF-A [21], although it was later found that it was the same molecule which had been found by Dr Harold Dvorak and coworkers 6 years earlier and at that

time it was the molecule had already been named vascular permeability factor (VPF), due to its ability to permeabilize the tumor vasculature [22]. The molecule is often referred with both names, but more commonly as VEGF-A.

VEGF consist of a whole family of proteins. Reading publications were simply VEGF is written and not VEGF family, the authors most likely refers to VEGF-A. The VEGF superfamily consists of VEGF-A, -B, -C, -D, and PlGF (figure 3). Since PlGF is so interesting and a bit controversial, I will describe it later in a whole section.

VEGF-A is known to induce irregular, highly leaky and torturous vessels. Alternative splicing of the human mRNA gives rise to several different isoforms of 121, 145, 165, 183, 189, and 206 amino acid residues. The mouse isoforms are one amino acid shorter, respectively. VEGF-A₂₀₆ is expressed during embryonic development, whereas the five other isoforms are expressed during adulthood. The smallest isoform VEGF-A₁₂₁ is a circulating protein, due to its absence of the heparin binding domain, which exists in all the other five isoforms, with the larger isoforms having more such domains and therefore an increasing affinity for heparin. When heparin interact with proteoglycans VEGF-A₁₆₅, VEGF-A₁₈₉, and VEGF-A₂₀₆ (although not VEGF-A₁₄₅) will be deposited into the ECM at a decreasing distance from the producing cell. This is causing a chemotactic gradient inducing the ECs to migrate and proliferate towards the VEGF-releasing site. Without the gradient the ECs will not respond in such a highly directional manner. If added alone the 165- isoform causes the most efficient gradient and is thereby the most efficient angiogenic inducer. This particular isoform is most commonly used in research, including this thesis.

Let us focus on VEGF-A, since to our knowledge up to date, VEGF-A is, as mentioned above, the most potent angiogenic factor [23]. What is the actual function in the body? To start with the embryonic development; VEGF-A is vital for developmental angiogenesis, as seen in the knockout (KO) mice which are embryonically lethal [24]. During embryonic development, endothelial progenitor cells arising from the mesoderm differentiates into endothelial cells and produces a vascular network. This process is VEGF-A dependent, the endothelial development, migration and proliferation is mediated by VEGF-A interaction with the VEGF receptor 2 (VEGFR2) [25]; and the tube formation is induced by VEGFR1 upon VEGF-A binding [26].

In addition to embryonic development, VEGF-A is indeed also expressed in almost all adult tissues, primarily by in the pericytes and stromal cells. It seems like VEGF-A have different functions in different tissue, depending on the ratio of expression. In tissues where a barrier is needed, such as the blood barrier in the brain, retina, and the testis, very few cells express VEGF-A, to limit the permeability. In the cardiac and skeletal muscle tissues, an intermediate VEGF-A expressions is needed for the ability of hypertrophy due to muscular exercise [27]. The richest ratio of VEGF-A expression is in fenestrated vessels, particularly in the epithelia, and special organs: In the secreting organs including pancreas, adrenal gland, prostate, and testis. VEGF-A is expressed in the kidneys of the fenestrated glomerulus for urine filtration. The fenestrations of liver have a VEGF-A expression due to both secretion and filtration [28].

Two isoforms of VEGF-B has been identified by Prof Alitalo and coworkers (Finland) [29]; VEGF-B₁₆₇ and VEGF-B₁₈₆. VEGF-B₁₆₇ has a strong affinity for heparin sulfate, which VEGF-B₁₈₆ is lacking. Both isoforms can heterodimerize with

VEGF-A₁₆₅ [30, 31]. VEGF-B is expressed mainly in muscle tissue, but also in embryonic tissues [32].

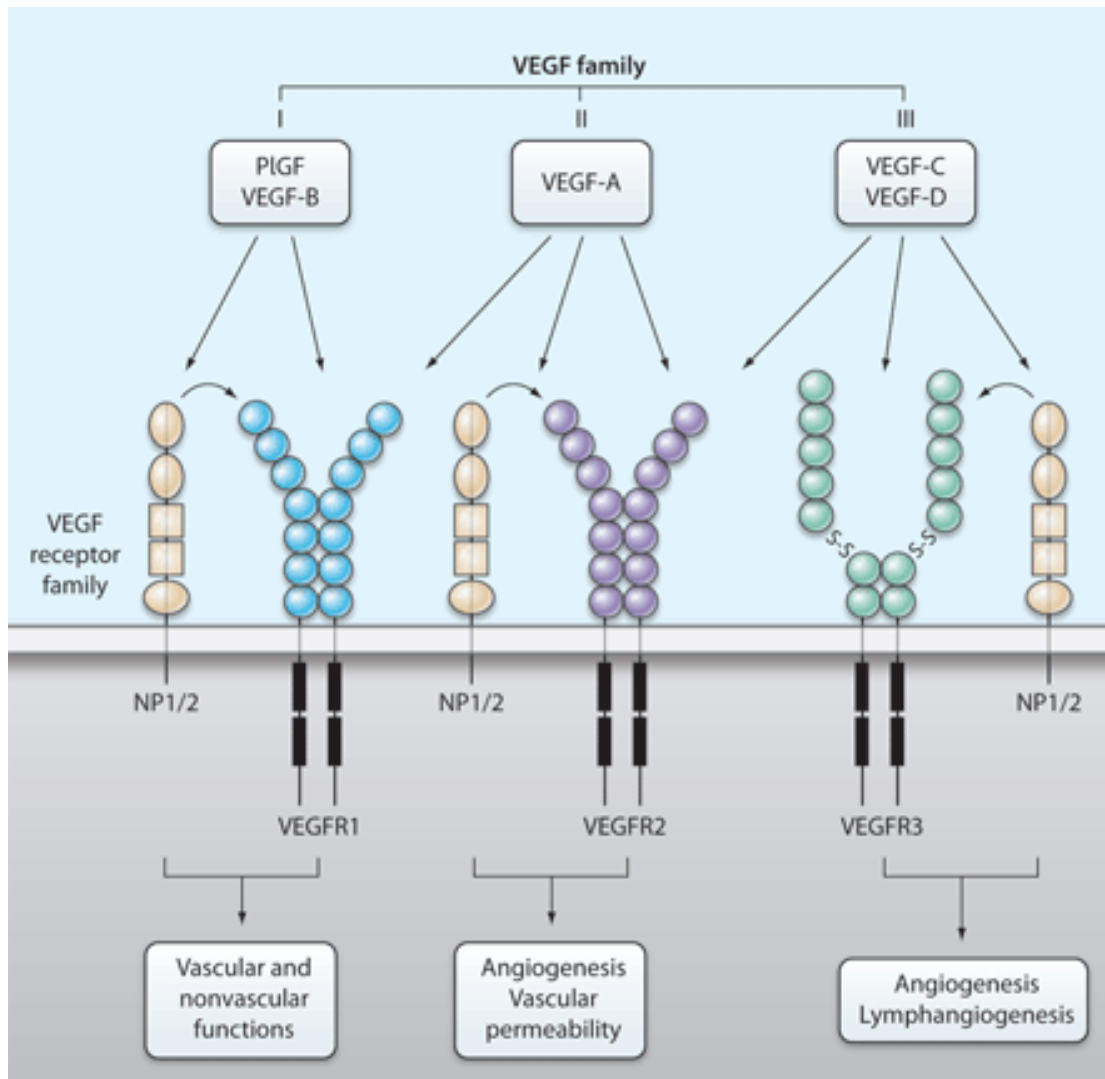


Figure 3. VEGF family [33].

VEGF-C and VEGF-D are lymphangiogenic factors, potentially inducing the growth of lymph vessels [34-36]. In mice where *vegfc* has been replaced by *lacZ* gene; *Vegfc*^{-/-} embryos die during development in utero because of an accumulation of fluid in the tissue, due to the lack of lymphatic sprouts and thereby lymphatic vessels [37].

I would also like to mention VEGF-E. It was found in 1992 by Dr Lyttle et al in an orf virus (OV) which affects sheep, goats and sporadically, humans. This virus causes

a brief angiogenic response; capillary endothelial proliferation, in the skin. It was reported that the sequence has high homology of the human VEGF [38]. The molecule was named VEGF-E, and was shown by Professor Shibuya in 1998 to bind VEGFR2 but not VEGFR1 and furthermore VEGF-E lack the affinity for heparin [39]. Since VEGF-E is not produced in mammals, a transgenic mouse was generated to express VEGF-E, by using a K14 (keratin-14) promoter, it was found that these mice had more reddish skin color and the subcutaneous tissue exhibits altered although still organized angiogenesis. In comparison, in the same transgenic (tg) system, K14:VEGF-A₁₆₅ tg mice had various symptoms like perinatal death and altered vascularization with tissue edema, as a consequence of hyperpermeabilisation and subcutaneous hemorrhage[40]. VEGF-E might therefore be useful for pro-angiogenic therapy instead of VEGF-A, since it might not be related such severe risks of edema [41]. As a curiosity, one more protein with homology to the VEGF-family has been found in snake venom and has got the name VEGF-F [42].

1.5.1.2 Placental growth factor (PlGF)

As the name implies, PlGF was found in the placenta and it was shown to have a high homology to VEGF, and is part of the VEGF family [see below]. The two predominant isoforms of PlGFs are PlGF-1 (PlGF₁₃₁), and PlGF-2 (PlGF₁₅₂), although there are four PlGF isoforms, named PlGF-1 to PlGF-4 [43-47]. The mouse only have one isoform, representing PlGF-2. PlGFs bind VEGFR1, whereas PlGF-2 also binds neuropilin (NrP), due to a 21 amino acid insertion. PlGF-1 and PlGF-3 lack the heparine binding domain and are thereby diffusible, compared to PlGF-2 and -4 which bind heparin [48, 49].

