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ANGIOGENIC MECHANISMS IN ADIPOSE TISSUE AND TUMOR

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To those who believe…

ABSTRACT

Angiogenesis is involved in the development and progression of many human diseases, including cancer, cardiovascular diseases, chronic inflammation, and metabolic diseases. Despite differences in microenvironment under various pathological settings, angiogenic blood vessels share some common features in numerous diseases. This thesis reveals novel molecular mechanisms of angiogenesis in tumors and adipose tissues, as well as defining potential therapeutic targets for treatment of cancer and obesity-associated metabolic diseases.

In Paper I, we showed that PDGF-BB is a tumor-derived vascular remodeling factor that promotes tumor growth through activation of stromal fibroblasts and perivascular cells in tumor microenvironment. Tumor-derived PDGF-BB activates stromal fibroblasts to produce erythropoietin (Epo), which in turn triggers extramedullary hematopoiesis thereby enhancing oxygen perfusion in tumor vasculatures leading to an accelerated tumor growth rate. Epo is also known as a potent angiogenic factor which acts directly on endothelial cells (ECs) to induce tumor neovascularization. Therefore, PDGF-BB modulates tumor angiogenesis, vascular remodeling and hematopoiesis, via activation of the Epo signaling pathway, thus facilitating tumor growth, invasion and possibly reduces drug responsiveness. Understanding the role of Epo in promoting tumor growth and angiogenesis not only provides novel mechanistic insights into the complex interplay between various signaling pathways involved in the stimulation of angiogenesis, but also highlights the risk associated with using Epo in treatment of cancer-associated anemia.

In Paper II, we used mouse tumor models to propose a novel mechanism underlying the combination therapy consisting of anti-angiogenic and chemotherapeutic agents commonly used in human patients. We showed that tumor-derived VEGF induces severe aplastic anemia in mice, and delivery of chemotherapeutics to these tumor-bearing mice led to an earlier demise due to the synergistic or additive suppression of bone marrow hematopoiesis by VEGF and chemotherapy. Switching to a sequential delivery of anti-angiogenic drugs prior to administration of chemotherapeutics drugs resulted in significant recovery of bone marrow hematopoiesis, and thus markedly increased tolerance to chemotoxicity. Given the fact that a significant number of cancer patients die of chemotoxicity, our findings provide an important mechanism in which anti-angiogenic drugs decreases chemotoxicity.

In Paper III, we discuss the novel methods we developed for the study of adipose angiogenesis, which are becoming increasingly used by other scientists. In Paper IV, we showed for the first time that cold acclimation of mice markedly activates an angiogenic phenotype via sympathetic upregulation of VEGF expression. Importantly, inhibition of angiogenesis significantly modulates adipose metabolism. This work provides the first example where targeting adipose vasculature might provide a novel therapeutic approach for the treatment of obesity and metabolic diseases.

LIST OF PUBLICATIONS

I. Xue Y, **Lim S**,Yang Y, Wang Z, Jensen LD, Hedlund EM, Andersson P, Sasahara M, Larsson O, Galter D, Cao R, Hosaka K, Cao Y. PDGF-BB modulates hematopoiesis and tumor angiogenesis by inducing erythropoietin production in stromal cells. Nature Medicine. 2011 Dec 4;18(1):100-10

II. Zhang D, Hedlund EM, **Lim S**, Chen F, Zhang Y, Sun B, Cao Y. Anti-angiogenic agents significantly improve survival in tumor-bearing mice by increasing tolerance to chemotherapy-induced toxicity. Proc Natl Acad Sci U S A. 2011 Mar 8;108(10):4117-22

III. Xue Y*, **Lim S***, Bråkenhielm E, Cao Y. Adipose angiogenesis: quantitative methods to study microvessel growth, regression and remodeling in vivo. Nature Protocols. 2010 May;5(5):912-20 *Equal contribution

IV. Xue Y, Petrovic N, Cao R, Larsson O, **Lim S**, Chen S, Feldmann HM, Liang Z, Zhu Z, Nedergaard J, Cannon B, Cao Y. Hypoxia-independent angiogenesis in adipose tissues during cold acclimation. Cell Metabolism. 2009 Jan 7;9(1):99-109

LIST OF RELATED PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. Yang X, Zhang Y, Yang Y, **Lim S**, Cao Z, Rak J, Cao Y. Vascular endothelial growth factor-dependent spatiotemporal dual roles of placental growth factor in modulation of angiogenesis and tumor growth. Proc Natl Acad Sci U S A. 2013 Aug 20;110(34):13932-7

II. Dong M, Yang X, **Lim S**, Cao Z, Honek J, Lu H, Zhang C, Seki T, Hosaka K, Wahlberg E, Yang J, Zhang L, Länne T, Sun B, Li X, Liu Y, Zhang Y, Cao Y. Cold Exposure Promotes Atherosclerotic Plaque Growth and Instability via UCP1-Dependent Lipolysis. Cell Metabolism. 2013 Jul 2;18(1):118-29

III. Hosaka K, Yang Y, Seki T, Nakamura M, Andersson P, Rouhi P, Yang X, Jensen L, **Lim S**, Feng N, Xue Y, Li X, Larsson O, Ohhashi T, Cao Y. Tumour PDGF-BB expression levels determine dual effects of anti-PDGF drugs on vascular remodelling and metastasis. Nature Communications. 2013 Jul 8;4:2129

IV. **Lim S**, Honek J, Xue Y, Seki T, Cao Z, Andersson P, Yang X, Hosaka K, Cao Y. Cold-induced activation of brown adipose tissue and adipose angiogenesis in mice. Nature Protocols. 2012 Mar 1;7(3):606-15

V. Cao Y, Arbiser J, D'Amato RJ, D'Amore PA, Ingber DE, Kerbel R, Klagsbrun M, **Lim S**, Moses MA, Zetter B, Dvorak H, Langer R. Forty-year journey of angiogenesis translational research. Science Translational Medicine. 2011 Dec 21;3(114):114rv3

VI. Xue Y, Chen F, Zhang D, **Lim S**, Cao Y. Tumor-derived VEGF modulates hematopoiesis. Journal of Angiogenesis Research. 2009 Dec 23;1:9

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LIST OF ABBREVIATIONS

Ang	Angiopoietin
BAT	Brown Adipose Tissue
BMI	Body Mass Index
CTX	Cyclophosphamide
DIO	Diet Induced Obesity
EC(s)	Endothelial Cell(s)
Epo	Erythropoietin
EpoR	Erythropoietin Receptor
FDA	Food and Drug Administration
FDG	[^{18}F]fluorodeoxyglucose
HGB	Hemoglobin
HCT	Hematocrit
HIF	Hypoxia Inducible Factor
iBAT	Interscapular Brown Adipose Tissue
iWAT	Inguinal White Adipose Tissue
MCC	Metastatic Colon Cancer
NE	Norepinephrine
NST	Non Shivering Thermogenesis
OECD	Organization for Economic Co-operation and Development
PDGF-BB	Platelet-Derived Growth Factor-BB
PDGFR-(α/β)	Platelet-Derived Growth Factor Receptor-(alpha/beta)
PET-CT	Positron Emission Tomography-Computerized Tomography
PGC-1α	Peroxisome Proliferator-Activated Receptor-Gamma Coactivator-1 alpha
PPAR-γ	Peroxisome Proliferator-Activated Receptor-Gamma
RBC(s)	Red Blood Cell(s)
rhEpo	Recombinant Human Erythropoietin
TKI(s)	Tyrosine Kinase Inhibitor(s)
TKR(s)	Tyrosine Kinase Receptor(s)
TSP(s)	Thrombospondin(s)
UCP1	Uncoupling Protein 1
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WAT	White Adipose Tissue
WBC(s)	White Blood Cell(s)
WHO	World Health Organization

1 INTRODUCTION

1.1 ANGIOGENESIS

Angiogenesis, the process of the formation of new blood vessels from existing vasculature, is vital in growth and development in both physiological and pathological conditions[1,2]. It can be divided into two types, sprouting and intussusceptive angiogenesis[2-4]. In healthy adults, blood vessels are usually quiescent until activated during processes such as wound healing, the female reproductive cycle and pregnancy[5,6]. Angiogenesis is highly governed by the balance of pro-angiogenic stimulators and inhibitors, therefore pathological conditions including cancer, diabetes and macular degeneration arise when this balance is tipped[7,8]. The most well-known angiogenic stimulators and inhibitors are vascular endothelial growth factor-A (VEGF-A) and thrombospondins (TSPs), respectively (see 1.1.2.1 and 1.1.3.1).

1.1.1 Angiogenic process

Angiogenesis is a dynamic and complex process. Angiogenic response is mediated by regulators such as growth factors, cytokines, matrix metalloproteinases (MMPs), laminins and integrins[9-11]. Blood vessels are composed of at least two cell types; 1) endothelial cells (ECs), which form the lumen of the blood vessels, and 2) mural cells that surround the blood vessels to ensure its maturation and the stability.

Hypoxia is one of the most potent triggers of angiogenesis. When ECs sense a low level of oxygen in the local environment, they upregulate hypoxia inducible factor (HIF) which in turn upregulate VEGF[12,13]. Neighboring quiescent ECs respond to the VEGF gradient, and compete to become either tip cells or supporting stalk cells. Tip cells typically have long filopodia which sprout and migrate towards a high level of VEGF concentration, whereas stalk cells that precede tip cells are highly proliferative to "push" the tip cells closer to the VEGF gradient[14,15]. Tip and stalk cell formation are highly regulated by VEGF and Notch signaling pathways. VEGF binds to vascular endothelial growth factor receptor-2 (VEGFR-2) on tip cells and stimulates delta-like 4 production[16,17]. Delta-like 4 then binds to transmembranal Notch receptors on nonsprouting stalk cells. Simultaneously, when the dynamic angiogenic response is triggered, anti-angiogenic factors such as endostatin, angiostatin and TSP are upregulated[18-21]. Activated ECs also secrete MMPs to breakdown the basement membrane of blood vessel walls, thereby allowing ECs to proliferate and migrate[11,22].

The sprouting tip cells proliferate to form a capillary tube through anastomosis. The newly formed lumen allows blood flow that provides oxygen to the hypoxic tissue. Increased oxygenation cancels the hypoxia signal, thereby reducing VEGF secretion[12,13,23]. Meanwhile, tip cells secrete platelet derived growth factor-BB (PDGF-BB), which recruits platelet derived growth factor receptor-beta (PDGFR-β) positive mural cells to ensure maturation and stability of the newly formed capillary[24,25].

1.1.2 Angiogenic stimulators

Positive regulators of angiogenesis can range from growth factors to cytokines that switch on the angiogenesis process in quiescent vessels[1,2,5,6,19]. Many angiogenic stimulators have been purified and characterized, including VEGF (see 1.1.2.1), PDGF (see 1.1.2.2), Angiopoietin (Ang)/Tie2 (see 1.1.2.4), erythropoietin (Epo) (see 1.1.2.3), hepatocyte growth factor, fibroblast growth factor and MMPs.

1.1.2.1 Vascular endothelial growth factor (VEGF)

VEGF is one of the best-characterized angiogenic growth factors involved in regulations of both physiological and pathological angiogenesis[23]. In 1983, Senger et al. purified a 38 kDa protein known as vascular permeability factor from pigs' liver tumor, which was later determined to be similar to VEGF purified by Ferrara et al. in 1989[26,27].

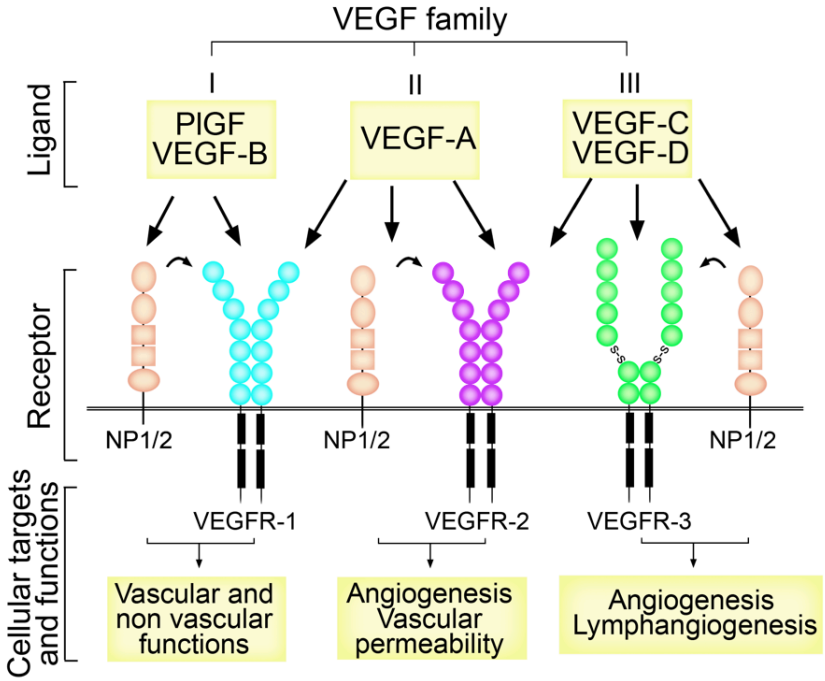

Figure A. The VEGF family and VEGF receptors

The VEGF family of proteins consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF[28-30]. VEGF proteins bind to the tyrosine kinase receptors (TKRs), VEGFR-1,

-2, and -3, triggering downstream signaling cascades that stimulate growth, proliferation, and migration of ECs[28-30] (Figure A). Different VEGF-A exists in isoforms as a result of alternative splicing. VEGF-A binds to VEGFR-1, VEGFR-2, neuropilin 1 (NP1) and neuropilin 2 (NP2). VEGFR-1 and VEGFR-2 are primarily expressed on ECs and other cell types including neurons and hematopoietic stem cells.

VEGF-A binds VEGFR-1 with higher affinity, but most of the biological effects are exerted through interaction with VEGFR-2 due to the weaker kinase activity of VEGFR-1. It is therefore postulated that VEGFR-1 acts as a decoy receptor that reduces binding of VEGF-A to VEGFR-2, hence dampening the angiogenic response. While VEGFR-1 knockout mice are embryonic lethal due to defects in hemangioblast differentiation and impaired vascular formation, VEGFR-2 knockout mice are embryonic lethal due to defects in hematopoietic precursors[31-33]. The expression of VEGF is driven by environmental cues such as hypoxic conditions under which hypoxia inducible factor (HIF) is upregulated, as well as factors such as leptin, insulin, oncogenes and tumor suppressor genes[13,23,34,35].

1.1.2.2 Platelet derived growth factor (PDGF)

Members of the PDGF family and their receptors, PDGFRs, have been reported to influence tumor growth and invasive progression through interaction of tumor cells and their surrounding stroma[24].

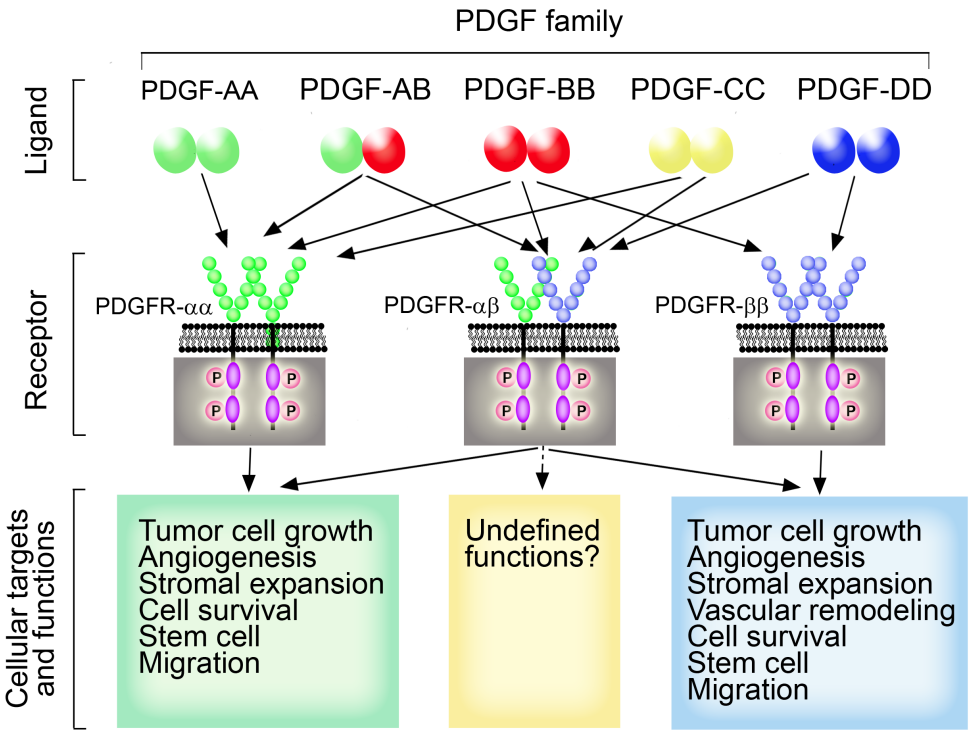

Figure B. The PDGF family and PDGF receptors

The PDGF family consists of five homodimers or heterodimers, PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC and PDGF-DD which bind to their respective TKRs (Figure B), PDGFR-alpha (PDGFR-α) and PDGFR-beta (PDGFR-β), thereby activating downstream signaling pathways resulting in the modulation of ECs survival, growth and migration[25,36].

PDGF-BB/PDGFR-β signaling pathway is crucial in mediating pericyte recruitment to blood vessels[37,38]. PDGF-BB is secreted as a homodimer by ECs of sprouting tip cells, which recruit PDGFR-β positive pericyte onto the nascent vasculature. The recruitment of pericytes to blood vessels is essential in maintaining the normal function of vessels. Lack of pericyte coverage, as seen in PDGF-BB or PDGFR-β deficient mice, results in unstable, leaky vessels and irregular vascular network[38,39]. Increased PDGF and PDGFR expression have been reported in different cancer types including glioma, prostate cancer and pancreatic cancer[40,41].

The PDGF/PDGFR signaling cascade has been shown to play an essential role in invasion and metastasis by promoting epithelial mesenchymal transitions in colorectal and breast cancers[42,43]. Nilsen et al. reported that coexpression of PDGF-BB and fibroblast growth factor results in a synergistically induced tortuous vascular network[44]. PDGF-BB can also induce angiogenesis via upregulation of Epo (see Paper I). Anti-PDGF drugs are currently used in the clinic for treatment of leukemia and mastocytosis[45,46]. However, it was shown in a recent finding that the anti-PDGF drug imatinib mesylate inhibited PDGF-BB/PDGFR-β signaling cascade in high PDGF-BB producing tumor, but promoted the repulsion of pericytes from tumor vessels in low producing tumor, causing implications in tumor invasion[47]. Therefore, anti-PDGF drug treatment should be administered based on PDGF-BB levels in patients.

1.1.2.3 Erythropoietin (Epo)

Epo, a cytokine hormone with a molecular weight of 34 kDa, controls erythropoiesis, the process of red blood cell (RBC) production. The kidney is the primary source of Epo[48]. Epo binds to Epo receptor (EpoR) on erythroid progenitors cells triggering downstream signaling pathways resulting in the proliferation, maturation and differentiation of RBCs[49]. In the clinic, recombinant human Epo (rhEpo) is used as a treatment regimen against anemia observed in patients suffering from kidney disease, HIV, and in cancer patients receiving chemotherapeutic drugs[50,51]. Epo delivery improves the level of RBC, thereby improving oxygenation[51,52]. However, the administration of rhEpo to patients should be monitored closely as EpoR is also found

on different cell types, and the Epo/EpoR axis has been shown to induce proliferation, angiogenesis, and inhibit apoptosis thereby supporting tumor invasion[49,53,54].

1.1.2.4 Angiopoietin and Tie receptors

Ang ligands and Tie receptors play essential roles in vascular development, remodeling and maturation. The Ang family consists of Ang-1 and Ang-2. Ang-1 acts as an agonist while Ang-2 acts as an antagonist for the Tie2 receptor[55]. Ang-1 binds to Tie2 receptor on ECs to induce autophosphorylation of Tie2, which stimulates ECs survival and angiogenesis. On the other hand, Ang-2 binds to Tie2 receptors on ECs with similar affinity to that of Ang-1, resulting in competitive binding and inhibition of vascular maturation and stabilization. Ang-1, Ang-2 and Tie2 receptors are crucial in early vascular development, and deletion of Ang-1, Ang-2 or Tie2 causes defects in embryonic vascular development[56,57].

1.1.3 Angiogenic inhibitors

Angiogenic inhibitors can be categorized into endogenous and exogenous. Endogenous inhibitors include soluble VEGFR-1, Ang-2, TSP, angiostatin, integrins and endostatin, which exert their effect through inhibition of ECs survival, proliferation and migration[10,18-21,55,58]. Angiostatin and endostatin seem to be the most promising amongst all endogenous inhibitors, however, the beneficial outcome of administering exogenous angiostatin and endostatin can be difficult to predict[20,21,58,59].

1.1.3.1 Thrombospondin (TSP)

TSP was the first endogenous angiogenesis inhibitor reported in tumors[60]. The TSP family is comprised of five multimeric glycoproteins, which regulate cell-cell and cell-matrix interactions[18]. In the tumor environment, TSP-1 and TSP-2 serves as potent endogenous inhibitors of angiogenesis by activating transforming growth factor beta, thus suppressing tumor angiogenesis[61]. TSP-1 binds to VEGF, which prevents the release of VEGF from extracellular matrix. Deletion of TSP-1 results in enhanced angiogenesis[62].

1.1.3.2 Angiostatin and Endostatin

Several angiogenic inhibitors are proteolytic fragments of larger proteins. For example, angiostatin is a fragment of plasminogen and was first isolated in 1994 from serum and urine of tumor-bearing mice[21]. Angiostatin has been reported to bind to several

receptors on the ECs including adenosine triphosphate synthase, annexin II angiomotin and integrin $\alpha_v\beta_3$[21].Angiostatin was reported to inhibit tumor angiogenesis and growth by inhibiting ECs migration, proliferation and via induction of apoptosis[58].

Endostatin, a 20 kDA protein fragment of collagen XVII, is a member of the endogenous anti-angiogenic factors. The naturally produced endostatin, first described by O'Reilly and Folkman in 1997, was reported to inhibit angiogenesis and shrank tumor growth significantly[20]. Endostatin inhibits angiogenesis by downregulating pathways involving tumor necrosis factor-alpha, nuclear factor kappa B and ephrin which results in inhibition of survival and migration of ECs. This led to the belief that endostatin was the "ideal anti-cancer weapon" to eradicate cancer. In 1998, Dr James Watson said: "*Judah Folkman is going to cure cancer in two years*." However, after a phase II trial, it was concluded that endostatin did not result in a significant tumor regression in patients with advanced neuroendocrine tumors[59,63]. In conclusion, the use of these endogenous inhibitors warrants further investigations.

1.2 CANCER

1.2.1 Tumor angiogenesis

In 1971, Dr Judah Folkman, a prestigious cancer surgeon, revolutionized our understanding of cancer with his remarkable breakthrough when he coined the concept of tumor angiogenesis – the process of blood vessels formation in a tumor[7]. He postulated that in order for a tumor mass to grow beyond 2 to 3 mm^3, new blood vasculature (neovascularization) is required to supply oxygen and nutrients, as well as remove metabolic waste products[1]. Therefore, anti-angiogenesis can be an effective therapy against tumor growth if deprivation of tumor cells from oxygen and nutrients is effectively achieved[64,65]. Since then, it has stirred immense interest in the research community, and many therapeutic drugs have been developed to target angiogenesis dependent diseases including cancer, ophthalmic diseases, arthritis, psoriasis, obesity and obesity-related metabolic diseases[5-8,19,65,66].

Cancer is a complex disease. Tumor angiogenesis – the sophisticated process employed by cancer cells to grow blood vessels to support their own growth – is as intricate as a Gordian knot. Angiogenesis is essential, and crucial for physiological processes including regulation of fetal development, the menstrual cycle, and wound healing[5-7,67,68]. Similar to healthy tissues, the growth and progression of cancer is highly dependent on angiogenesis[1,7,8,69].

Tumor angiogenesis is one of the most important steps in mediating tumor progression and development. When the tumor mass is small, tumor cells can rely solely on the host blood vessels for oxygen, nutrients and the removal of metabolic waste products. However, angiogenesis is required to further support its growth beyond 2 to 3 mm^3 [1,7,8]. Tumor cells trigger angiogenesis via sprouting angiogenesis and intussusceptive angiogenesis[70,71]. Unlike blood vessels in healthy tissues, tumor vessels are usually highly dilated. Tumor vessels are also highly disorganized, and arterioles, venules and capillaries become difficult to classify. Tumor vessels are highly permeable and leaky due to increased endothelial fenestrations[72]. These abnormal characteristics of tumor vessels caused improper delivery of oxygen and nutrients resulting in a hypoxic environment, which further activate the angiogenic cascade[12,13] (see 1.1.1). Intussusceptive angiogenesis, also known as vessel splitting, is initiated when ECs from opposite sides of the capillaries protrude towards each other, which creates a lumen and further fuses to complete the formation of a functional neovasculature[71].