In the early 90s, PlGFs were found to bind to VEGFR1 but not VEGFR2. Compared to VEGF-A, which is a permeability inducer; neither PlGF-1 nor PlGF-2 could induce

significant vascular permeability. Further it was also found that the two PIGF isoforms could enhance the effect of low but not high concentrations of VEGF [50].

Surprisingly PIGF null mice were healthy, fertile, and exhibited only slightly decreased remodeling of retinal vasculature and decreased number of vessels in corpora lutea. Furthermore, PIGF^{-/-} mice had an increased level of VEGF compared to the wt mouse [51] (worth mentioning is that in the publication the heterodimerization between PIGF and VEGF-A in the wt mice was not discussed). In the adult, PIGF is expressed in ECs, but mainly in the placenta although it can also be found up-regulated in malignancies, such as choriocarcinoma.

1.5.1.3 VEGF receptor (VEGFR)

I have now described the growth factors, but how about their corresponding receptors? There are three VEGF receptors. The VEGFR1 is denoted Flt-1, and VEGFR2 is also known as KDR in humans and Flk-1 in mice, whereas VEGFR3 is denoted Flt-4. The VEGFRs are a group of tyrosine kinase receptors, which are activated upon ligand binding, leading to receptor dimerization and activation of intracellular signaling. During the dimerization the intracellular domains gets phosphorylated and thereby trigger the intracellular signal transduction. The ligand VEGF-A binds both to VEGFR1 and 2, whereas PIGF and VEGF-B binds VEGFR1 alone. Further VEGF-C and -D both bind to VEGFR3, which induces lymphangiogenesis [52]. VEGF-D also binds to VEGFR2 (with poor angiogenic activity), although only in humans, not in mice [53].

Receptor 1 and 2 have different expression pattern, have different roles to play during embryogenesis, give rise to different signal transduction responses [54], and also give

rise to different angiogenic responses, although, the function of receptor 1 has not clearly been found out yet. The field contains different and contradictory reports of the functionality of VEGFR1, where it is claimed that the VEGFR1 also induces angiogenic response in the same fashion as the well-established angiogenic inducer VEGFR2 [55]. VEGFR1 has a higher affinity for VEGF-A, compared to VEGFR2 which has low affinity, although tyrosine kinase activity of VEGFR1 is 10-fold weaker than VEGFR2 [56, 57].

All three receptors are expressed on endothelial cells, whereas VEGFR1 is further expressed on monocytes/macrophages, pericytes, SMCs, osteoclasts, dendritic cells, bone marrow progenitor cells, hepatocytes, neural cells, and various tumor cells [33, 56-60]. The function of the receptor in these cells is not yet defined. VEGFR2 is further expressed on hematopoietic stem cells, neuronal cells, retinal progenitor cells, megakaryocytes, osteoblasts, and pancreatic duct cells. Simultaneous expression of VEGFR1 and VEGFR2 is found on cells in human testicular tissue, Leydig cells in the mouse testis, and in the myometrium. VEGFR1 and R2 are expressed on the ECs of the embryo (both human and mouse). In mouse, VEGFR2 is first expressed at day 7.0 on the mesodermal blood island progenitors and then in the angioblasts surrounding the blood islands in the early embryogenesis [4]. At E7.5 the VEGFR2 positive cells can be found in the tail region and from where they migrate toward the head region and yolk sac, where the cells differentiate to primitive endothelial cells [61]. VEGFR1 is expressed first at day 8.5 in the angioblasts. The expression level of the receptors are high, but decreases after day 14.5 to 16.5. The VEGFR2 expression levels continue to decrease and diminish at the end of gastrulation [4] and in the adult mouse the VEGFR2 expression is mostly found in the vascular ECs and hematopoietic cells, which could be the progenitors for ECs [61]. On the contrary,

VEGFR1 expression is increased in the newborn mouse and in the adult mouse the expression level is relatively high [4].

To study the receptor functionality from another point of view, the *VEGFR1 (Flt-1)* null mutant mice die at the embryonic stage E 8.5-9.0, due to an overgrowth of disorganized vessels, proving the important role of VEGFR1 as a negative regulator of vasculogenesis. The *Flt-1 TK* mice, lacking the tyrosine kinase domain of the receptor are healthy with relatively normal blood vessels [62], although these mice have a defective migratory response of macrophages toward VEGF-A. VEGFR2 KO embryos die at E8.5-9.0 due to lack of vasculogenesis and a hampered hematopoietic development [57]. These facts, that the VEGFR1 is important as an embryonic inhibitor of the vasculature and that VEGFR2 is important for the initiation of vasculogenesis, shows that both VEGFR1 and R2 are essential for the development of the vascular blood system.

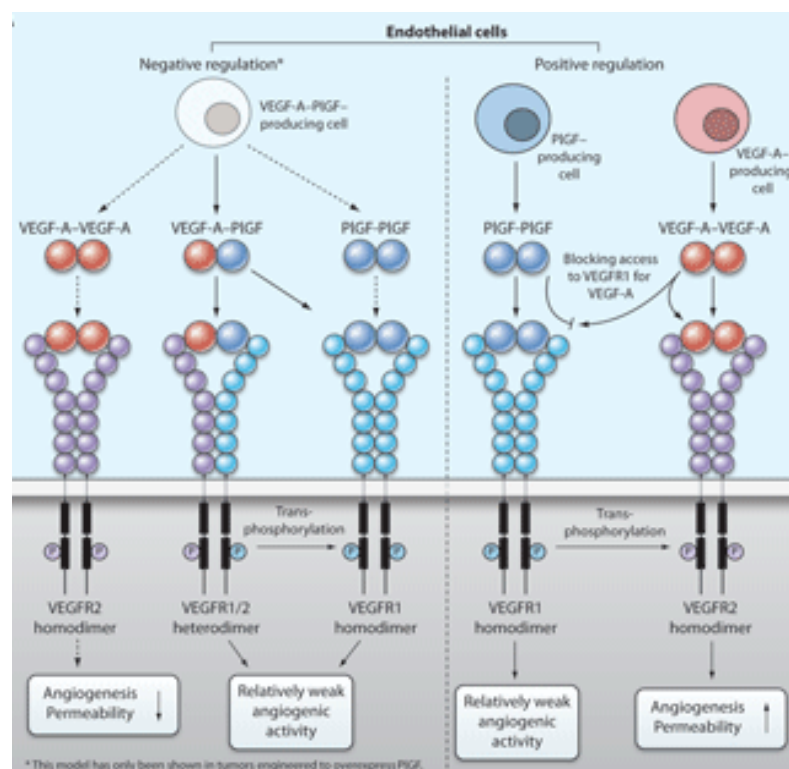


Figure 3. Positive and negative regulation. (Modified from [33])

1.5.1.3.1 Signal transduction pathways

Upon ligand binding the VEGFR2 is activated by phosphorylation, this will lead to an activation of phospholipase C γ (PLC γ), which in its turn leads to inositol trisphosphate (IP3) generation and activation of the protein kinase C (PKC) pathway, as well as calcium mobilization, and PKC will also stimulate the c-Raf-MEK-MAP-kinase cascade, ending up in DNA synthesis. The VEGFR2 tyrosine kinase activation is unusual in the way that the Ras pathway is not truly activated. Further, phosphoinositide-3 kinase (PI3K) pathway is activated through the VEGFR2 activation, leading to cell survival. Interestingly, the Src pathway is also activated upon VEGF-A ligand binding in ECs lacking the SMC coating, which lead to actin cytoskeletal rearrangements and migration. VEGFR1 is not as clearly described as for the previous receptor, when it comes to signal transduction. The PI3K pathway is activated as well as PLC γ [57], and it has been found that IP3 is generated under certain conditions [61]. The reason in why the signal transduction pathways for VEGFR1 have not been clearly defined is due to the weak induction of the VEGFR1 activity *in vitro* and also because different ligands will give rise to different cellular responses. The ligands do not always bind the VEGFRs alone but also bind the co-receptors. These co-receptors can be another reason for the complexity of studying VEGFR1.

1.5.1.4 Soluble VEGFR (*sVEGFR*)

VEGFR1 has a soluble form, called sVEGFR1, which lack the transmembrane and intra-cellular parts of the receptor, which is pivotal for the intracellular signaling. The sVEGFR1 is found in the human EC, SMC, placental trophoblast, corneal epithelial cells, proximal tubular cells of the renal epithelia, and activated peripheral blood monocytes. In the diverse cell types, sVEGFR1 is produced as different splice

variants, suggesting multiple functionality. The different functions of these splice variants remains to be elucidated [63]. Further, this soluble receptor is embryonically conserved in different species [64], which tells us of its essentiality function in basic *in vivo* biology.

The functionality of sVEGFR1 is not defined yet, but it is believed to be involved in at least two mechanisms: 1) to act as a decoy-receptor, by binding the VEGF-A molecule, and thereby prevent it to bind transmembrane receptors and transduce further signals, and 2) to heterodimerize with the transmembrane receptor, and thereby prevent homodimerization and thus any further signal transduction [63].

Not only sVEGFR1 are produced by alternative splicing, but also sVEGFR2 and R3. Recently sVEGFR2 has been shown to be secreted in the plasma of the mouse, but also in the human plasma. By the use of several different antibodies directed against the soluble form of VEGFR2, the receptor could be detected in conditioned media from different human and mouse ECs [65]. sVEGFR2 and R3 have been previously shown to inhibit EC proliferation [66-68]. Although not much is known about these receptors and further investigations need to be done.

1.5.1.5 VEGFR1 in tumor angiogenesis

VEGFR1 can be involved in both positive and negative regulation of tumor induced angiogenesis.