Cancer cells fully exploit the blood vessels to succinate their survival and mediate their progression and dissemination[67,68,73,74]. Blood vessels not only provide the necessary nutrients to support tumor growth, they also act as a passageway for cancer cells to leave the primary site, enter the circulation and metastasize to a new site[67,68,73,74]. Therefore, understanding the role of angiogenesis in cancer development has pivotal role in the circumvention of cancer[65].

1.2.2 Cancer

Cancer is one of the major causes of mortality and morbidity worldwide, where one in four individuals would be affected. The World Health Organization (WHO) reported in 2008 that approximately 7.9 million deaths (13% of all death) were due to cancer. The WHO also projected that the number of deaths from cancer will increase 45% from 2007 to 2030. Fortunately, with the advancement in modern science and technology, we are getting closer to unravel this complex disease. However, continuous effort is required to understand this complex disease as cancer cells develop resistance mechanisms to therapy, which is one of the major hurdles in the treatment of cancer.

1.2.3 Cancer progression

Cell division and proliferation are usually tightly controlled and highly governed by DNA damage repair genes, apoptotic genes, tumor suppressors and immune cells.

When these processes become unregulated, it results in an abnormal uncontrolled cell growth, which is a characteristic of cancer[8,67,68]. The progression and development of cancer is a long process that begins when a cell in a tissue or organ evades the usual process of apoptosis and continues to multiply uncontrollably. Perpetual propagation of these cells result in cancer. Exposure to chemical carcinogens or ionizing radiation as well as infection by viruses such as human papillomavirus, hepatitis B and C, and Epstein-Barr virus can induce genomic instability through accumulation of consequential mutations[75-77].

The word "tumor" is derived from the Latin "tumere", which means to swell. Tumor can be classified as benign or malignant. Tumors that are not cancerous are classified as benign. Fortunately, not all tumor or neoplasm masses develop or progress into cancer. This form of tumor is not life threatening, but may pose problems to local tissue due to the pressure they exert on neighboring tissues and organs. Malignant cancer on the other hand is life threatening as they not only invade the local primary site, but also extravasate to distal organs by metastasis[67,68,73]. Cancer cells are malleable and can adapt to best exploit the host and eventually destroy it. The process of metastasis is a long process that involves different steps: Malignant cancer cells have to first intravasate into the blood stream, survive in the blood stream, invade a new site and start proliferating in the new niche[67,68,73].

It is well accepted that tumor environment is complex due to the interactions of various cell types such as fibroblasts, ECs, mesenchymal and immune cells, each of which play their part in tumor growth, development and progression by secreting a myriad of cytokines and growth factors into the tumor microenvironment[67,68,72-74,78]. Cancer cells that are capable of developing resistance to drugs are probably assisted by various cell types in the tumor microenvironment[79,80]. It was reported that only minority of cancer deaths is caused by the primary tumor, whereas most cancer patients die from metastasis at the later stages of their malignancy[81]. Cachexia manifestation in cancer patients contributes to 20% of cancer deaths[82]. Taken together, this suggests that an "off tumor target regimen" could be a better approach to circumvent and improve the survival of these cancer patients[83].

1.2.4 Anti-angiogenic therapy in cancer

To date, many anti-angiogenic drugs have been developed and approved by the Food and Drug Administration (FDA) for the treatment of many types of cancer based on the humdinger concept proposed by Dr Judah Folkman[1,7,64]. Anti-angiogenic drugs can be

classified into subtypes: 1) Monoclonal antibodies such as bevacizumab (see 1.2.4.1), 2) ligands targeting the VEGF pathway such as aflibercept and ramucirumab, 3) small molecule tyrosine kinase inhibitors (TKIs) such as imatinib mesylate (see 1.2.4.2), sunitinib (see 1.2.4.3), sorafenib, pazopanib, and 4) inhibitors of mTOR kinases such as everolimus. Anti-angiogenic drugs are commonly administrated in combination with conventional chemotherapy[84] (Figure C).

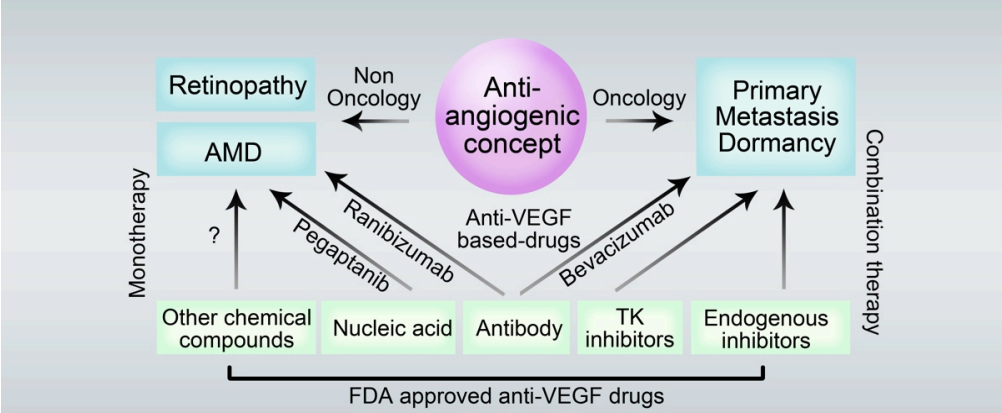

Figure C. Anti-angiogenic drugs targeting VEGF and VEGF receptors

Similar to most drugs, anti-angiogenic drugs cause side effects including gastrointestinal perforation, hypertension and proteinuria[65] (Figure D). It is proposed that anti-angiogenic drugs to normalize blood vessels to allow higher efficacy of drug delivery[85]. In Paper II, we hypothesized that sequential delivery of anti-angiogenic drug prior to chemotherapy could protect bone marrow against chemotherapy-induced deficits in hematopoietic regeneration.

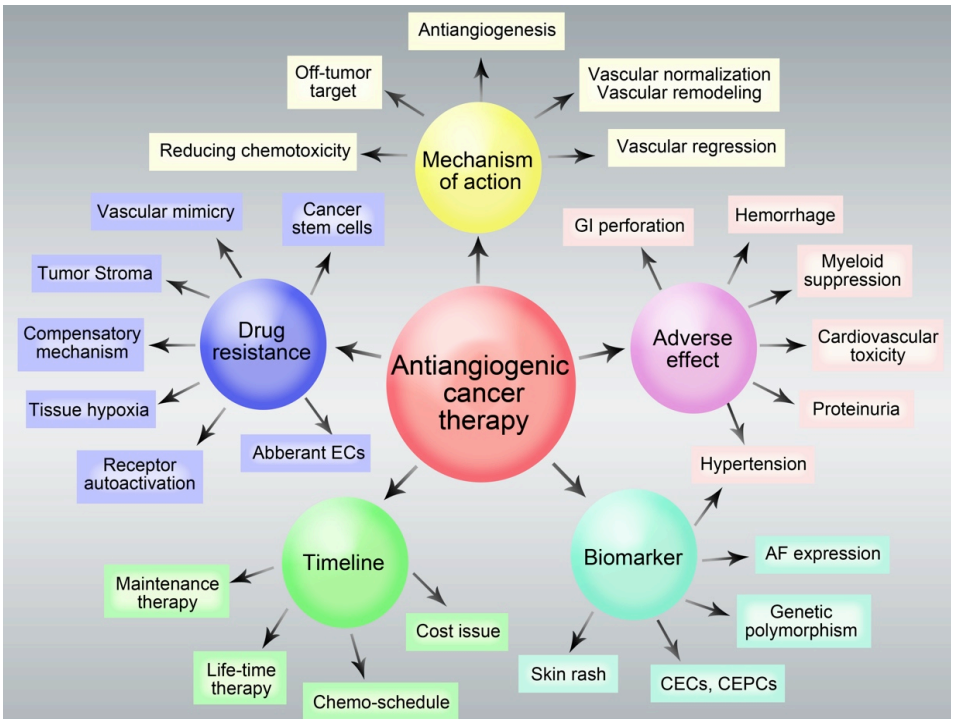

Figure D. Common side effects of anti-angiogenic therapy

1.2.4.1 Bevacizumab

Bevacizumab, also known as Avastin® (Genentech) is the first humanized monoclonal antibody, which recognizes and binds specifically to VEGF[86] (see 1.1.2.1). Bevacizumab prevents VEGF from binding to receptors, thereby inhibiting the downstream signaling cascade, preventing angiogenesis and depriving tumor cells from oxygen and growth factors needed for growth[86]. Bevacizumab was first approved in 2004 by the FDA as a first-line therapy for metastatic colorectal cancer (MCC) in combination with chemotherapy drug[84,87]. Bevacizumab administration to MCC patients improved overall survival by five months and progression free survival for four months. In 2006, bevacizumab was approved in non-small cell lung cancer used in combination with chemotherapy, carboplatin and paclitaxel[88]. In 2008, FDA granted the use of bevacizumab in metastatic HER2-negative breast cancer[89]. Bevacizumab was further approved in 2009 for patients with metastatic renal cell carcinoma[90].

It looked promising until 2011, when FDA withdrew the use of bevacizumab in metastatic HER2-negative breast cancer. Bevacizumab, like all other drugs, has side effects, the most serious of which includes gastrointestinal perforation, hemorrhage, hypertension and incomplete wound healing[91]. Unfortunately, in the case of metastatic breast cancer, the side effects of bevacizumab outweighed the beneficial effects observed in these cancer patients[92].

1.2.4.2 Imatinib mesylate

Imatinib mesylate, also known as Gleevec® or STI571 (Norvatis) is a small molecule TKI. Imatinib mesylate prevents proliferation by competively binding to tyrosine kinases, hence preventing the binding of substrates to the kinase sites. Imatinib mesylate binds to tyrosine kinase such as bcr-abl, c-kit and PDGFR[93-95]. It was first approved by the FDA in 2002 for the treatment of gastrointestinal stromal tumors and subsequently, approved for the treatment of leukemia and mastocytosis. Imatinib mesylate causes adverse effects including diarrhea, hypertension, hand-foot skin reaction and skin rash[96].

1.2.4.3 Sunitinib malate

Sunitinib malate, or Sutent® (Pfizer) is a TKI approved by the FDA for the treatment of gastrointestinal stromal tumor, pancreatic cancer and renal cell carcinoma[97-99]. Sunitinib malate targets the intracellular domain of tyrosine kinase including VEGFRs, PDGFRs, c-kit, Ret, CSF-1R and Flt-3[93,100]. Sunitinib is approved by the FDA for the treatment

of gastrointestinal stromal tumor intolerant to imatinib mesylate. Adverse effects of sunitinib includes gastrointestinal toxicities, hypertension, hair depigmentation and dermatologic sensitivity[93].

1.2.5 Chemotherapy

Chemotherapy primarily aims to kill rapidly dividing cells such as cancer cells by targeting their RNA and DNA, hence preventing their division[101]. Chemotherapy can be categorized into different classes based on the mechanisms to kill cancer cells and their chemical structure (i.e. platinum-based drugs, taxanes and vinca alkaloids)[102]. Most chemotherapy belongs to a class of drugs known as alkylating agents, which prevent cancer cells from growing and thus killing them. However, chemotherapy is unable to differentiate between dividing cancerous and noncancerous cells, therefore resulting in adverse effects. As many chemotherapeutic drugs induce severe impairment in bone marrow hematopoietic regeneration, most chemotherapy is given as a combination therapy with other anti-cancer drugs[103,104]. In this thesis, we set out to ask if anti-angiogenic drugs administered as neoadjuvant threapy to cancer-induced bone marrow impaired patients would first allow the bone marrow to have time to recover before further exposure to chemotherapy.

1.2.5.1 Carboplatin

Carboplatin, also known as paraplatin, is a cisplatin that binds to DNA and kills proliferating cancer cells[101]. Carboplatin was approved by the FDA to use as a single agent therapy or in combination with other drugs for the treatment of non-small cell lung cancer and recurrent ovarian cancer[105,106]. Common side effects of carboplatin include decrease in RBC counts, low platelet count and neutropenia which causes anemia, increased risk of bleeding and increased risk of infections, hair loss, nausea and vomiting[103,104].

1.2.5.2 Cyclophosphamide

Cyclophosphamide or more commonly known as CTX has been approved for the treatment for lymphoma, leukemia, multiple myeloma, breast and ovarian cancers[107]. CTX elicits its effect by crosslinking with DNA therefore inhibiting cell proliferation. CTX causes possible adverse effects such as increased risk to infection due to decrease in white blood cell (WBC) counts, bleeding in the bladder, hair loss, nausea and vomiting[103,104,107].

1.3 ADIPOSE TISSUE

Obesity and over weight are first world health epidemics that requires immediate attention due to the implicated major chronic diseases that are tagged along with this metabolic disorder caused by excessive fat (adipose tissue) accumulation[35,108,109]. Obesity and overweight is also escalating in the developing countries. The prevalence rate of obese individuals has escalated at least two-folds within the last 30 years. According to the WHO, an individual is classified as overweight when body mass index (BMI), calculated by weight in kilograms divided by the square of height in meters, is equal to or more than 25; with a BMI of more than 30, an individual is classified as obese.

The WHO projected that in 2015, approximately 2.3 billion adults will be overweight and 700 million will be obese. The fact that childhood obesity has tripled in the past 30 years is becoming an urgent issue to tackle. Childhood obesity predicts serious and imminent problems on physical and psychological health. Excessive accumulation of fat from high food intake and low physical activities attributes to health impairing diseases such as type II diabetes, cardiovascular diseases, stroke and certain types of cancers[35,110-114]. Therefore, therapeutic intervention of obesity requires immediate attention.

1.3.1 White adipose tissue and brown adipose tissue

Similar to other organs, the growth and regression of adipose tissue is highly regulated by blood vessels[115]. Adipose tissue in humans and other mammals can be broadly categorized into white adipose tissue (WAT) and brown adipose tissue (BAT). Substantial evidence now suggests a third type of adipose tissue, the brite (brown-in-white) / beige adipose tissue[110-112]. WAT is present in the subcutaneous and visceral abdominal fat, stores energy as large fat droplets in the form of triglycerides, which upon lipolysis releases fatty acids and glycerol. Aside serving as mechanical support for several organs in our body, WAT is also a rich source of adipokines[35,109,113,116].

In contrast, BAT, present in smaller amount in the cervical and supraclavicular areas, consists of multilocular droplets that are rich in mitochondria (see 1.3.3). BAT is essential for facultative and adaptive thermogenesis, and is believed to be promising for combatting obesity[117-119]. Accumulation of visceral fat is considered to be more harmful due to its high correlation with metabolic disorders such as glucose intolerance, hypertension, dyslipidemia and insulin resistance[120].

1.3.2 Vascular functions and adipose derived factors

WAT and BAT are highly vascularized, with almost each adipocyte typically surrounded by a blood vessel, which provides oxygen and nutrients for growth and maintenance, as well as the removal of metabolic waste products[35,121,122]. Most people are more familiar with WAT – the blob or rather blobs of unwanted fat accumulation in undesirable places of the body, (something) we yearned to get rid of – without fail appears on our top ten New Year's resolution. And if they are gone, oh good riddance, we praise. The intimate interaction between adipocyte and blood vessels is crucial for the regulation of homeostasis in the adipose tissue[35,113-115,122]. Vascular density in the adipose tissue is highly correlated to the metabolic statues of the adipose tissue; BAT has significantly higher blood vessel density to cope with its high metabolic demands, whereas the metabolically less active WAT is also less vascularized. Blood vessels are also sources of growth factors, cytokines, hormones, stem cells and inflammatory cells that are essential for the maintenance of the adipose tissue[35,109,113-116] (Figure E).

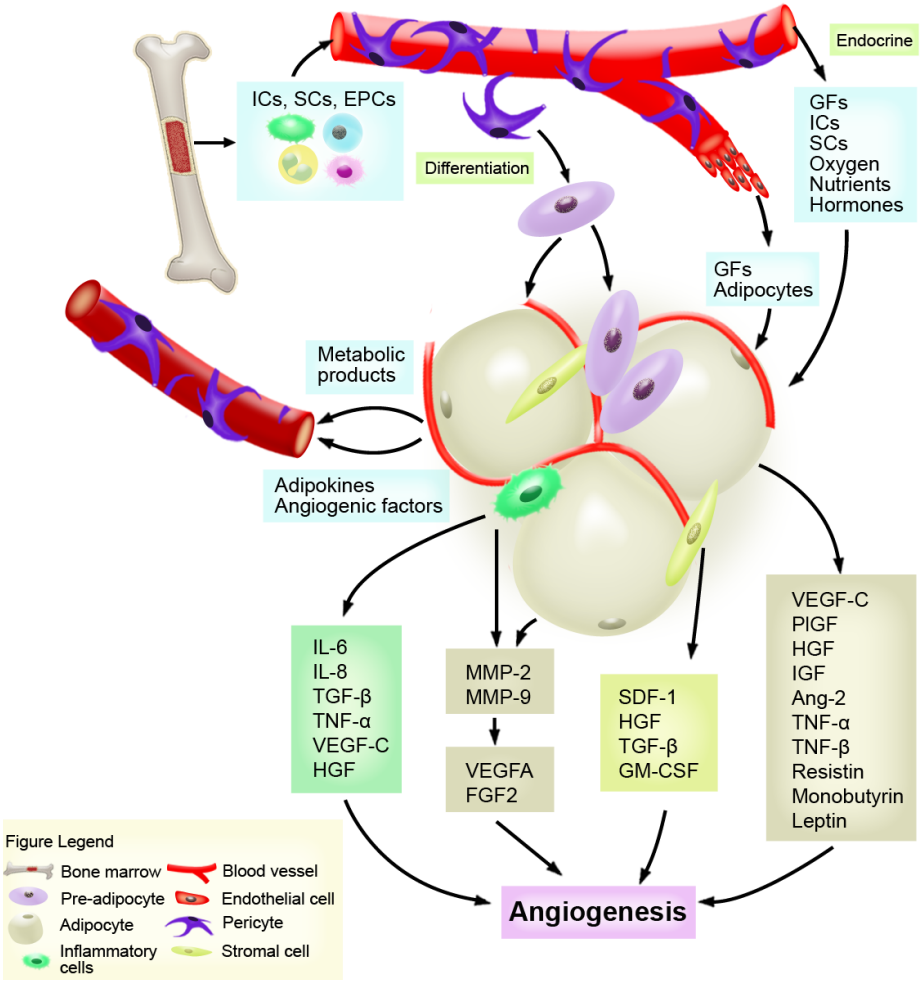

Figure E. Vascular functions and adipose-derived factors (adapted from Angiogenesis in Adipose tissue, Springer book, 2013).

Adipose tissue is one of the most plastic organs in the body. Its capability to expand and regress up to 10-fold throughout adulthood is highly dependent on blood vessels, suggesting that adipose tissue has the ability to maintain and also to recruit new vessels[115,116]. Adipose tissue is now accepted as an endocrine organ, which secretes a myriad of cytokines and growth factors – collectively known as adipokines[109]. Apart from adipocytes, adipose tissue comprised of ECs, preadipocytes, macrophages, lymphocytes and fibroblasts. The hypoxic conditions resulting from the expansion of adipocytes drives HIF-1α, which further induced expression of VEGF, leptin, tumor necrosis factor-alpha, and plasminogen activator inhibitor-1, thereby initiating neovascularization[12,35,113,114,116]. Adipose tissue produces angiogenic inhibitors such as adiponectin, which inhibits angiogenesis. Adiponectin expression levels negatively correlate with obesity. Adipose tissue also produces other inhibitors such as endostatin, TSP-1 and VEGFR-2[123,124].

1.3.2.1 VEGF in adipose tissue homeostasis

VEGF is highly expressed in adipose tissues and function as the main angiogenic response. Adipocytes and resident macrophages have been reported to be the main sources of VEGF[34,125]. The expression and production of VEGF in adipose tissue is driven mainly by insulin and hypoxia[34,126]. Genetically modified mouse with adipose tissue specific VEGF knockout (aP2-Cre) resulted in abnormal decrease in blood vasculature in adipose tissue[127]. It was reported that blockade of VEGF or VEGFR-2 function inhibits WAT expansion in diet-induced obesity (DIO)[128]. Independent research groups reported that hypoxia is experienced in obese individuals during the expansion of adipocytes (hypertrophy), which in turn stimulates angiogenesis[126]. However, Pasarica et al. demonstrated that VEGF level does not correlate with expanding adipocytes in human[129]. Modulation of VEGF in adipose tissues could be a therapeutic regimen for treatment of obesity and obesity-related metabolic diseases[122,130].

1.3.3 Brown adipose tissue and non-shivering thermogenesis

When mammals are exposed to cold, the initial mechanism is to shiver to defend their body temperature[119]. However, with time (adaptive thermogenesis), they will recruit non-shivering thermogenesis (NST), which increases their metabolism and heat production through activation of BAT[131]. In mice, norepinephrine (NE) is injected to investigate NST that originates from BAT (Paper IV). Cold exposure stimulates

sympathetic activation and release of NE, which then binds to the adrenergic receptors on plasma membrane on BAT. The downstream signaling activated as a result triggers the lipolysis of triglycerides into free fatty acids and glycerol. Free fatty acids then bind to uncoupling protein 1 (UCP1) stimulating respiration in the mitochondria. BAT, differs from its counterpart WAT, by its unique ability to burn fat to produce heat upon stimulation[119].

UCP1 is a transmembrane protein, virtually exclusively expressed in the inner membrane of mitochondria of BAT[132]. BAT has been reported to be the only organ in the body that is capable of adaptive thermogenesis[119]. Ablation of UCP1 results in obese phenotype in mice demonstrating the importance of UCP1 in regulating diet-induced adrenergic thermogenesis[133]. Recently publications also show the importance of BAT in regulating glucose homeostasis and plasma triglyceride clearance[134,135]. BMI was reported to negatively correlate to the amount of BAT. Taken together, these suggest that the activation of BAT in obese individuals may be a therapeutic intervention for obesity since merely 40-50 g of fully activated BAT is able to burn up to 4 kg of WAT in a year[136,137]. Despite all the investigations on BAT and thermogenesis, little is known about the role of blood vessels in regulating this highly metabolic tissue.

1.3.3.1 Refuting the dogma – Existence of brown adipose tissue in human adults

Research of BAT can be dated back to the 1960s where several groups had described the role of BAT in cold-induced thermogenesis in small mammals[138]. After 40 years, the longstanding dogma that adult human does not have functional BAT was being definitively refuted in 2009. BAT is believed to play an essential role only in neonates but not in adults. Interestingly, in 2009 three independent back-to-back publications in NEJM demonstrated the existence of functional BAT through [^{18}F]fluorodeoxyglucose (FDG) positron emission tomography and computerized tomography (PET-CT) – usually used to detect cells or tissues with high uptake of glucose. Cypess et al., van Marken Lichtenbelt et al. and Virtanen et al. used FDG PET-CT which demonstrated an increased glucose uptake in BAT of human adults exposed to cold (exposure at 16˚C for 2 hours)[136,139,140]. Virtanen et al. reported up to 15-fold increase in the glucose uptake in cold-exposed adults, and detection of the BAT specific UCP1 protein usually specific to the BAT was increased in the WAT of these cold-exposed human adults[139]. The detection of active FDG uptake in BAT of human adults indeed revoked a surge of interest in the scientific community to further elucidate methods to demonstrate the

activity of BAT in adult human, with the hope of exploiting metabolic properties of BAT in order to generate novel therapeutic treatments for obesity. Since then, many researchers have been demonstrated the possibility to increase BAT or brite adipose tissue activity in human adults[110-112,141-143].

Yoneshiro et al., reported that cold exposure (19˚C) of healthy young for 6 weeks, resulted in an increase the FDG uptake in the supraclavicular BAT demonstrating BAT activity. This increased in cold-induced increments of energy expenditure thereby contributing to the reduction in body fat mass[110,144,145]. Interestingly, dietary intake of capsinoids mimics the effect of cold exposure, in increasing BAT activity[143].

A recent study showed that administration of high concentration of ephedrine, an adrenergic agonist, increases the activity of BAT in lean individuals but not in obese individuals[146]. However, adrenergic agents when used in high dosages caused side effects including high blood pressure and heart rate. Therefore, the use of adrenergic agents such as ephedrine as an anti-obesity drug still requires further investigations.