When the tumor cells overexpress VEGF-A, homodimers of the ligand will bind VEGFR2 on the EC and stimulate angiogenesis. Tumor cells which release homodimers of PlGF will cause weak angiogenic stimuli, due to the activation of VEGFR1. In the case when the tumor is releasing PlGF and VEGF-A from different cells, PlGF will homodimerize and bind VEGFR1, and thereby shifting VEGF-A to mainly bind VEGFR2, causing a strong angiogenic response, with vessels of typical

irregular, and torturous structure with a high amount of vascular plexus formation. In tumor cells transformed to overexpress P-IGF it has been shown, that a heterodimerization will occur between PlGF and VEGF-A ligands. The homodimers will bind the corresponding receptor, whereas the heterodimer will bind to a heterodimerization of the receptors, VEGFR1/2. The receptor shift of the ECs will be toward the receptor-heterodimer and less VEGFR2 homodimer is thus activated by VEGF-A homodimer ligands. In this scenario, the angiogenic response is very low, and the vessels have a so called normalized phenotype [33].

1.5.1.6 *Neuropilin (NrP)*

Neuropilins are broadly expressed, transmembrane glycoproteins and function primarily as co-receptors for semaphorins, which are responsible for axon guidance during the development of the nervous system [69]. Further, NP also acts as a co-receptor for VEGFRs. There are two isoforms, NP-1 and NP-2, and both are expressed on EC, but differ in which ligands they bind. Both NPs bind VEGF-A₁₆₅, VEGF-B, VEGF-E, and PlGF-2 [4, 69, 70]. VEGF-A₁₄₅ only binds NP-2 and VEGF-A₁₂₁ does not bind the NPs at all. Transgenic mice overexpressing NP-1 had increased amounts of capillaries, dilated blood vessels, and hemorrhage. NP-1^{-/-} ko mice, die at E12.5-13.5 due to impaired cranial and spinal fibers, neural vascularization, irregular development of branchal arch arteries, large vessels, and disorganized vascular network in the yolk sac. NP-2^{-/-} on the other hand, did not have a normal vasculature. In mice where both NPs are KO (NP-1^{-/-} NP-2^{-/-}) they die in E8.5 with severe defects of the blood vasculature in the yolk sac and in the embryo, with higher severity of vascular malformations compare to the single KO, proving that both NPs are important for the development of the vasculature [69, 70].

1.5.1.7 Fibroblast growth factor (FGF)

Fibroblast growth factor (FGF) family has two pre-dominant isoforms known as acidic FGF and basic FGF, named after the purification extraction [71]. They are presently called FGF-1 and FGF-2, respectively. The human FGF family consists of 23 different isoforms. FGF does not have the cytoplasmic sequences for extracellular transport and the mechanism behind extracellular release of FGFs is still not known [72]. However what is known is that the FGF ligands have a high affinity for heparin sulfate proteoglycans (HSPGs), which binds both FGF and one of the FGF receptors (FGFRs). The extracellular FGFs are deposited in the HSPGs. Alone the HSPG have no biological effect on FGF, although in contact with the FGFR, the FGF ligand is efficiently 'presented' to and bound by the receptor. A complex is formed consisting of HSPGs, ligands and receptors. The ligand-bound receptors will dimerize, leading to autophosphorylation, and this will be followed by a rapid internalization [73]. Activated signal transduction of the receptors includes Ras-Src-PI3K, and the PLC pathways [72]. FGF-2 deficient mice have cerebral cortex impairments, yet the mice are viable. Further, these mice had regular vessels but a decreased blood pressure [74]. By using an adenovirus expression vector encoding of dominant-negative and truncated form of FGFR1 in mice embryos, it was demonstrated that these abnormal embryos had extra vascular development and that FGFR1 is needed for the maintenance of the mature vasculature in the embryo [75].

The receptors are tyrosine kinases and are expressed on most cell types [76], and have a wide range of biological roles, such as angiogenic activity, stimulation of stem cell differentiation, tissue repair, neurotrophic activity, and osteogenesis [77]. FGFRs are often expressed in tumors, and overexpression and expression of mutated forms of FGF is also common in tumors [78]. FGFR activation of ECs leads to angiogenesis.

FGF-2 overexpression in non-small cell lung cancer and bladder carcinoma, have correlated with poor prognosis [79, 80].

1.5.1.8 Platelet derived growth factor (PDGF)

The Platelet derived growth factor (PDGF) family consists of four isoforms, PDGF-A, -B, -C, and -D. The isoforms exist in their active and extracellular form as homodimers or as the heterodimer PDGF-AB. The ligand binds its receptor, the PDGF receptor (PDGFR), which has two isoforms PDGFR α and PDGFR β . PDGF-A, -B, and -C binds to the PDGFR α and PDGFR β are bound by the B- and C-chains it gives rise to different receptor-ligand combinations. The intracellular pathways activated by the two receptors give rise to similar but not identical cellular responses. The activation of PDGFR $\alpha\beta$ heterodimers and homodimers of PDGFR β causes chemotactic responses of SMCs and fibroblasts. On the contrary, inhibition of chemotaxis is caused by homodimers of the PDGFR α receptor [8]. The involved signal transduction pathways are the PI3K and PLC γ which are activated in chemotaxis. The Erk-MAP and Src pathway are induced in cell proliferation. Further, the Ras pathway and STAT family are activated upon ligand-receptor binding. The receptors are also inactivated by peroxiredoxin type II, a cellular peroxidase that eliminates H₂O₂ [81].

It has been demonstrated that the endothelial tip cell expresses high amounts of PDGF-B mRNA, simultaneously with VEGFR2 mRNA [82]. As described above, VEGFR2 of the tip cell is stimulated by the chemoattractant. The role of the expressed PDGF-BB is to recruit the pericytes or SMCs, to stabilize the endothelial stalk cells, which are in close relation to the tip cell. PDGFR β are expressed by mural cells and stromal cell, both in healthy tissue and in tumor. The first time PDGFR β

was described as in colorectal cancer year 1993 [83], and has been seen in most solid tumors. Because, the PDGFRs are so widely expressed in different tumors, they are the focus of extensive research, and further the receptors are also targeted in cancer therapy. The mechanism of mural recruitment/ablation of tumor vessels is not fully understood [8].

The PDGFs are expressed by many epithelial cells in different organs and the receptors in the nearby mesenchymal cells during embryonic development [84]. Even embryonic development it has been shown that PDGF and PDGFRs are involved in vessel maturation and recruitment of pericytes. In the PDGF-B knock out mice (PDGF-B^{-/-}) the vessels lack the pericyte coverages. These vessels were torturous, had micro aneurysms (a balloon like bulge structure filled with blood), and had variable diameter. In comparison, the wt mice had straight vessels of regular diameter. The mice have hemorrhage and edema, and they die during late embryogenesis [85, 86]. Overexpression of PDGFR in ovarian cancer has been associated with a poor outcome [87].

1.6 CANCER-ASSOCIATED RETINOPATHY (CAR)

Retinopathy is a non-inflammatory damage of the retina of the eye. There are several different types of retinopathies, including retinopathy of prematurity (ROP), diabetic retinopathy (DR), and cancer-associated retinopathy (CAR).

ROP can occur in infants born prematurely and it can progress into blindness. ROP will occur in two phases: Phase 1) the retina is only partially vascularized with vessels lacking in the peripheral zone up. When the eye is growing, the retina will not receive the correct amount of nutrient, growth factors, or oxygen; and it will cause a hypoxic environment. Phase 2) the hypoxia will increase VEGF-A release and induce

angiogenesis of low quality vessels [88, 89]. The treatment used of a cute-CAR, is laser therapy for removal of the avascular areas, although less destructive therapies are under evaluation, such as intravitreal anti-VEGF (bevacizumab) therapy, which is already used in adults [89].

DR will affect almost all diabetic patients after ten-years of time. The DR is a long process which starts with a vasoregression initiated by pericyte ablation, and it seems like Ang-2 is involved in this process. The vasoregression causes hypoxia and then retinal angiogenesis occurs to restore the oxygen delivery, cooperated by VEGF-A and Ang-2 [90]. The blood-retinal barrier breaks down and it causes plasma leakage into the central part of the retina, causing swelling of the retina. Further, vascular growth into the vitreous can hemorrhage the vitreous and all these defects can cause vision failure [91].

1.7 THERAPY

1.7.1 General cancer therapy

Cancer therapy can roughly be divided into two groups of local therapies and systemic therapies. The local therapies are further divided into: 1) Surgery is to remove either the tumor or tissues. These tissues are removed in prevention, since they most likely could turn into a tumor. 2) Radiation therapy, which is often used in combination with chemotherapy. Radiation therapy is to kill the cancer cells either before or after the main treatment. As an option, it is also used to decrease the size of a tumor when surgery is not possible, such as in some areas of the brain. It can also be an alternative to decrease pain. 3) Tumor ablation, which is to kill the cancer cells locally, without surgery or radiotherapy. This group includes radiofrequency ablation through electrodes. Also, it includes arterial chemo- or mechanical-embolization,

which refers to closure of a major artery to interrupt the blood supply of the tumor and simultaneous delivery of chemotherapy to the specific area [i].

Systemic therapies include chemo-, targeted-, hormone-, and biological therapy. A) Chemotherapy works by chemical destruction of the DNA or RNA machinery or other structures thereby interrupting the proliferation and/or survival of the cancer cells. B) Targeted therapy acts by targeting specific molecules, existing in both tumor and healthy tissues. The targeted molecules such which are often overexpressed or expresses in a modified form by the tumor. These targeted molecules are often critical for tumor growth and survival. C) Hormone therapy is directed or indirect toward the tumors, which needs hormones to grow, and successfully used in breast and prostate cancer, respectively. D) Biological therapy or biotherapy is an indirect cancer therapy, which works by targeting the immunesystem, which in turn could act on the cancer cells [i].

Also worth mentioning is palliative therapies. Palliative therapy or palliative care is to improve life quality, by removing pain and other discomfort symptoms [i].

1.7.1.1 Chemotherapy:

Chemotherapeutic agents work through the interference with DNA synthesis or function, and can be classified as alkylating agents, antimetabolites, anthracyclines, platinum, or anti-tumor antibiotics or non-DNA interference such as, monoclonal antibodies, or plant alkaloids [ii].