1.3.4 Brite adipose tissue

The adipose tissue scientific community has made concerted efforts to increase the activity of the BAT (or UCP1 expression) in human adults, and to characterize beige/brite adipose tissue which regulate energy expenditure, in hope to combat obesity[110,142,145,147]. Brown adipocytes seem to originate from the same precursor as the skeletal muscle that expresses the muscle factor Myf5, while white adipocytes originate from Myf5-negative precursors[148]. However, the origin of the brite adipocytes warrants further investigations even though they seem to originate from the Myf5-negative precursors[111,112]. Yoneshiro et al. reported that BAT activity decreases with age, and could be responsible for age related fat accumulation[110]. Rajakumari et al. discovered that early B cell factor-2 could switch on white to brown-like transition[149]. Overexpressing early B cell factor-2 in WAT resulted in increased metabolism in mice, demonstrating increased energy expenditure[149]. Another group demonstrated that mice with ablation of type 1A BMP-receptor were born with deficiency of BAT, but were still capable of maintaining normal temperature and preventing DIO by white to brite transition[150]. It was reported that the activity of sirtuins deacetylates peroxisome proliferator-activated receptor-gamma induced white to brown-like transition leading to increased metabolic activity[151].

Brite adipocytes can also be induced by chronic cold exposure (Paper IV) or through stimulation of β-adrenergic receptor agonists[110,131,143]. Upon chronic cold

exposure, these brite adipocytes upregulated expression of genes like *Ucp1*, *Cidea, Cox7a1*, which are otherwise specific to BAT[152,153]. Importantly, changes in the vascular system accompany the white to brite transition. Many angiogenesis related genes are upregulated during the transition of white to brite thereby stimulating angiogenesis to support the high metabolic demand (Paper IV). We speculate that increased vascularization in cold-induced transition further provide more angiogenic growth factors, cytokines, hormones, stem cells and inflammatory cells, which support the survival and maintenance of adipose tissue.

1.3.5 Potential anti-obesity therapies

Many research groups have shown that anti-angiogenic drugs such as TNP-470, angiostatin, endostatin, and anti-VEGF agents can inhibit adipose tissue mass in both DIO and genetically modified mouse models[122,130,154]. TNP-470, derived from fumagillin is an anti-angiogenesis inhibitor, which inhibits ECs proliferation, cell migration and angiogenesis. It has been reported to also inhibit DIO in genetically modified leptin deficient ob/ob mice models by reducing caloric intake and increasing energy expenditure. TNP-470 treatment decreases circulating levels of low-density lipoprotein and cholesterol. TNP-470 treatment also increases insulin sensitivity, suggesting that anti-angiogenic inhibitors might be able to prevent the development of type II diabetes[130]. Despite the benefits, TNP-470 caused neurotoxic effect in mice, therefore the use of TNP-470 as an anti-obesity drug in human needs further investigations.

Studies have shown the presence of functional BAT in lean human adults after cold exposure[110,136,144,145] Therefore, finding methods to increase the activity of BAT or increasing WAT to brite transition could be an important strategy against obesity. We were interested to investigate the role of blood vessels in modulating WAT to brite adipose tissue transitions. Would combination therapy of anti-angiogenic drugs therapy and stimulation of WAT to brite transition be an effective therapy to combat obesity and its related metabolic diseases?

2 AIMS

The overall aim of this thesis was to elucidate the roles of angiogenesis in both tumor development and adipose tissue metabolism, by exploring the potential interventions for treatment.

The specific aims were:

- To investigate the role of angiogenic factor, PDGF-BB in the tumor environment
- To study the systemic effect of angiogenic factor, VEGF-A, and to propose a neoadjuvant delivery of anti-angiogenic drug before chemotherapy in tumor
- To investigate the angiogenic switch in mediating white to brite adipose tissue transition

3 METHODS

In this section, I will discuss some of the key methods used in this thesis (detailed experimental procedures are elaborated in materials and methods of the constituent papers). The key and limiting factors will also be discussed in this section.

Mouse tumor model – syngeneic and xenograft

To investigate tumor angiogenesis and the interaction of different growth factors in the tumor microenvironment, we used syngeneic and xenograft mouse models (Paper I, II). Prior to tumor inoculation, mouse or human cell lines were cultured in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin streptomycin at 37˚C at 5% CO_2. At approximately 80% confluence, cells were washed twice with phosphate buffered saline (PBS) and trypsinized in 0.25% trypsin. Trypsin was neutralized with medium containing 10% FBS and cell suspension was well mixed and counted using a hemocytometer. Cells were resuspended in PBS and approximately one million cells were injected into the dorsal subcutaneously to C57 black 6 (C57/Bl6) or severe combined immunodeficiency (SCID) mice. Tumor size, once palpable was measured every other day with a vernier caliper. Tumor volume was calculated using the formula, $width^2 \times length \times 0.52$[155]. In blockade experiments, PDGFR-α (IH3) or PDGFR-β (2C5) (0.8 mg per mouse, three times a week, i.p., Imclone) antibodies, STI571 (1 mg per mouse, daily, i.p., LC laboratories), EPO neutralizing antibody (0.2 mg per mouse, three times a week, i.p., R&D systems) were administrated on day five after tumor inoculation (Paper I). CTX (62.5 mg/kg, i.p., Sigma-Aldrich), carboplatin (50 mg/kg, i.p., Hospira) or sunitinib malate (60 mg/kg, p.o., LC Laboratories) were administrated when tumor volume reached 0.5 cm^3 (Paper II). VEGFR-1 (MF1) or VEGFR-2 (DC101) (800 µg per mouse, i.p., Imclone) were administrated twice a week for four weeks (Paper IV).

Mouse cold adaptation assay

Prior to exposure of mice to 4˚C (cold), it is critical to adapt mice at 18˚C for at least one week. The adaptation period is dependent on the mouse strains (Paper IV). Thermoneutral zone is the temperature where the resting basal metabolic rate is at the lowest maximum gradient to maintain functionality of different organs. The thermoneutral zone in mice is approximately 30˚C; therefore sudden exposure of mice to 4˚C would result in death due to insufficient time for regulating heat loss to skin and

activation of the NST. Adaptation of one week is mandatory for wild-type mice to allow sufficient time for the mice to activate their NST. Knock-out mice such as UCP1$^{-/-}$ mice are incapable of NST, therefore they require at least three weeks of adaptation at 18˚C before transferring them to cold. During cold exposure experiments, ethical and humane considerations should be thoroughly reviewed to ensure the wellbeing of mice.

Whole mount antibody staining and immunohistochemistry

Hematoxylin and Eosin staining (H&E stain) is widely used in basic research laboratories as well as in clinical medical diagnosis to visualize and to diagnose different stages in cancer progression. Paraffin embedded tissues are rehydrated with decreasing concentrations of ethanol, cell nuclei are stained blue with hematoxylin and the cytoplasm are counterstained with eosin which gives a pink staining (Paper I, II, IV).

Whole mount staining and immunohistochemistry (IHC) methods are essential to analyze the angiogenesis effect and the inhibitory effect of anti-angiogenic drugs, as well as the expression of proteins in tissues of interest. Whole mount staining and immunohistochemistry methods were performed as described in (Paper III). Tumor or fat samples were freshly fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich), embedded in frozen O.C.T compound (Sakura Tissue-Tek) followed by whole mount staining or immunohistochemical stainings of paraffin embedded sections or cyrosections. These methods utilize suitable antibodies that bind to specific antigens and these signals can be visualized with fluorescence-labeled secondary antibodies. Primary antibodies specific for ECs such as mouse CD31-specific rat antibody (1:200 whole mount or 1:400 in IHC, BD Pharmingen), mouse CD34-specific rat antibody (1:400, Angio-proteomie), mouse endomucin-specific rat antibody (1:400, eBiosciences) and biotinylated isolectinB4 (1:500, Vector Lab) were used (Paper I, II, III). Antibodies specific to α-SMA (1:400, DAKO), NG2 (1:400, Chemicon) were co-stained with endothelial specific markers to investigate the interaction of mural cells in association to vasculature. Antibodies specific to Ki67 (1:1000, Novocastra), Ter119 (1:100, BD Pharmingen), EPO (1:100, Santa Cruz Biotech) (Paper I) were used and IHC sections were counterstained with an antifade reagent washed DAPI (4',6-diamindio-2-phenylindole, Vector Lab) to localize the nuclei and to allow us to keep the samples for months. In some cases, imaging was performed using a Nikon D eclipse C1.

Microscopy – importance in biomedical research

Imaging was performed using fluorescence or confocal microscopy. Confocal imaging enables the projection of individual layers of image to obtain a three dimensional image that allows us to investigate the vascular structure and the interaction of vascular cells with mural cells. Whole mount staining works on tissue samples as thick as 1 mm, and the use of confocal microscopy makes it possible to obtain images of greater detail. However, whole mount staining requires longer staining procedures and higher concentration of antibodies in comparison to cryosection or paraffin immunostaining. In constituent papers discussed in this thesis, we quantify the signals obtained from antibody staining performed using Image J or Adobe Photoshop to study angiogenesis and distribution of mural cells in tumor environment and fat tissue. Even though the vascular density of tumor mass is not used as a criterion to access the response to anti-angiogenic drug in the clinical setting, it is important to investigate vascular density and its association and interaction with perivascular and stromal compartments of the tumor as a way to study the underlying mechanism of angiogenesis in tumor microenvironment and fat tissue.

Fluorescence-activated cell sorting (FACS)

FACS allows the detection of different types of fluorescently labeled cells based on size (forward scatter), and granularity (side scatter) as they flow through a fluid stream. Liver and spleen tissues were harvested from mice mechanically minced and filtered through with a cell strainer. Blood was collected from the heart, and single cell suspensions were prepared. One million cells were blocked with mouse serum and incubated with PE-Ter119 (eBioscience) for 20 minutes on ice. Cells were washed with PBS and fixed in 1% PFA containing DRAQ5 (Alexis). In this thesis, flow analysis was performed by FACSort and analyzed with CellQuest (Paper I).

Complete blood count test

A complete blood count test is a commonly used in the clinics to diagnose conditions such as anemia, RBC count, hemoglobin (HGB) and hematocrit (HCT) levels, infection and leukemia (WBC level). Towards the late stages of malignancy, patients usually develop cancer-associated syndrome including impairment of several organs. The impairment in organs such as kidney and bone marrow affects the RBC and WBC population. In this thesis, complete blood count tests were performed using Mindray auto hematology analyzer. Approximately two drops of blood were removed from the

tail vein and blood count test includes RBC, HGB, HCT and WBC (Paper I, II). The hematology analyzer is both time and cost effective in comparison to manual cell counting; however, the machine may not be able to differentiate the cell types accurately due to cell clustering.

Transduction of specific plasmid, siRNA and shRNA interference

To elucidate protein function and interaction, gain and loss of function techniques are essential to substantiate the findings. In this thesis, angiogenic growth factors were overexpressed in selected target cell lines through transduction with retro or lenti-virus encoding the growth factor of interest (Paper I, II). In *in-vitro* knock down experiments, small interfering RNAs against mouse *Atf3, Klf5, Jun, Sp1, Pdgfra or Pdgfrb* were used (Dharmacon RNAi Technologie) (Paper II).

Elisa

Enzyme-linked immunosorbent assay (Elisa), compared to other immunoassays are highly specific and sensitive to detect protein. In this thesis, we measure circulating levels of soluble VEGFR-1, PDGFBB, EPO (Paper I, II) using sandwich Elisa.

Vascular permeability assays

Several assays are available to access the perfusion and permeability of vessels. Miles permeability assay is a classical method to investigate vascular permeability performed by injecting Evan's blue dye into the tail vein of the mouse, followed by measuring the extravasated dyes by spectrophotometer. However, this method is not very accurate and usually produces high variation. To obtain accurate ultrastructure of the endothelium, labeled bioparticles such as ferritin and colloidal gold particles can be injected, followed by detection using electron microscope. However, this method is expensive and time consuming. Fluorescent-labeled molecule such as lysine fixable dextran of different molecular weights (70 kDa or 2 000 kDa) can be preinjected into mice intravenously, and circulate in the vessels before sacrificing the mice. Tissues of interest are then fixed in 4% PFA, followed by whole mount staining with anti-CD31 antibody to evaluate permeability and perfusion respectively (Paper III).

4 RESULTS

This thesis consists of four papers, which represent a concerted effort to understand the role of angiogenesis in tumor (Paper I and Paper II) and adipose tissue (Paper III and IV).

4.1 PDGF-BB MODULATES HEMATOPOIESIS AND TUMOR ANGIOGENESIS BY INDUCING ERYTHROPOIETIN PRODUCTION IN STROMAL CELLS (PAPER I)

In our investigation to unravel the roles of PDGF-BB in tumor angiogenesis and its tumor environment, we implanted two different cell lines (fibrosarcoma (T241) and Lewis Lung Carcinoma (LLC)) overexpressing PDGF-BB into mice dorsally. We observed that PDGF-BB overexpressed tumors grew significantly faster than control tumors in both tumor cell lines (Figure 1a). Excision of tumor followed by immunohistological staining with several antibodies including ECs specific anti-CD31, isolectinB4, and vessels perfusion with dextran revealed that the vascular density was higher in these PDGF-BB tumors as compared to the controls (Figure 1c).