Alkylating chemotherapy, which is the most common strategy used anti-cancer treatment, act by crosslinking of the DNA strands to cause abnormal base pairs, and thereby prevention the cell division process. Alkylating agents are used in a large variety of cancers and are most efficient in slow growing cancer, such as solid tumors

and leukemia. Side-effects of long-time use can be infertility of both sexes and secondary cancers, such as Acute Myeloid Leukemia. Cyclophosphamide (CTX) is an example of an alkylating agent [ii].

Anti-metabolites work by incorporation in the DNA or RNA and interfere with the process of cell division. The structures of the compounds in this group of chemotherapeutics are similar to structures naturally occurring in the body such as vitamins, amino acids, or precursors of DNA or RNA. Anti-metabolites are commonly used in treatment of leukemia, and also in some cases of tumors of the gastrointestinal tract, ovary, and breast [ii].

Anthracyclines form free radicals which break the DNA strands, or integrate in the DNA or RNA between the base pairs, or inhibit topoisomerase II and thereby prevent DNA transcription and replication. The anthracyclines are derived from the *Streptomyces* bacteria. This group of chemotherapeutics is the most efficient anti-cancer treatment and they are used in a large variety of tumors such as breast, ovarian, leukemia, and lung cancers. Side effects of these drugs are quite severe; the heart muscle can be damaged [92, 93]. Anthracyclines are sometimes classified as a separate own group of chemotherapeutic but also sometimes are listed as a subgroup of the anti-tumor antibiotics [ii].

Anti-tumor antibiotics are also developed from *Streptomyces* bacteria, and have many described similarities as anthracyclines. These chemotherapeutic agents form radicals, breaking the DNA strands, stopping proliferation, although not presumably killing the cell. Anti-tumor antibiotics are used widely for the treatment of different cancers, such as testicular cancer and Hodgkin's lymphoma. Treatment is usually combined with other chemotherapeutic agents. The most common side-effect seems to be limited to the lungs [ii].

Platins cross-link subunits of DNA and prevent various DNA functions. Cisplatin was first generated but had high toxicity and can give rise to damaged kidneys. The second-generation, called Carboplatin is less toxic but still has side effects on the kidneys. A third generation, Oxiplatin has even less side-effects however, it can cause severe neuropathies. Cisplatin is used in the treatment of testicular cancer (highly successful), non-small cell lung cancer, small cell lung cancer, lymphomas and myelomas. Another drawback of Cisplatin that is it to some extent causes resistance. Since Carboplatin is so similar in reactivity, it is used in the same types of cancers, but in particular ovarian cancer. Oxiplatin is capable of overcoming cisplatin resistance and is used in colorectal cancers and is in clinical trials for several other cancer types [94] [ii].

Monoclonal antibodies work in different ways, by binding antigens either in the tumor which can then be recognized by the immune system, or by binding to important growth factors or their receptors and thereby prevent the tumor growth. These antibodies are used in colon, head, neck, lung, and breast cancers [ii]. An example of these antibodies is Bevacizumab (Avastin).

Plant alkaloids are derived from plants and are cell-specific. They work by interfering the cell-cycle in different ways. The plant alkaloids are further grouped into four categories and they are used in leukemia and many other cancers [ii].

1.7.1.1.1 Carboplatin

Carboplatin (Paraplatin®) is used as initial or palliative treatment of non-small cell lung carcinoma, ovarian carcinoma, and lung, bladder, head and neck, and cervical cancer, and others. Even though carboplatin was developed to reduce the side-effects, treatment it still leads to severe bone marrow suppression, with decreased white blood cell counts, and can cause anemia [94, 95] [iii].

1.7.1.1.2 CTX

One of the most successful anti-cancer drugs is Cyclophosphamide (CTX or Cytosan®). It is used in treatment of lymphomas, sarcomas, and breast, germ cell, and testicular cancer. CTX works by crosslinking the DNA, and in this way interfering with cell proliferation. High dose CTX treatment the side-effects include bone marrow suppression; causing low blood counts, which can give rise to anemia, bleeding, and infection. Further CTX can suppress the immune system and is therefore used in autoimmune diseases. Side-effects such as leukopenia, thrombocytopenia, anemia, cardiotoxicity, and gonadal failure (mostly for females) are common in high-dose administration of CTX [96, 97] [iii].

1.7.2 Anti-angiogenic therapy:

The anti-angiogenic therapies can generally be divided into three groups. 1) **Monoclonal antibodies**, which are directed against specific pro-angiogenic factors and/or the corresponding receptors. The Food and Drug Administration (FDA) of USA has approved Bevacizumab and Cetuximab. 2) **Small molecule tyrosine kinase inhibitors** (TKIs) of a multitude of pro-angiogenic growth factor receptors. Three TKIs used clinically are Imatinib, Sunitinib, Sorafenib, and Vandetanib. 3) **Inhibitors of mTOR**, the mammalian target of rapamycin in the PI3K-AKT-mTOR signaling transduction pathway. 4) A group of other anti-angiogenic agents, e. g. targeting Endostatin and Interferon alpha [98].

1.7.2.1 Bevacizumab

Bevacizumab (Avastin®) is the first approved anti-angiogenic agent. Used in the treatment of metastatic colon or rectal cancer, non-squamous, non-small cell lung

cancer, metastatic breast cancer, glioblastoma and of metastatic renal cell carcinoma and is mostly used in combination with chemotherapy. Therefore, it is generally not known whether the side-effects are caused by the chemotherapeutic agents or by the monoclonal antibody. The reversible side-effects, which disappear after end of treatment, generalized weakness, low white blood cell counts, proteinuria, nausea, pain, and constipation. Bevacizumab works by targeting VEGF-A, and thereby preventing it from binding to its receptors, and further thereby angiogenesis [98, 99] [iii].

1.7.2.2 Imatinib

Imatinib Mesylate (GleevecTM or STI-571) was the first small molecule TKIs approved. Imatinib is used in the treatment of chronic myelogenous leukemia (CML), gastrointestinal stromal tumors which are positive for c-kit, and myelodysplastic/myeloproliferative diseases associated with PDGFR. Side-effects include low blood counts, edema, and hemorrhage. Imatinib is a inhibitor of PDGFR, c-kit, CSF-1R, and Abl (a cytoplasmic and nuclear protein tyrosine kinase). Imatinib antagonizes the ATP binding site of the tyrosine receptor, preventing further intracellular signal transduction [98, 100, 101] [iii].

1.7.2.3 Sunitinib

Sunitinib (Sutent® or SU11248) is a small molecule inhibitor and is used in treatment of gastrointestinal stromal tumor (GIST) and advanced renal cell carcinoma. The generally low side-effects may include fatigue (predominantly), high blood pressure, and/or low blood counts. As a curiosity, the patient may gain a skin discoloration, which is possibly due to the strong yellow colour of the drug. Personal observations: the mice treated with Sunitinib have distinct yellow colour of the urine compared to PBS treated mice. Sunitinib is a multi-targeted receptor tyrosine kinase (RTK)

inhibitor, targeting the VEGFR, PDGFR, RET, colony-stimulating factor-1 receptor (CSF-1R), and the hematopoietic markers Flt-3 and c-kit [98, 102-104]. As Imatinib, Sunitinib inhibits the tyrosine kinases by competing with ATP to bind the intracellular domain, and thereby stop further signal transduction [104] [iii].

2 AIMS

The general aim of this thesis is to increase our understanding of the mechanisms of specific angiogenic growth factors and their function in tumors and cancer associated disease, and further to understand the effects of anti-angiogenic drugs in combinations with chemotherapy.

Specific aims:

- 1) To study the cross-talk between various angiogenic growth factors in promotion of tumor growth and metastasis (paper I)
- 2) To study the role of the VEGFR1 specific ligand PlGF in tumor angiogenesis, normalization and tumor growth (paper II)
- 3) To investigate the angiogenic growth factors and their receptors in cancer-associated disease (paper III)
- 4) To study non-tumor targets of anti-angiogenic drugs (paper IV)

3 METHODOLOGY

3.1 *IN VIVO* ASSAYS

Classical **tumor models** or **xenografts** were performed by tumor cells injection subcutaneously into the mid-dorsal region of the mice. When tumor reached a certain volume or after a fixed number of days, the mouse was euthanized, and blood, tumor and other tissues were collected. In the **metastasis assay**, the tumor was removed and skin stitched together of the anesthetized mice, and after a few weeks the mice were euthanized and examined for metastasis. The **tumor cell lines**, have been described previously, are transfected with cDNA coding for a human growth factor cloned into a vector containing EGFP [105]. The cells overexpress growth factors such as VEGF-A₁₆₅, PlGF-1₁₂₉, and PDGF-BB.

- This type of tumor models (syngeneic, orthotopic, or as xenografts) are a broadly used in the medical research, where the tumor effects of anti-cancer drugs can be studied. Tumor growth is studied by measuring the tumor size, over a period of time, which is quickly and easily performed by a caliper. This is not possible in the orthotopic model, where the tumors are not reachable and the tumor sizes of different individuals will be compared after sacrificing the mouse. Thereby the progression of the cancer can be followed. The vascular structures and other tissue-specific changes can be studied post sacrifice (paper I, II, III, and IV). When the primary tumor is removed, the mouse (or patient) may not heal from the cancer, but tumor cells may still remain and cause dangerous metastasis. The described metastatic model gives us proves for just how aggressive some overexpressing growth factors are in comparison to others (paper I).

The vascular **permeability** and **perfusion assays**, which were developed in this thesis, were performed when the tumors had reached a size of maximum 1 cm³, followed by a tail vein injection of lysine-fixable rhodamine labeled dextran (LRD) of different molecular sizes. The small size of 70 kDa circulated for 5 minutes and then the tumors were collected and immediately placed in a paraformaldehyde (PFA) solution, after whole mount staining and visualization in the confocal microscope the extravasated dextran could be analyzed. In the perfusion assay, 2000 kDa dextran were injected and tumors were collected after 15 minutes, following the same procedure as previously described, and analyzing the perfused vessels.

- Permeable agents of different molecular weights, one such as Evans Blue, have been used for the study of the vessel permeability, although after quantifications of the extracted dye, it reveals little of the actual microvessel structure-function relationship. When injection of fixable and fluorescent dyes, such as LRD, it is possible to stain the vessels, visualizing the areas where the leakage has occurred and also to quantify the number of perfused vessels, containing LRD in an accurate manner (paper II and III).

Mouse corneal micropocket assay was carried out by mixing the growth factors with sucralfate (a heparin-binding slow release substance) into a pellet, and then placing the small pellet in a micropocket in the cornea of the mouse [106]. The eyes were photographed regularly and lastly the corneas were collected and the vessels were visualized by whole mount staining and confocal microscopy.

- The cornea is an avascular and transparent tissue. By placing an agent which induces vascular growth into this tissue; all of the vessels visualized are newly formed (by angiogenesis). Further by monitoring the vessels over long time, the stringency of the particular agent is remarkable. Some growth factors will have only immediate time induction of vessels such as PDGF-BB, whereas other growth factors, such as PDGF-BB

in combination with FGF-2, will last a long time, even a year after the pellet implantation and even after the loss of the pellet [107] (paper I).

Immunohistochemistry were performed by using three different assays. **Whole mount staining** was used on PFA fixed fresh tissue, which were sliced into small pieces, from non-necrotic regions and digested in proteinase K, fixed in methanol and blocked in milk overnight. The pieces were then incubated in the primary antibody overnight, for an example (the most commonly used antibody in this thesis) rat anti-mouse CD31 (PECAM-1, BD Bioscience). After rigorously washing followed by blocking, the pieces were incubated with a secondary antibody e.g. Alexa 555-labeled goat anti-rat (Invitrogen). Further the tissues were washed and mounted in Vectashield mounting medium (Vector Laboratories) and lastly, visualized and analyzed by confocal microscopy (Zeiss or Nikon) (paper I, II, III, IV). **H&E** were performed on tissues fixed in PFA, dehydrated and embedded in paraffin. These samples were sectioned and immunostained by the use of biotinylated monoclonal antibodies. Further, peroxidase activity was developed with diaminobenzidine (Vector Laboratories) and the samples were counterstained by hematoxylin and eosin and lastly analyzed in a light microscope (paper I and IV). Immunofluorescent staining, so called **Cryosection staining**, was performed on tissue which immediately upon collection had been placed in a cryomold, and these tissues were sectioned in a cryostat, washed and blocked in serum, followed by incubation with specific antibodies. Further washes of the sections followed by secondary antibody incubation and lastly mounting. The tissues were examined under the confocal microscope (paper I).

- Whole mount staining is a widely used method to stain large 3-dimensional areas of the tissue. H&E and cryosection staining are performed on very thin slices, especially the cryosection which is an advantage if single cells need to be visualized and quantified. With the confocal microscope it is possible to visualize depths, which has limiting abilities in other types of microscopes. The disadvantage with the whole mount staining is the time consumption and that the samples have to be used within approx. 2 months after dissection of the mouse. The paraffin- and cryo-embedding will last for years. By the use of different antibodies of different species, it is possible to visualize three different targets, in one picture. For example, it is possible to stain endothelial cells, SMCs, and pericytes simultaneously.

3.2 IN VITRO ASSAYS

Cell isolation of primary cells was performed by using fresh lung tissue, to isolate pericytes. Aorta tissue was used to isolate SMCs. Tissues were collected and immediately resected into small pieces which were digested by collagenase II (Invitrogen). The red blood cells were lysed in ammonium chloride lysing reagent (BD Pharmingen). The cells were then incubated with primary antibodies (e.g. NG-2 for the pericytes) followed by a secondary fluorescent-labeled antibody and the cells were sorted with Fluorescent-activated cell sorting (FACS) (Becton Dickinson). The sorted cells were then cultured. For verification of the sorted cells, **cell staining** was performed, where the cells were seeded out and grown on coverslips and then stained with primary and a fluorescent secondary antibody, followed by visualization in the fluorescent microscope (Nikon) (paper I and IV).

- Isolating primary cells is of value, since they will act more natural than cell lines, which are modified to avoid senescence. Isolated primary cells will quickly lose their *in vivo* characteristics, by differentiation or entering senescence after a few passages. Therefore it is important to use the cells in experimental settings with as low passage number as possible. The markers used to separate the cells are of high importance as a marker needs to be chosen, which will not be expressed on another cell type in the same tissue. The FACS is a flow sorting technique, where single cells are separated and collected from a

heterogeneous cell population. The FACS separation is efficient, although not 100 % reliable. A few unwanted cells might appear in the culture and therefore it is important to further stain and confirm the newly sorted cells.

Western Blot was performed by treatment of cells with different growth factors at various time points. The cells were washed and lysed, followed by centrifugation and the supernatants, containing the proteins were collected. Equal amounts of proteins (determined by a modified Lowry method (Bio-Rad) and BSA as standard were loaded into gels (SDS/PAGE, Novex) and transferred onto nitrocellulose membranes and further blocked (BSA) overnight, followed by a primary antibody incubation overnight. Further, after washing the membranes, they were incubated with peroxidase-coupled secondary antibodies to reveal the bands and photographed in a Lumi-Imager. For the loading control β -actin (anti- β -actin, Sigma-Aldrich) was used (paper I).

Proliferation and migration

The **proliferation assays** was performed by seeding out cells in a plate with or without growth factors after 12 hours the medium was changed and new growth factors were added to some of the wells. The cells were incubated for another 60 hours and then the proliferated cells were counted in a Coulter counter (Beckman Coulter) (paper I). The **migration assay** or **chemotaxis assay** was performed in a classical Boyden chamber. Cells either pretreated or non-pretreated with growth factors were seeded in the upper chamber and a filter was placed in between the cells and the lower chambers in which there were medium with or without growth factors. After 4 hours the migrated cells on the lower side of the membrane was fixed, stained, and mounted, followed by a quantification of the cells through a light microscope. The number of migrated cells was counted as a criterion for chemotactic stimuli (paper I).

- One of the first steps in angiogenesis is the proliferation of ECs and later mural cells. To measure the induction of proliferation by different angiogenic growth factors, we used a simple cell counting assay, which measured by the number of cells that are dividing. The time of a cell cycle for most EC types are in the range of 18-26 hours. Therefore we pre-treated the cells with one growth factor for 12 hours, and then added the next round of growth factors, before the first division. This gives the cells a chance to respond to the first factor, in our case causing a changed receptor expression before being exposed to the second. The cells are then left to be able to divide a few rounds, before counting the cell number. The Coulter counter is an electronic particle counter which is using a size parameter for counting.
- Another early step in the process of angiogenesis is EC migration toward a chemotactic gradient, into the perivascular stroma. Further, a mural cell migrates in connection to the ECs. We used a classical transmembrane *in vitro* assay. The advantage of this system is the migration of cells due to a concentration gradient, and the result can be easily measured by counting the cells. The incubation time, is 4 hours, which is short enough for the cells to migrate, without any misleading proliferative effect. Within these 4 hours the gradient should not change significantly, but after longer time osmosis might occur and can destroy the gradient. Chemokinesis, the random movement of cells, will be counted as a background, and subtracted from the counted value such that the final value will indicate the number of migrated cells due to chemotaxis. We used cells which were both non- and pre-treated with growth factors, to see whether the pre-treatment and the chemotactic factor had a higher migratory response, compared to the non-pre-treated cells.

4 RESULT AND DISCUSSION

4.1 FGF-2 AND PDGF-BB SYNERGISTICALLY PROMOTE TUMOR ANGIOGENESIS AND METASTASIS

Angiogenesis is induced by VEGF-A, but tumors produce a diversity of other angiogenic factors and in some cases no expression of VEGF-A can be detected. Previously Cao *et al* reported that the combination of VEGF-A with FGF-2 or with PDGF-BB, gave no further angiogenic response in the cornea, whereas a combination of FGF-2 and PDGF-BB induces severe angiogenesis, which lasted 7 months after pellet implantation (which was lost at day 12 post operation). Treating the mice with VEGFR-2 neutralizing antibodies, did not decrease the angiogenic response of the corneas [1]. Thereby it was shown to be a VEGF-A independent mechanism.

In this paper, we show a synergistic interaction between two angiogenic growth factors, affecting the endothelial cells (ECs) and smooth muscular cells (SMCs) in a reciprocal action to induce tumor neovascularization and metastasis. Tumors of mixed tumor cells, overexpressing either FGF-2 or PDGF-BB, respectively, grew with increased growth rate compared of each tumor cell type alone. Tumors give rise to visual pulmonary metastasis in 30% of the mice, but after histological analysis, tumor cells in the lungs could be found in as much as 44% of the mice, whereas none could be found in the other groups, of control or single factor expression. The tumor vessel structure had a remarkable changed phenotype, with huge irregular vascular plexus and tip cells all over the micro vessels. Interestingly, the SMCs were less attached to the vessels, considering PDGF-BB is an SMC attractant, when expressed by the ECs. Although one can speculate, that in the tumor tissue the PDGF-BB is

expressed all over the tissue in a non-gradient manner, which does not makes the SMCs respond in a regular manner. In the combined FGF-2 and PDGF-BB expressing tumors, the SMCs have a slightly increased association with the vessels compared to the PDGF-BB group alone. Although the combined group has significantly decreased vascular association of SMCs compared to the vector.