Moreover, we demonstrated using H&E staining that PDGF-BB tumors have increased infiltration of the stroma (Figure 1b). We sought to further examine the stromal tissue in PDGF-BB tumors by immunohistological staining with antibodies to categorize the stromal subtypes. We used antibodies specific to: PDGFR-β, chondroitin sulfate proteoglycan 4 (NG2) and alpha-smooth muscle actin (α-SMA) to identify pericytes, vascular smooth muscle cells (VSMCs), and myofibroblasts respectively (Figure 1b). We clearly showed an increase in the density of PDGFR-β positive staining in PDGF-BB overexpressing tumors but not in the control tumors (Figure 1e), indicating an infiltration of stromal cells in the PDGF-BB overexpressing tumors.

We were intrigued by the liver and spleen enlargement in these PDGF-BB tumor-bearing mice when we performed necropsy analysis. Hepatosplenomegaly suggested possible hematopoiesis occurring in the liver and spleen. Regular H&E histology stainings also showed expansion of red and white pulp in the spleen and presence of hematopoietic foci in liver of PDGF-BB tumor-bearing mice. We next investigated whether the erythroblasts in these hematopoietic foci were actively proliferating by co-staining with antibodies specific to Ki67 and Ter119 in the livers and spleens of PDGF-BB tumor-bearing mice (Figure 1g, h). Indeed, an increase in the Ki67 and Ter119

double positive population in the liver and spleen of PDGF-BB tumor-bearing mice was observed (Figure 1i, j). We have also evidently showed that PDGFR-α and PDGFR-β positive stainings are in the stromal compartment instead of hepatocytes and splenocytes of the liver and spleen (Figure 2g-i). The data collectively showed that the implantation of tumor cells overexpressing PDGF-BB led to systemic effects such as enlargement of liver and spleen, suggesting that tumor-derived PDGF-BB enters the circulation and it exerts its effect on peripheral organs. As such, we measured the circulating plasma level of PDGF-BB, and a level of 1.2 ng/ml was detected in PDGF-BB overexpressing tumor-bearing mice (Figure 1f).

We next characterized the erythroblasts populations to further confirm that hematopoiesis is caused by extramedullary hematopoiesis in the liver and spleen using fluorescence-activated cell sorting (FACS). We demonstrated the increased in RBC precursor population of erythroid burst forming units (BFU-Es) and mature BFU-Es in PDGF-BB tumor-bearing mice by colony forming assay (Figure 2). These results showed that PDGF-BB indeed induce extramedullary hematopoiesis but not hematopoiesis in bone marrow.

To further investigate the effects of extramedullary hematopoiesis, we measured the complete blood count in mice. Interestingly, in PDGF-BB tumor-bearing mice, the levels of RBC, HCT and WBC were significantly higher, suggesting that PDGF-BB induced hepatosplenomegaly induces extramedullary hematopoiesis resulting in an increase of RBCs and HCT that might the animal protect against tumor-associated anemia (Figure 6h). We were particularly intrigued by the increased levels of RBC and HCT, which led us to further measure the circulating erythropoietin (Epo) level. Indeed, the circulating plasma levels of Epo increased three-fold in PDGF-BB tumor-bearing mice (Figure 3b). The endocrine effect of PDGF-BB was further verified with a tumor-free model. Administration of adenovirus PDGF-BB into mice caused splenomegaly, leading to an increase in the circulating plasma levels of Epo, RBC and HCT as seen in the tumor model (Figure 6).

Treatment of PDGF-BB tumor-bearing mice (Figure 5a-d) and PDGF-BB adenovirus (Figure 6a-d) delivered mice with STI571 (Imatinib) and PDGFR-β-specific antibody significantly reduced tumor growth rate and vascular density (in the tumor model), hepatosplenomegaly, circulating levels of Epo, RBC and HCT. Taken together, we show that PDGF-BB exerts its paracrine effect by binding to PDGFR-β on the stromal compartment, thereby increasing the expression of EPO and increased

angiogenesis. Treatment of PDGF-BB tumor-bearing mice with Epo specific antibody also showed significantly reduced tumor growth rate and vascular density suggesting the direct effect of Epo on vascular cells (Figure 5d-f). In addition, we performed *in-vitro* stimulation of ECs with Epo, which caused proliferation, cell migration and tube formation, supporting the direct effect of Epo on vascular cells (Figure 5g, h).

Meanwhile, we performed a series of *in-vitro* experiments to delineate the molecular pathways involved. Stimulation of a stromal fibroblast cell line (S17) with PDGF-BB led to stromal cells adopting spindle-like fiber morphology (Figure 3a). Subsequently, we performed Affymetrix gene array analysis of S17 stromal cells treated with or without PDGF-BB and showed that Atf3 was amongst the top up-regulated transcription factors (Figure 3e, f). However, sequence analysis shows that Atf3 binding sites are absent from the EPO promoter region, suggesting that Atf3 could elicit its effect by forming a complex (Figure 3g, h). Consistent with this notion, we showed using siRNA and chromatin immunoprecipitation (ChIP) assays that upon binding of PDGF-BB to PDGFR-β, Sp1 recruited Atf3 and c-Jun thereby promoting transcription from EPO promoter (Figure 3i, j).

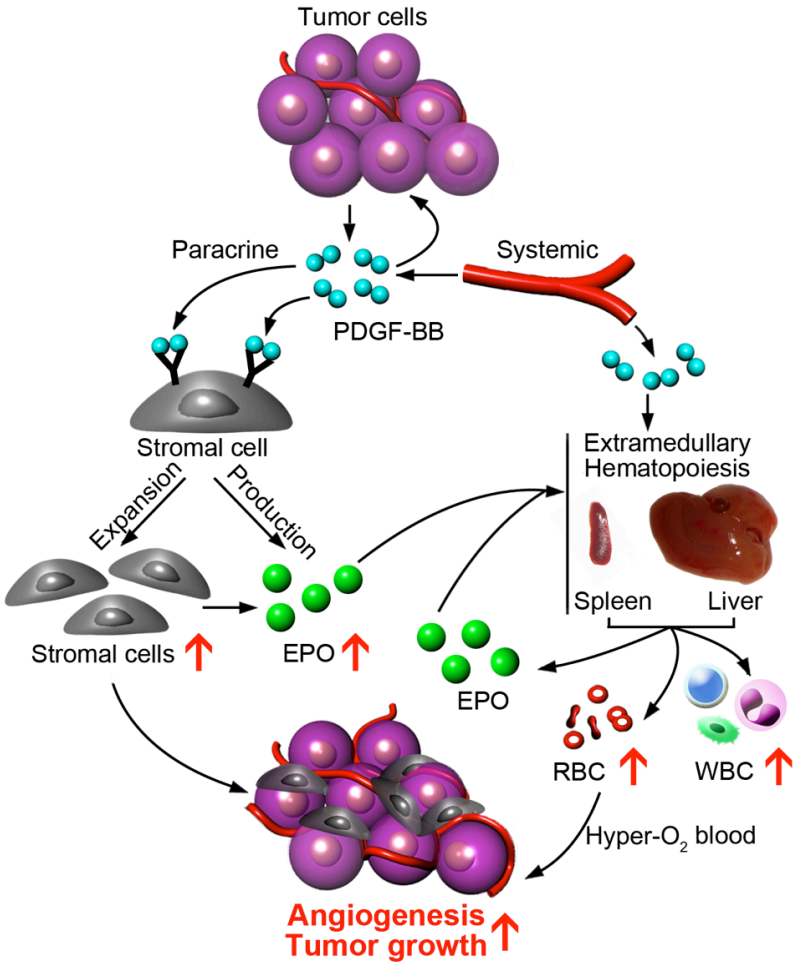

Figure F. Mechanisms of PDGF-BB in promoting tumor angiogenesis (adapted from Paper I)

In conclusion, our findings can be summarized in the schematic diagram (Figure F) where we show that tumor-derived PDGF-BB enters the circulation and binds to PDGFR-β stromal cells that are expressed on liver and spleen. PDGF-BB causes an increase in EPO expression and activates hematopoiesis in the liver and spleen, thereby increasing the RBC level, which further supports tumor growth by increasing oxygenation. We also demonstrated that Epo protects the host against tumor-associated anemia. Epo acts directly on ECs stimulating proliferation, cell migration and tube formation.

4.2 ANTI-ANGIOGENIC AGENTS SIGNIFICANTLY IMPROVE SURVIVAL IN TUMOR-BEARING MICE BY INCREASING TOLERANCE TO CHEMOTHERAPY-INDUCED TOXICITY (PAPER II)

Chemotherapy is widely used in the treatment of cancers. Chemotherapy may be given as combination therapy, and can also be administered at different stages of cancer development. Another example is the neoadjuvant chemotherapy, where other procedures such as surgery are employed after the tumor has been shrunk. However, chemotherapy causes a plethora of side effects such as myelosuppression, anemia, hair loss and inflammation of the gastrointestinal tract[107,156]. Cancer patients usually suffer from cancer-associated systemic syndrome towards the late stage of cancer malignancy including impairment of the bone marrow, anemia, and multiple organs dysfunction[157-160]. Thus, chemotherapy delivery on these patients with cancer-induced bone marrow function impairment could further impair their bone marrow leading to a more detrimental effect. Therefore, we hypothesized that neoadjuvant therapy of anti-angiogenic drug following chemotherapy could protect the bone marrow from chemotherapy induced bone marrow suppression.

To investigate this, we established a B16 melanoma mouse tumor model treated with an anti-angiogenic drug sunitinib, followed by a chemotherapeutic drug, carboplatin. Even though administration of sunitinib alone did not reduce tumor volume in B16 tumor-bearing mice, more importantly it did not cause any death (Figure 1a, b). This reflects the clinical scenario that anti-angiogenic drug improves overall survival. However, delivery of chemotherapy such as carboplatin, to these mice resulted in significant reduction of survival rate, where only 20% survival rate was observed after 10 days treatment with carboplatin (Figure 1b), suggesting the side effects of chemotherapeutic drugs. H&E staining on the bone marrow displayed severe

depletion of bone marrow hematopoietic niches, suggesting that chemotherapy, when used alone, decreased overall survival via bone marrow suppression (Figure 1e-g).

Next, we asked if pretreatment with an anti-angiogenic drug could reverse the high death rate caused by chemotherapy. We pretreated B16 tumor-bearing mice with sunitinib for five days followed by delivery with carboplatin alone, or a combination treatment of carboplatin and sunitinib, and observed that pretreatment with sunitinib significantly improved the survival rate (Figure 1d). In addition, pretreatment with sunitinib followed by carboplatin improved the bone marrow hematopoietic niches. Since chemotherapy causes myelosuppression leading to anemia, we sought to measure the complete blood count in mice receiving different regimens. Expectedly, tumor-bearing mice receiving carboplatin alone showed significant decrease in the levels of RBC, HGB and WBC. In contrast, sunitinib treatment neither caused suppression of hematopoietic niches nor decreased levels of RBC, HGB and WBC, indicating that sunitinib could have a protective effect on bone marrow (Figure 1h-j).

We have shown previously that high circulating levels of VEGF in tumor-bearing mice resulted in several cancer associated systemic syndrome, especially bone marrow suppression[158]. Due to the side effects of chemotherapy, we postulated that chemotherapy treatment of mice with bone marrow impairment would synergistically suppress the bone marrow function. Thus, we hypothesized that pretreatment with sunitinib to VEGF-induced hematopoietic niches suppressed mice could reverse and improve synergistic bone marrow suppression caused by VEGF and chemotherapeutic drugs. To validate this, we injected tumor cell line with overexpression of $VEGF_{165}$ to mice and treated them with sunitinib. Administration of sunitinib to VEGF tumor-bearing mice not only improved survival rate (Figure 2b, d), it restored bone marrow hematopoietic niches (Figure 2e, f) thereby alleviating the anemic syndrome (improved RBC and HGB levels) (Figure 2g-i). As expected, treatment of VEGF-induced bone marrow impair tumor-bearing mice with carboplatin dramatically suppressed the bone marrow of VEGF tumor-bearing mice (Figure 3a, c-g). We validated the impact of chemotherapeutic drug with another widely used chemotherapy drug cyclophosphamide (CTX). Conceivably, CTX decreased the survival rate and inhibited bone marrow hematopoiesis suppression, demonstrating the general side effect of chemotherapy (Figure 3b).

We speculated that anti-angiogenic drug could improve survival rate and VEGF or chemotherapy impaired bone marrow hematopoietic niches. These VEGF tumor-bearing mice received either a combination of carboplatin and sunitinib from the

beginning, or were first pretreated with sunitinib, followed by buffer, or carboplatin alone, or a combination of sunitinib and carboplatin. Expectedly, sequential regimen of pretreatment with sunitinib followed by chemotherapy improved hematopoiesis and anemia (Figure 4). Surprisingly, pretreatment with sunitinib followed by combination therapy of sunitinib and carboplatin did not rescue the bone marrow impairment. In clinical settings, anti-angiogenic drugs and chemotherapeutic drugs are administrated simultaneously. Here, our findings demonstrate that pretreatment with sunitinib can protect the bone marrow against chemotherapy-induced toxicity, and improve survival. Therefore, we propose a different regimen for anti-angiogenic and chemotherapy in cancer patients, especially in cancer patients with high circulating VEGF level.

Figure G provides a schematic summary on how anti-angiogenic drug protects bone marrow suppression and improves survival. Tumor produced VEGF acts directly on ECs to promote its survival, proliferation and migration, hence inducing tumor angiogenesis and tumor growth. VEGF also enters the blood circulation and target peripheral organs such as bone marrow, which causes hematopoiesis and myelogenesis suppression. Similarly, chemotherapy drugs also cause impairment in hematopoiesis and myelogenesis. If chemotherapeutic drugs are administrated to cancer patients with high circulating VEGF level, it will result in a synergistic detrimental impairment of bone marrow. However, if an anti-angiogenic drug is administered before the chemotherapeutic drug, the angiogenic drug could prime the bone marrow and improve bone marrow tolerance to chemotherapy-induced toxicity.

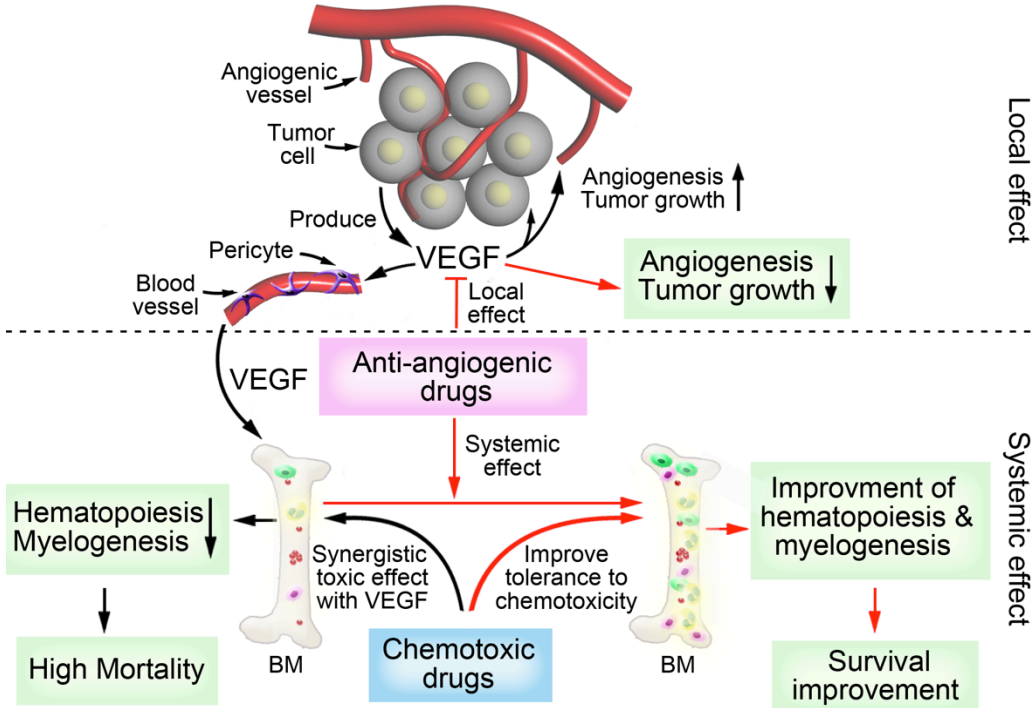

Figure G. Mechanisms underlying anti-angiogenic and cytostatic drugs (adapted from Paper II)

4.3 ADIPOSE ANGIOGENESIS: QUANTIFICATION METHODS TO STUDY MICROVESSEL GROWTH, REGRESSION AND REMODELING IN VIVO (PAPER III)

Similar to pathological conditions such as tumor angiogenesis (Paper I and II), blood vessels are spatiotemporally coupled to the expansion and regression of adipose tissues[115]. Adipose tissue remains highly plastic throughout life, with the capacity to increase and decrease its mass up to 10-fold[120]. This expansion and regression is highly dependent on vascularization. The protocol that we have established enables the investigation of blood vasculatures in the white adipose tissue (WAT) and interscapular brown adipose tissue (iBAT) in association to adipocytes. These detailed procedures give a robust and a reliable method to investigate microvessel growth, regression and remodeling using mouse model.

We established a detailed step-by-step protocol including all the materials and reagents used, and a troubleshooting table that includes most of the commonly encountered problems during preparation and staining of adipose tissue sections. In this protocol, we used WAT and iBAT of C57Bl/6 wt and FOXC2 transgenic mouse strains exposed only to room temperature. However, the immunohistological staining methods can also be used on adipose tissues of other mouse strains under different conditions such as exposure to thermoneutral temperature (30˚C) and cold (4˚C) (see Paper IV and Reference 159).

We described three different staining methods: whole mount staining, immunohistochemical (IHC) staining on cyrosections and paraffin tissue sections that revealed the blood vasculature in WAT and iBAT. Whole mount staining is preferred over IHC stainings when investigating the three-dimensional structure of blood vessels in association to vascular smooth muscle cells is required. IHC is preferred over whole mount staining when it is needed to reveal the interaction on the single cell level, and to quantify the number of vessels per adipocyte.

Slight modifications can be made to this protocol. For example, BODIPY used to stain lipid droplets in adipose tissue to identify adipocytes can be replaced with guinea-pig anti-mouse perilipin antibody (1:400 in PBST) (Procedure 7(A) ix), followed by secondary antibody, fluorescence Alexa 647 labeled hamster anti guinea-pig (1:400 in PBST) (Procedure 7(A) xii). Costaining with a rat anti-mouse F4/80 antibody or a rat anti-mouse Iba1 antibody with LYVE-1 antibody can be used to identify LYVE-1 positive inflammatory cells in adipose tissues. The subpopulation of M2 macrophages can also be characterized using a rat anti-mouse CD206 antibody.

4.4 HYPOXIA-INDEPENDENT ANGIOGENESIS IN ADIPOSE TISSUES DURING COLD ACCLIMATION (PAPER IV)

Independent research groups have demonstrated that cold activates an increase in metabolic activity in iBAT but no one has investigated the role of blood vessels in this cold-activation. We hypothesized if cold would switch on an angiogenic phenotype. If so, what are the mechanisms involved in this cold-induced phenotypic change?

We acclimatized two groups of mice at two temperatures, one group at their thermoneutral temperature 30˚C and the other group at 4˚C. We performed necropsy analysis of mice exposed to 4˚C and 30˚C for five weeks, and observed an obvious change in color of inguinal white adipose tissue (iWAT) (Figure 1a). We asked if this gross macroscopic change in adipose tissue was attributed to the increase in blood vessels density. We then performed immunohistochemical staining on these tissues with ECs specific marker, anti-CD31 and anti-isolectinB4 antibodies. Indeed, we observed a significant increase in the density of microvessels in iWAT and iBAT of 4˚C exposed group (Figure 1 c-h). We did a regular H&E histology staining and were intrigued by morphological changes seen in the iWAT and iBAT of 4˚C exposed group (Figure 1b). Multilocular droplets with high cellular content usually only observed in iBAT were now present in the iWAT of the mice exposed to 4˚C. We further stained these tissues with an antibody against prohibitin and observed a significant increase in the number of prohibitin positive mitochondria in the iWAT of the 4˚C exposed group. These data evidently suggested that exposure of mice to 4˚C caused increased vascular density and increased mitochondria content in the iWAT. Next, we asked if these increased vessels in the iWAT of the 4˚C exposed group were actively proliferating. Immunohistological staining of iWAT of 4˚C and 30˚C exposed to one week with a proliferating marker Ki67, and an ECs marker anti-CD31 clearly demonstrated that these vessels were actively proliferating (Figure 2a, b).

To further elucidate the molecular mechanisms involved in the angiogenic switch, we performed Affymetrix gene array on the iWAT of 3 groups of mice exposed to 30˚C, 4˚C for one week and five weeks. The four most upregulated genes in iWAT of 4˚C exposed groups were *Elovl3*, *Ucp1*, *Fabp3* and *Cpt1b* (Figure 3a, b). These genes are usually strongly associated to BAT rather than WAT, suggesting that cold exposure resulted in a change in gene expression towards BAT. We showed with Northern blot that UCP1, a BAT specific marker was increased more than 500-fold in iWAT after one week exposure to 4˚C. It should be emphasized that *Vegfa* was significantly upregulated after one week exposure to 4˚C, but after longer exposure of five weeks the

expression level reverted the level back to that of 30˚C. We were intrigued by the transient upregulation of VEGF. We further validated this transient increased VEGF expression with northern blot and indeed, one week of exposure at 4˚C led to a two-fold increase in VEGF (Figure 3f). We also observed a transient upregulation of peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) after one week of cold exposure. In contrast, an angiogenesis related inhibitor, TSP was significantly downregulated after cold exposure (Figure 3b). Similarly, the level of PGC-1α was validated by qRT-PCR (Figure 3c).

Since *Vegfa* and *Epas1* (HIF2) expressions were found to be upregulated in Affymetrix gene array analysis, we were interested in understanding the role of hypoxia in cold-induced angiogenesis. We measured tissue hypoxia using a hypoxyprobe stain (pimonidazole), and there was no evidence of hypoxia in iWAT and iBAT of mice living at 30˚C (Figure 4). However, in iWAT and iBAT of mice exposed to 4˚C, we saw hypoxia especially in the iBAT of mice exposed to 4˚C. We sought to look at role of UCP1, a BAT specific protein essential for thermogenesis, in cold-induced angiogenesis. We housed UCP1 ablated mice at 4˚C, and used hypoxyprobe and vessel staining with anti-CD31 antibody to show that there was no obvious detection of hypoxia in iWAT and iBAT. In addition, cold-induced angiogenesis was not observed in the iWAT and iBAT of mice exposed to 4˚C. This demonstrated that cold-induced angiogenesis is hypoxia independent.

We set out to ask which of the two VEGF receptors was responsible for the increased angiogenesis seen after cold exposure. VEGFR-1 and VEGFR-2 neutralizing antibodies were injected to 4˚C exposed mice. VEGFR-2 antibody, and sunitinib administration, followed by whole mount staining with the endothelial specific anti-CD31 antibody clearly showed decreased vascular density in iWAT and iBAT of mice exposed to 4˚C. In contrast, VEGFR-1 antibody treatment exhibited opposing effect. This data evidently show that cold-induced angiogenesis was triggered by VEGF/VEGFR-2 signaling pathway while VEGFR-1 inhibits cold-induced angiogenesis (Figure H).