The question now, is what actually has happened to the mural cells, since they are very important for the vascular support and functionality. *In vitro* experiments were performed, such as proliferation and migration, since it is the first steps in the process of angiogenesis. ECs had an increased proliferation due to FGF-2 stimuli, but not a migratory effect. PDGF-BB does not induce any direct effect on ECs, but here we show that, pre-treatment with FGF-2, followed by PDGF-BB treatment (as a chemoattractant) induced ECs migration. Comparing to the SMCs, pre-treatment with PDGF-BB followed by a treatment with FGF-2 increased the proliferation but not migration. FGF-2 further increased the migratory ability of the SMCs. Here we have an indication that ECs which express FGFR1 will proliferate upon FGF-2 receptor binding but need the presence of PDGF-BB for migration. On the other hand the proliferation of SMCs needs activation by the PDGF-BB pathway, followed by FGF-2 stimulation, which confirms the previous findings that in SMCs PDGF-BB induces FGF-2 release and thereby FGFR1 activation [2]. How is the ECs response of growth factor stimuli? When the promoters of PDGF receptors α and β , respectively, had been fused with a luciferase gene as a reporter system, then the promoter activity of both receptors were increased upon treatment with FGF-2. Further in comparison, when the SMCs were treated with PDGF-BB the FGFR1 promoter had an increased activity. Taken together, these result shows that upon PDGF-BB stimulation of SMCs it will cause a FGFR1 expression. The ECs will express the PDGFRs upon FGF-2 stimulation.

To further validate the findings, the mRNA expression was detected via *in situ* hybridization of corneas, and showed that the FGF-2 induced vasculature had elevated high levels of PDGFR expression, whereas the PDGF-BB induced blood vessels had relatively low levels of the receptors, in comparison of the controls. Lastly, after the receptor activation, the signal transduction pathways are to be elucidated. Here we show, in activated ECs, FGF-2 pre-treatment followed by PDGF-BB treatment, activated the ERK-pathway leading to DNA transcription.

The mechanism behind the severe malformation of the tumor vasculature, and increased incidence of metastasis in mice carrying tumors expressing both FGF-2 and PDGF-BB is due to FGF-2 will bind and activate the FGFR1 on the ECs, which leads to proliferation, but also to an expression of PDGFRs, and their activation upon PDGF-BB binding lead to EC migration. The ECs alone will cause the angiogenic response. Simultaneously, the PDGF-BB will bind and activate the PDGFRs on SMCs, followed by an increased FGFR1 expression, which will be activated by FGF-2 causing proliferation. FGFR1 activation by FGF-2 binding will also cause the SMCs to migrate. As a result, vascular remodeling will occur. The synergism between the growth factors in the tumors will lead to cross communication of the cells leading to an elevated but mis-guided vessel growth in combination with a lack of proper SMC coverage and maturation, causing severe tumor growth and induction of metastasizes. This could be an important feature to consider for cancer therapy, especially since increased tumor vasculature and ablated mural cells, have been shown to be associated with metastasis and poor prognosis even in cancer patients [5].

4.2 PLGF PROMOTES NORMALIZED TUMOR VASCULATURE

The VEGF-family consists of the different members of VEGF-A, -B, -C, D, and PlGF. These members have different functions and causes different morphological structures of the vessels. The actions of PlGF is not as well defined as the other members of the family. PlGF which binds exclusively to VEGFR1, might have another function than VEGF-A, which binds to both VEGFR1 and R2. VEGF-A mainly causes the potent angiogenic response via binding to VEGFR2. Here, we would like to investigate the function of PlGF in association to tumor vascularization in mouse models, and further compare to human malignancies expressing endogenous PlGF.

Comparing tumors overexpressing the angiogenic growth factors VEGF-A, PlGF-1, and PlGF-2, with the empty vector, we show that the VEGF-A fibrosarcoma (T241) tumors have an increased vessels density, the structure is irregular, torturous, full of vascular plexus, and the vascular branch points are more than a 5-fold higher compared with the vector. The vascular structure induced by both PlGF-1 and -2, are completely of a different character; a normalized phenotype. The vessels are long, straight and have few branch points. Another feature which differs in the PlGF in the induced vasculature is the size of the vessels. The diameter is increased approximately 3-fold, giving rise to a large lumen. The vessels of the vector and VEGF-A groups have a smaller diameter, due to an abundance of microvessels and tip cells.

The pericytes are important for the guidance of proper vascular growth, support, stability, and prevention of leakage. We show the pericyte association of vessels to be sparse and only approximately half of the vessels have pericytes attached in the vector group. The VEGF-A induced tumors vessels, have even poorer amount of pericytes, and some are not attached to the vessels. In the PlGF tumors on the other hand, the pericytes cover most of the vessels and almost the full entire endothelial-surface. Then what about the functionality of these vessels? Through the injection of dextran, of small molecular weight, we were able to evaluate the extravasation (the leakage) of the vessels. The vector tumors proved to have highly permeable vessels leading were approximately half of the dextran being extravasated. In comparison, the VEGF-A overexpressing tumors had even more dextran leaked out into the extravascular space. The PlGF induced vessels, with the high mural coverage and normalized feature, had much less vascular leakage. Further, will the tumor vasculature be functional? Through the injection of a high molecular weight dextran, it was shown that almost 100% of the vessels in the PlGF tumors were perfused with blood. In contrast, only a third of the VEGF-A induced vessels were perfused, proving the non-functionality of this tumor vasculature.

To further evaluate if the findings of the PlGF vascular remodeling occurs in other tumor types, Lewis Lung Carcinoma (LLC) cells overexpressing the very same growth factors as the fibrosarcoma cells were used. LLC is a more aggressive tumor type but also commonly used in research. The LLC tumor overexpressing PlGF-1 and -2 showed the same vascular pattern as fibrosarcoma PlGF-1 and -2, with vascular normalization and high pericyte coverage. The vasculature in LLC-VEGF-A tumors also followed the same pattern as fibrosarcoma-VEGF-A, with a high number of branch points and pericyte dissociation. Comparing the pericyte coverage with

fibrosarcoma, LLC had a bit higher percentile coverage, which has to do with the LLC tumors itself. It can be speculated that other factors, are expressed in these tumors, which attract pericytes and lead to higher pericyte coverage.

To analyze further whether this vascular normalization actually occurs in human tumors, we studied a human choriocarcinoma (JE-3) which intrinsically express high amounts of homodimers of PlGF, high amounts of heterodimers of PlGF-VEGF-A, and low amounts of homodimers of VEGF-A. This tumor vasculature showed to be of normalized phenotype with almost 100% pericyte coverage corresponding to the PlGF-induced vessels in fibrosarcoma and LLC. In comparison, we used a human squamous cell carcinoma (A431), which expresses extremely low amounts of homodimers of PlGF and heterodimers of PlGF-VEGF-A, but high levels of VEGF-A homodimers. Interestingly, the A431 tumor vasculature had extremely large vascular plexuses and only a quarter of the vasculature had pericyte coverage, similar to the VEGF-A producing fibrosarcoma and LLC tumors, although the A431 vascular plexuses were of larger morphology. The functionality of the vessels in both JE-3 and A431 remains to be studied.

To evaluate the normalized vascular remodeling by PlGF, which transmits the signal through VEGFR1, we injected a VEGFR1 neutralizing antibody into the tumor bearing mice. To our surprise the PlGF-induced vessels remained in a normalized manner with full pericyte coverage and few branch points. Interestingly, the diameter had decreased into the size found in vector tumors. Further, the tumor growth rate had not changed in any of the PlGF- or vector- tumor groups. Further, vector tumors treated with the VEGFR2 neutralizing antibody, showed a reduced tumor growth rate

and a normalized vascular structure, with fewer branch points, but with unaffected pericyte coverage and vessel diameter, compared to PBS treated tumors.

Here we show that the main function of PlGF induced vascularization through VEGFR1 is to dilate the vessel. In agreement to the observed phenotype in VEGFR1 KO mice, which die during the early embryonic development, due to disorganized and overgrown vasculature [62], our results indicate that VEGFR1 have a negative regulatory role in angiogenesis. Further, the Flt-1 TK transgenic mice, which lack the intracellular domain of VEGFR1 and only express the sVEGFR1, are also embryonically lethal, but exhibit a normal neovascular phenotype [62]. In the mice carrying PlGF overexpressing tumors treated VEGFR1 neutralizing antibodies the normalized tumor vascular structure (although decreased lumen), could be due to the fact that PlGF heterodimerize with VEGF-A, and thereby inhibit the VEGFR2 induced angiogenesis via VEGF-A homodimers. The same vascular pattern was seen in anti-BEGFR1 treated compared to PBS treated vector tumors, which intrinsically express small amounts of mouse VEGF-A and PlGF. In conclusion, PlGF is causing vessel dilation and PlGF through dimerization with VEGF-A and thus neutralization of VEGF-A homodimer-induced VEGFR2 activation, induces normalization.

4.3 PERICYTE REMOVAL MEDIATED THROUGH VEGFR1 IN THE RETINAL VASCULATURE

Cancer-associated retinopathy (CAR) is a non-inflammatory damage of the retina of the eye; the research has been focused on tumor-induced autoimmune destruction of the retina with the focus of the photoreceptor layers. In other types of retinopathy, such as diabetic retinopathy (DR) and retinopathy of prematurity (ROP), are extensive

research regarding the retinal vasculature and the importance of VEGF-A [48-51]. Here, we would like to investigate the retinal vessels, and the vascular changes due to tumor expressed angiogenic factors, and further the underlying mechanisms.

To start, we found that the retinal distribution of VEGFR1 and VEGFR2 was found to be on different cell types. The retinal vasculature surprisingly lacks VEGFR2 expression, but rather expresses VEGFR1. The VEGFR2 expression was found on the nonvascular cells, the ganglion cells and photoreceptors, seen by the overlap staining of anti-VEGFR2 and a glial marker. The VEGFR1, on the other hand, was well distributed on the retinal blood vessels, which were also covered by pericytes. Since the mural cells, pericytes and SMCs are important for the stability and prevention of vascular leakage, and these cells were investigated for the VEGFR1 expression. First, to identify these isolated primary cells, they were stained positively for PDGFR- β , which is known to be expressed on both pericytes and SMCs [8]. The isolated pericytes expressed NG-2, a pericyte marker, and a small subpopulation expressed alpha smooth muscle actin (α SMA), and the contrary; SMCs expressed α SMA and a subpopulation expressed NG-2. Both primary cell types were expressing VEGFR1, and further the pericytes and ECs also expressed the VEGFR1 mRNA confirmed by reverse-transcriptase PCR analysis. The signal transduction pathways induced in the pericytes after VEGF-A-stimulation, were confirmed to be the ERK-pathway and cell survival pathway through Akt. Erk activation could be inhibited by the anti-VEGFR1 treatment. Taken together, here we have shown the direct activation of VEGF-A via VEGFR1 on pericytes in the retinal vasculature.

VEGF-A binds both VEGFR1 and 2, and a known effect is to disperse the pericyte and decrease coverage, as seen in tumors overexpressing VEGF-A. If we then

compare with PlGF which binds VEGFR1 alone, which has been proven to induce high pericyte coverage in the tumor-vasculature, would the retinal vasculature respond in the same pattern, in spite of the fact that tumor microvessels express both VEGFR1 and VEGFR2, and the retinal vasculature lack the VEGFR2 expression? In the investigation, of whether only VEGF-A activation of the VEGFR1 on retinal vessels or if PlGF also induce the VEGFR1 induction of pericyte ablation; the micro-pocket assay were used. We placed small pellets containing VEGF-A or PlGF in the cornea and both VEGF-A and PlGF induced pericyte ablation, and even increased ablation after longer time stimulation of the growth factors. Further in systemic delivery of adenoviral vector expressing VEGF-A also ablated the pericytes of the retina vasculature, compared to the empty vector.

Pericytes are known to respond and migrate toward a chemo-gradient of PDGF-BB, via activation of PDGFR β , therefore we needed to compare the retinal vasculature of retinas from mice treated with a neutralizing antibody against PDGFR β (anti-PDGFR β), and imatinib, the tyrosine kinase receptor inhibitor of PDGFR β (STI571). After administration of these two inhibitors there was a clear pericyte ablation. Although after 25 days, the ablation was not as high compared to the VEGF-A administrated retinas.

For more relevance of tumor induced retinopathy, retinas of tumor-bearing mice were examined. Two different tumor types (T241 and LLC) overexpressing VEGF-A, induced a pericyte loss in the retinal vasculature, cooperating the effects on the tumor vasculature seen in previous experiments. Further, the same ablation could be seen in the retinal vasculature of PlGF-T241 bearing mice. That PlGF have the same

induction of pericyte loss, leads to the confirmation that VEGFR1 induces the ablation process.

Pericyte ablation would probably lead to decreased stability and functionality of the retinal vasculature. We, therefore, evaluated the retinal vascular leakage by dextran injection into the tumor bearing mice. The VEGF-A-tumor mice had large areas of vascular leakage compared to the lack of leakage in the retinal areas of the wt- or vector-tumor mice. This indicates that the vasculatures with the ablated pericytes are less leaky, which is not healthy and can lead to tissue edema.

The retinas of mice with T241-VEGF-A tumors had increased pericyte coverage of the retinal vessels when treated with anti-VEGFR1 and anti-VEGF. The anti-VEGFR2 treatment had no effect on the pericyte coverage. To validate these findings in a model, where the expression levels of angiogenic factors has not been manipulated; a mouse model which spontaneously develops mammary adenocarcinoma was used. The retinas also had increased and recovered pericyte association of the vessels when treated with anti-VEGFR1. Further, no such loss could be detected in the anti-VEGFR2 treated retinas.

In conclusion, we have found a new mechanism of rescuing the impaired retinal vasculature in CAR in several different mouse cancer models. The damaged vasculature, by the VEGF-A induced pericyte loss; could be inhibited by systemic administration of anti-VEGFR1, but not anti-VEGFR2 antibodies.

4.4 ANTI-ANGIOGENIC DRUGS INCREASE SURVIVAL AGAINST CHEMOTHERAPY TOXICITY

Chemotherapy is an efficiently used cancer treatment, although the severe side-effects can be lethal. One of the most severe side-effects is bone marrow (BM) suppression, which could cause severe anaemia. Anti-angiogenic drugs are often administered simultaneous with the chemotherapeutic agents. There is previously not much known of the mechanisms behind the benefits and of co-administering anti-angiogenic drugs and chemotherapy. Here we show that tumor-VEGF-A induced BM suppression, can be inhibited by the anti-angiogenic drug.

To assess the question whether the combination of the anti-angiogenic drug sunitinib and the chemotherapeutic drug carboplatin would improve the survival compared to the carboplatin treatment alone, we used a B16-melanoma mouse model. The mice treated with sunitinib alone, had a full survival until the end of the experiment, in comparison to the control treated mice which had a 70% survival, and further as few as 20% of the carboplatin treated mice survived.

When the mice were treated with sunitinib for 6 days then followed by the administration of both sunitinib and carboplatin, the survival improved to 80%. Interestingly, primary treatment with sunitinib, followed by carboplatin treatment alone resulted in 100% survival. These findings, did not accord with the tumor growth, showing that the poor prognosis of carboplatin therapy is not due to tumor size.

Patients treated with chemotherapeutic drugs often suffer from severe side-effects including the life-threatening BM suppression [3]. Previously, Xue et al has shown

that VEGF-A-induced impaired murine hematopoiesis could be improved by the treatment with Bevacizumab (anti-VEGF-A) and an anti-VEGFR2 antibody, respectively [4]. Therefore we were interested in examining the BM of these mice. In comparison to the non-tumor bearing mice, in those with tumors, the BM had a scattered appearance of the hematopoietic islet. As expected the melanoma mice had slightly decreased Red Blood Cell (RBC) counts, and surprisingly increased numbers of White Blood Cells (WBC). Carboplatin suppresses the BM in patients, and when carboplatin was used as a single treatment, there were large areas of empty extracellular spaces and the hematopoietic islands contained fewer cells compared to buffer treated BMs. When treating the melanoma mice with sunitinib alone, the BM was not suppressed, but rather contained more BM cells in comparison to the buffer treated mice. When pre-treating the mice with sunitinib, the carboplatin induced BM suppression was blocked, both in regard to the number of hematopoietic islets and number of circulating RBCs and hemoglobin (HGB).

Looking at the tumor vascularization of these mice, we found that sunitinib alone led to decreased angiogenesis in comparison to the buffer treated mice. The treatment with carboplatin alone showed the same amount of tumor vessels as the buffer treated mice. When sunitinib and carboplatin were used in combination the amount of tumor vessels was unchanged when compared to sunitinib alone. Following consecutive treatment with sunitinib and carboplatin, the vascularization was increased. This shows, that after the stop of sunitinib treatment, the process of angiogenesis will continue and that carboplatin has no anti-vascular effects.

As mentioned above, VEGF-A can damage the BM. Can sunitinib treatment prevent this damage? To investigate this, fibrosarcoma expressing an empty vector or a VEGF-A-expressing vector, were injected and grown on mice. The survival of the

VEGF-A group were low, only 40% were still alive after 18 days, whereas every single mouse in the vector-group had survived. The fast growing VEGF-A tumors caused severe BM damage. Very few small hematopoietic islands were visible and the blood contained much less of RBCs and HGB, in comparison to the vector. Could sunitinib rescue the BM and perhaps even increase the survival? To investigate this, we started treating mice with sunitinib at the time the mice were about to become ill. This would more closely reflect the time point, when a patient seeks help for their illness. Interestingly, the VEGF-A induced death could be delayed with several days due to the sunitinib administration. Further, this group had a rescued BM cell population, and hematopoiesis. The vector-tumor bearing mice treated with sunitinib showed none such effect of the BM. Here we showed that sunitinib can prevent the VEGF-A induced BM suppression.

Since, VEGF-A suppresses the BM, would the combination of such tumor with chemotherapy increase the BM suppression even more, even though these agents with high probability work through different, VEGF-unrelated mechanisms? One of the most common chemotherapies used is carboplatin, due to its efficiency. The liability, is the severe BM suppressing side-effects. The dramatic result from the carboplatin treatment of VEGF-A tumor bearing mice, was only 20% survival after 5 days of chemotherapy administration. We wondered if this low survival was only an effect of carboplatin, specifically, or if other chemotherapeutic agents would have the same synergistic effect. We tested CTX, which also causes BM suppression, although not as severe as that seen in patients treated with carboplatin. This combination of VEGF-A expressing tumors treated with CTX, gave a 30% survival. Both chemotherapeutic agents induced decreased BM cells and extremely small hematopoietic islands with enormous areas of non-cellular space in the VEGF-A bearing mice. The blood counts

showed decreased RBC and HGB in both groups. There is a synergistic effect of two different kind of chemotherapeutic drugs with VEGF-A, on BM suppression, leading to early death.

The severe synergistic effects of the chemotherapy and VEGF-A expressing tumors, could perhaps be decreased with an anti-VEGF-A agent. If this agent is given before chemotherapy, would it not then give the BM a chance to recover, before the chemotherapy-induced suppression? When sunitinib was administered for 5 days, followed by carboplatin, the mice exhibited an 80% survival rate. Sunitinib followed by a combination of sunitinib and carboplatin led to a 50% survival rate. Sunitinib followed by buffer or a combination of sunitinib and carboplatin without any pre-treatment, gave a very poor survival of 10 % in both experimental set-ups. Remarkably, the BMs of sunitinib followed by carboplatin alone had as many BM cells as single treatment of sunitinib. This consecutive treatment had a healthier phenotype of the BM, compared to sunitinib followed by combination of sunitinib and carboplatin. The RBC and HGB values were increased in all cases where sunitinib had been in pre-treatment. These data proves that sunitinib rescues the chemotherapeutic agent induced BM suppression, when administered before the agent.

Further, sunitinib treated tumors had less vascularization while the treatment was maintained but; when sunitinib was removed then the angiogenesis continued. When sunitinib was administered during the whole experiment, where carboplatin was added later on, the vascularization was normalized, but the BM was not. Thereby this kind of treatment caused a higher death amongst these mice.

To further evaluate if sunitinib can rescue chemotherapy induced BM suppression by another agent, we performed the same experiment with CTX instead of carboplatin. We found that pre-treatment of sunitinib did improve the survival, compared to combination of both sunitinib and CTX simultaneously. The BM cell amounts were clearly increased in the sunitinib pre-treatment group. In conclusion, sunitinib given before the chemotherapy will serve as a protection toward the chemotherapy induced BM suppression.

5 CONCLUSION AND FUTURE PERSPECTIVES

5.1 PAPER I:

Here we studied the interplay between two angiogenic factors commonly expressed in advanced tumors. The vasculature is composed of both ECs and mural cells. The EC forms the vessels and the mural cells gives structural support but also control the blood flow within the vessel. Since ECs express the FGFR1 constantly, in presence of the ligand FGF-2 two actions will occur; receptor activation followed by expression of PDGFRs, and EC proliferation. When PDGF-BB is present, then the ECs start to migrate. In the cases where this is induced by regulate chemotactic stimuli, the migration would occur in strictly regulated manner, where one EC form a so called tip cell, and proliferation of the stalk ECs will form a lumen. In tumors when FGF-2 and PDGF-BB are present everywhere; the ECs form vessels which are instead irregular and full of vascular plexuses. This mechanism might be able to be inhibited by the mural cell-EC interactions, although mural cells too, respond to these growth factors. The SMCs express PDGFRs, which upon PDGF-BB ligand binding are activated and causes FGFR1 expression. When the FGF-2 is bound to its receptor, it will cause the SMCs to migrate. Since the SMCs migrating away from the vessels, toward the tumor cells which are producing FGF-2, the ECs are exposed to the growth factors. Co-culture of SMCs and ECs will inhibit the EC proliferation, which otherwise will [108]. Thus in tumors expressing both of these factors, vascular remodeling and destabilization will occur. These vascular structures are highly permeable and migratory tumor cells can easily enter the blood stream, and spread to and colonize other tissues, and have thereby caused a metastasis. We found that 30%

of the mice had metastasis in the lungs, and further 40% of the lung tissues did carry the tumors cells. It can be speculated, that over time even the remaining mice might also develop metastasises. It has been shown, in patients that ablated mural cells in the tumor vasculature, is associated with increased metastasis and poorer prognosis of the cancer patient [14].

We can look at these findings with two perspectives. 1) When both PDGF-BB and FGF-2 are expressed in tumors, it could lead to induced tumor growth, and metastasis. 2) The beneficial effects of pro-angiogenesis. The combination of these two factors could be used as a new approach in the therapeutic pro-angiogenesis research field. In heart ischemic tissue, all single pro-angiogenic factors have failed, and not induced any new highly functional, arterial vessels. This has caused a halt in the pro-angiogenic research. We have shown in an ischemic rat-hind limb model that FGF-2 and PDGF induced long lasting and functional vessels [109]. Further, we showed in a pig myocardial infarction model that FGF-2 and PDGF-BB could improve the myocardial function, by establishment stable collateral networks, which induced improved blood flow [110]. Hopefully, this will lead to an increased interest in the pro-angiogenic research field, and if this synergism of FGF and PDGF could be further investigated. Maybe it could be used as a treatment for patients with myocardial infarcts in the future.

Returning to the anti-angiogenesis aspect, further studies need to be performed, to evaluate anti-angiogenic therapeutic drugs. It would be interesting to try whether imatinib or sunitinib would normalize the vasculature of these tumors, or just decrease the effect to a single-factor induced angiogenesis. Since both drugs target the PDGFRs on both ECs and SMCs, it is most likely to result in full normalization, and thereby reduced tumor growth. Maybe in the future patients could be scanned for

both factors and it would most probably be a marker for poorer prognosis, and perhaps an indication of which treatment to use.

5.2 PAPER II:

We studied fibrosarcoma overexpressing PlGF and showed that the tumor have a normalized vasculature, with very few branches. In comparison the vector tumor had an increased amount of vascular branches, and further the potent angiogenic factor VEGF-A had fully irregular and tortuous vessels. The PlGF induced microvessels had enlarged lumens and the vessels had extensive pericyte coverage. This coverage is highly important for the vascular stability, permeability and functionality. These vessels were fully functional and had extremely low leakage in comparison to the vector tumor, where about half of the vessels lacked perfusion.

Interestingly, studying choriocarcinoma, a cell line derived from a clinical tumor sample and which intrinsically express high levels of PlGF, we found that the vascular structures resemble those in PlGF expressing fibrosarcoma. The microvessels of choriocarcinoma had few branch points, high pericyte coverage, and a normalized phenotype. As shown in the fibrosarcoma cell line, the normalized vessels were functional. What also is so interesting, is that Choriocarcinoma has a very good prognosis and is highly sensitive to chemotherapy [111]. This is probably due to the highly functional tumor vessels, as the chemotherapeutic agents are thus better able to reach the tumor tissue. In the case of the chaotic tumor vessel structure, seen in VEGF-A producing tumors, the functionality of these vessels are very low. That means that the therapeutic drugs might not even reach some areas of the tumor tissue. Taken together with the fact that these VEGF-A-induced vessels are highly

leaky, causing high interstitial fluid pressure (IFP) in these kinds of tumors, it would make it difficult for any kind of exchange from healthy to tumor vessels [15]. An example of this is the lacking ability of tumor vessels to support sufficient oxygen exchange, which makes the tumor tissue hypoxic. Anti-angiogenic therapy causes normalization, and that could be the cause of why anti-angiogenic drugs show clinical benefits together with chemotherapy. Since anti-angiogenic drugs like sunitinib, normalizes the vasculature, and PlGF do the same, therefore PlGF could be used as a biomarker, for when the tumor would be sensitive for chemotherapy. It would be very interesting to pay more attention to this. What would the outcome be if, we treated these fibrosarcomas expressing the empty vector or PlGF with chemotherapeutic agents? Sunitinib, had no beneficial effect on the PlGF expressing tumors, but would the targeted receptor antibody have some? Further, the choriocarcinoma cells are also worth studying more, since these human tumors are sensitive for chemotherapy; there might be other important knowledge to be revealed regarding what makes some tumors sensitive and others not.

5.3 PAPER III:

Cancer-associated retinopathy is currently a non-treatable disease, even though it is uncommon, and therefore it is important to find promising therapeutic targets. Since anti-VEGF-A treatment has not been reported, to be applied in the treatment of CAR, it would be a new approach. Although it should be a treatment targeting VEGFR1 which are expressed on the vasculature and not VEGFR2, since VEGFR2 are expressed on the photoreceptors. The photoreceptors which already are affected in CAR and are therefore weakened in for the patient, these cannot sustain to be further attacked by some kind of treatment. Therefore the further research in this field has to

be conducted with care. The effects of VEGF-A binding to the VEGFR2 on these photoreceptors need further investigation. It would be interesting to study the vascular effects of bevacizumab, targeting VEGF-A, in CAR since it is used in other ocular diseases. Although then P-IGF would not be affected, but VEGFR2 on the photoreceptors cells would. Therefore it would be of great interest to further investigate the effects on retinal vasculature by targeting VEGFR1 directly.

Our research is focused on the vasculature. The malfunctioning retinal vessels in CAR are in need of evaluation, and here we have found that these vessels do not express VEGFR2, but exclusively VEGFR1. Further, these vessels are impaired, lacking protecting pericyte coverage and are leaky. This leak can cause retinal hemorrhage and edema. Therefore further investigations are needed, to evaluate if VEGFR1 could be targeted as a therapeutic approach.

5.4 PAPER IV:

Chemotherapy is one of the most efficient cancer treatments of certain tumor types. The drawback of chemotherapeutic drugs, are the severe side-effects. Hair loss, nausea, kidney cytotoxicity, bone marrow (BM) suppression, is just a few of the many side-effects caused by chemotherapeutic agents. The BM suppression, alone could lead to severe and life-threatening anemia, hemorrhage, and decreased blood clotting, decreased immune response, which can cause death by a simple infection. Anti-angiogenic drugs are simultaneously administered with the chemotherapeutic agents. There is not much known of the mechanisms behind the benefits of this combination and here we show that tumor-VEGF-A-induced BM suppression, can be inhibited by the anti-angiogenic drug sunitinib. Further, the chemotherapeutic drugs induces BM suppression, and in combination with tumors releasing VEGF-A, there

will be a synergistic and much more severe BM suppression. This increased suppression, causes therapy-induced mortality. That could be translated to patients with VEGF-A expressing tumors, as they would have much more severe and maybe even be lethal bone marrow toxicity when treated with chemotherapy. But for patients much more is needed to be investigated. Here, we show that if the anti-VEGF drug is given before the chemotherapies, the BM will be more normalized and this will rescue the mice, and prevent the increased mortality induced by the chemotherapies. Sunitinib which is a multi-tyrosine kinase inhibitor induces this rescue of the BM cells, and in previous publications we have shown, that anti-VEGFR2 would also prevent the VEGF-A induced BM damage. It needs to be further investigated whether other inhibitors, e.g. bevacizumab which is used in the clinical treatment of cancers, could cause the same rescue. Perhaps then these drugs could be used in other cancer types. Further, since we here show that VEGF-A should be suppressed before the chemotherapies, to give the BM a chance to recover, maybe patients with known VEGF-A-expressing tumors or if we screen patients for tumor-VEGF-A expression, they could be treated with the anti-VEGF-A drugs as a pre-treatment. Even though, advanced tumors should be treated as aggressively, as soon as possible, maybe if the patient receives these “recovering” drugs first, the patient have a chance to be able to withstand the severe and sometimes lethal side-effects induced by the chemotherapies. Further, when a patient have shown to have high VEGF-A values in the blood, perhaps it should be considered to give other chemotherapeutic drugs, which is not known to have highly BM suppressing side-effects. Another point of view is that, anti-angiogenic drugs far from always have any beneficial effect on the tumor growth per se. Here we showed in two cases, of different chemotherapeutic agents, that sunitinib had either no or a beneficial (normalizing) effect on the vasculature, but in both cases rescued the BM suppression. Although, much more is

to be investigated, here we propose a mechanism behind the beneficial effects of anti-VEGF drugs, and a schedule for their delivery in combination with chemotherapy which maybe ought to be considered when designing clinical trial with such drugs in the futures.

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