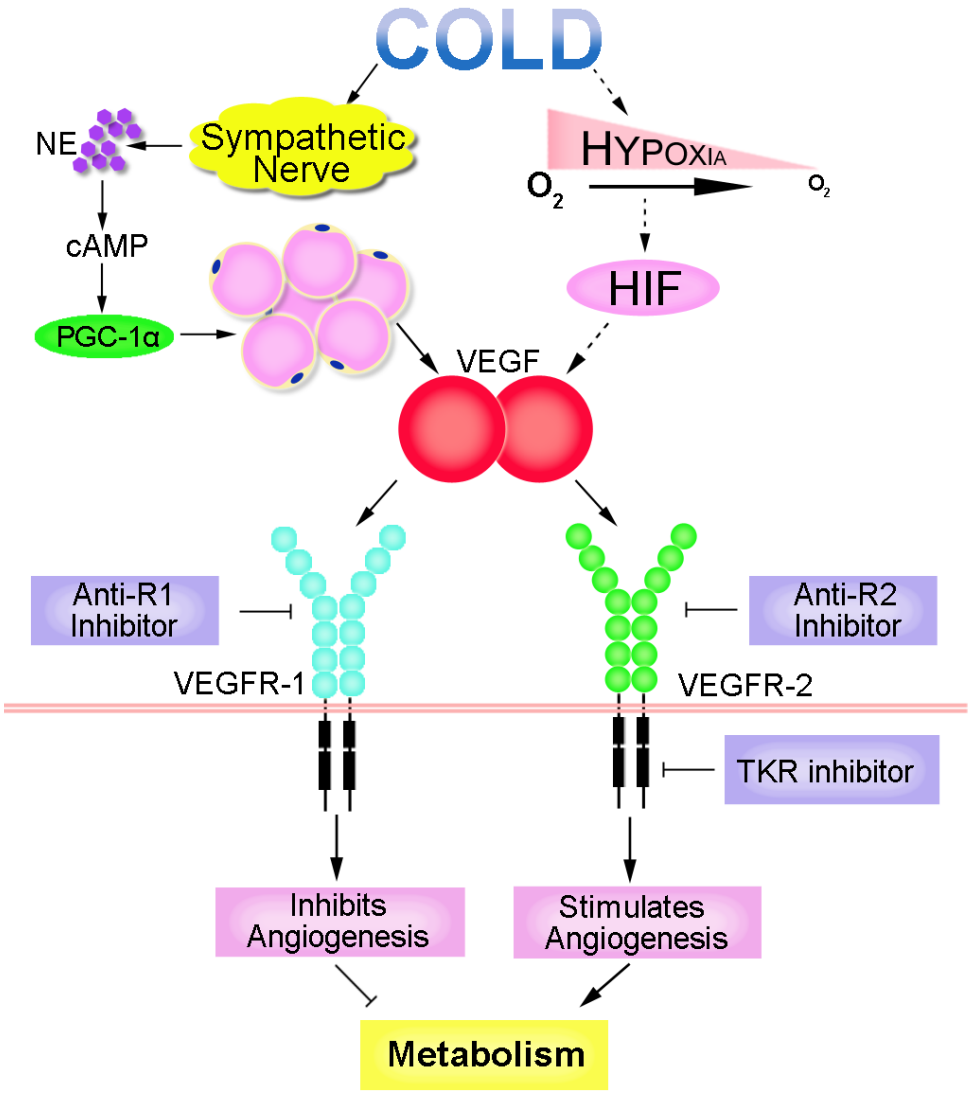

Figure H. Cold-induced angiogenic signaling pathway in adipose tissue (adapted from Paper IV)

Surprisingly, treatment with VEGFR-2 antibody caused behavioral alterations. Mice that received VEGFR-2 antibody seemed to be less tolerable to cold; as they accumulated more nesting materials, preferred to stay in their nest and shivered more than the VEGFR-1 antibody administrated mice (Figure 6a, b). These behavioral alterations stimulated us to ask if tissue metabolism was altered in cold exposed mice treated with VEGFR-1 and VEGFR-2 antibodies. Mice that received VEGFR-2 antibodies had a lower and a delayed response to NE injection, suggesting that VEGFR-2 inhibited neovascularization, hence decreasing the capacity to NST. In contrast, administration of VEGFR-1 antibody led to an even higher NE response in comparison to the buffer treated group, which clearly show the opposing role of VEGFR-1 in cold-induced angiogenesis.

5 DISCUSSION

In this thesis, we established two mouse models: 1) tumor-bearing mouse model to study the local and systemic impact of tumor-derived factors: PDGF-BB (Paper I) and VEGF (Paper II), 2) cold exposed mouse model to unravel the signaling pathways that mediate the cold-induced angiogenic switch in white to brite transition in adipose tissue (Paper IV).

In Paper I, we implanted PDGF-BB overexpressed tumor cell lines into mouse dorsally and demonstrated how PDGF-BB signaled through the stromal compartment of the tumor microenvironment to promote tumor growth and angiogenesis. In Paper II, we investigated how VEGF-A led to a synergistic suppression of bone marrow hematopoiesis on bone marrow impaired mice that have undergone chemotherapy. We then proposed that neoadjuvant therapy with sunitinib before chemotherapy could have a protective effect and allow the recovery of cancer-induced bone marrow suppression caused by VEGF-A.

In Paper III, we developed a novel and detailed protocol to study angiogenesis in adipose tissue. In Paper IV, we acclimated mice at lower temperature, which activated the cold-induced angiogenic switch in iWAT. Further treatment with either VEGFR-2 antibody or sunitinib inhibited angiogenesis and regulated metabolism, demonstrating that VEGF/VEGFR-2 signaling is involved in cold-induced angiogenesis. The cold-activated angiogenic switch in the iWAT of mice resulted in increased vasculature, as well as higher expression of brown-tissue specific proteins, such as UCP1 and PGC-1α[161].

There have been arguments that using genetically similar and homogenously inbred mice is not ideal as a cancer model to recapitulate the effect of drug blockade in human. One of the key arguments is the rapid tumor formation in syngeneic and xenograft models that have little variations resulting in high reproducibility, which do not adequately reflect the tumor heterogeneity and complexity associated with tumor microenvironment in cancer patients. The method of determining the response and effect of anti-cancer drugs in mouse xenograft models include analyzing parameters such as tumor growth rate, tumor volume, retardation and percentage of inhibition of tumor growth rate, which differs from that in cancer patients where overall survival and cancer progression-free survival are used as benchmarks[162]. This discrepancy can be seen by most of the positive data from many drugs that have been tested in mice throughout the years, which during later stages of clinical trials in humans have failed

to have any significant effect in prolonging the life of cancer patients. In the recent years, more and more researchers are trying to shift the current syngeneic and xenograft mouse models toward orthotopic mouse models. Orthotopic mouse models may be more time consuming as they require surgery and skillful tumor inoculation techniques, however this results in a model that might better represent the condition in cancer patients. Unfortunately, there are still no models that exist to study tumor angiogenesis and the responses to drugs therefore it is important that we researchers do not overestimate the therapeutic outcome of drugs that are tested in mouse models.

5.1 PDGF-BB MODULATES HEMATOPOIESIS AND TUMOR ANGIOGENESIS BY INDUCING ERYTHROPOIETIN PRODUCTION IN STROMAL CELLS (PAPER I)

We demonstrated that tumor-derived PDGF-BB binds to PDGFR-β cells in the stromal compartment, and mediates transcription factor Atf3 to form a complex with c-Jun and Sp1, thereby driving the expression of Epo promoter. This PDGF-BB mediated Epo production stimulated angiogenesis and tumor growth. Extramedullary hematopoiesis seen in the liver and spleen resulted in increased oxygenation through elevation of RBC, HGB and HCT levels further supporting tumor growth. We showed that PDGF-BB tumor-bearing mice have a circulating PDGF-BB plasma level of 1 ng/ml, which is clinically relevant to patients stricken with cancer such as glioblastoma, lymphoma, sarcoma and epithelial cancers[163,164]. Therefore, we proposed that treatment with anti-PDGF-BB drugs and anti-Epo drugs would yield better effects, especially in cancer patients with high circulating levels of PDGF-BB.

However, anti-Epo treatments with patients in the clinic may be controversial as Epo is an erythropoiesis-stimulating agent that is used in clinics to treat patients with chronic renal failure and cancer-induced anemia[48-51,165-167]. Using rhEpo as a treatment for patients with renal failure led to severe side effects, including cardiovascular complications and stroke[167]. In addition, rhEpo administration to cancer-induced anemic patients caused thromboembolism[168]. With evidence that the use of Epo in patients may lead to the stimulation of underlying small tumor masses, we proposed the use of anti-Epo to cancer patients[169]. However, due to unknown adverse effects that may be caused by anti-Epo delivery in cancer patients, the use of anti-Epo as a treatment requires more investigation[53,170].

Several drugs targeting the PDGF signaling pathway have been approved by the FDA and are currently used as treatment for different types of cancers by targeting the

autocrine signals in tumor cells[171,172]. It is known that PDGF-BB is essential for pericytes recruitment that aids the maturation and stability of vessels. Therefore would inhibit the PDGF signaling result in leaky vessels with poor pericyte coverage, thereby facilitating metastasis? It was demonstrated recently that anti-PDGF (Imatinib mesylate) treatment in low PDGF-BB producing tumor led to increased tumor cell dissemination and metastasis, whereas treatment in high PDGF-BB producing tumor prevented vascular leakage and decreased tumor dissemination and metastasis[47].

In Paper I, we demonstrated that the administration of anti-Epo antibody to PDGF-BB tumor-bearing mice with high circulating levels of PDGF inhibited tumor growth. In conclusion, the use of drugs targeting the PDGF signaling pathway needs to be investigated more thoroughly. Based on our study, we speculated that therapy using drugs targeting both the PDGF signaling pathway and Epo pathway could be an alternative therapy in cancer patients with high circulating levels of PDGF-BB. However, the administration of anti-Epo to reduce cancer-induced anemia may raise controversial debates, since Epo is used to treat patients suffering from cancer-induced anemia[48,49,165-167]. In conclusion, the use of anti-Epo with the current anti-PDGF therapy definitely requires further investigations.

5.2 ANTI-ANGIOGENIC AGENTS SIGNIFICANTLY IMPROVE SURVIVAL IN TUMOR-BEARING MICE BY INCREASING TOLERANCE TO CHEMOTHERAPY-INDUCED TOXICITY (PAPER II)

Chemotherapy is widely used in the treatment of cancers. Chemotherapy may be given as combination therapy at different stages of cancer development[103,156,173]. Another example is the neoadjuvant chemotherapy, where other procedures such as surgery are employed after the tumor has shrunk. However, chemotherapy causes a plethora of side effects such as bone marrow suppression, which causes the reduction of RBCs production and platelet formation, resulting in bleeding and bruising tendencies. In addition, WBC level is also decreased, causing the patients to be susceptible to secondary infections.

Our group has previously reported that high circulating plasma levels of VEGF-A in tumor-bearing mice caused severe systemic effects including anemia and the depletion of bone marrow hematopoietic niches[158]. Knowing the adverse impact of chemotherapy on bone marrow, we asked if further treatment of these VEGF-induced bone marrow impaired mice with chemotherapy would cause a synergistic impairment of the bone marrow (Paper II).

Conventional regime of chemotherapy drug is used as neoadjuvant therapy, or in combination with other anti-cancer drugs including anti-angiogenic drugs[173]. In this paper, we proposed an alternative regime where we first treated the cancer-induced bone marrow using an anti-angiogenic drug to allow bone marrow recovery before administrating the chemotherapy treatment. This protects the bone marrow from chemotherapy-induced suppression, thereby improving survival.

In this study, we used murine melanoma as it produces high levels of VEGF-A, thereby mirroring cancer. The choice of chemotherapy drugs (CTX and carboplatin) and sunitinib as an anti-angiogenic drug was based on the accessibility to the drugs. Therefore, it would be interesting to validate our findings using human cell lines that produce high levels of VEGF naturally, and to use the relevant chemotherapy drugs that have been approved clinically for the treatment against the different types of cancer. Nevertheless, we have demonstrated that pretreatment with an anti-angiogenic drug followed by a sequential delivery of chemotherapy would yield better survival than combination therapy.

5.3 HYPOXIA-INDEPENDENT ANGIOGENESIS IN ADIPOSE TISSUES DURING COLD ACCLIMATION (PAPER IV)

The expansion and regression of adipose tissue throughout life is highly dependent on angiogenesis and the remodeling of blood vessels. Alterations in vasculature to modulate the supply of oxygen, nutrients and metabolites may be an effective therapeutic intervention for obesity and its related metabolic disorders. We developed a detailed protocol to study the vasculature in adipose tissues (Paper III), and further extended the protocol to study the role of vasculature in adipose tissue and adipose tissue metabolism[174].

In this study, we demonstrated that it was the angiogenic switch in adipose tissue that modulated the cold-induced iWAT to brite adipose tissue transformation. Organ vasculature is dependent on its metabolic status, as shown by the great number of blood vessels in the highly metabolically active BAT so as to provide the tissue with sufficient metabolites. Cold acclimation not only further increased vascular density in BAT to support its demand for oxygen during NST, it also increased vasculature in iWAT. In addition, cold acclimation caused iWAT to acquire the characteristics of BAT. In this paper, we show that cold-stimulated angiogenesis is highly mediated by the VEGF-A/VEGFR-2 signaling pathway. Anti-VEGF pathway drugs, specifically sunitinib and VEGFR-2 antibody, could inhibit the cold-induced angiogenic switch.

Administration of VEGFR-1 antibody on the other hand, caused an opposite effect where blood vasculature was increased and an increased response upon NE stimulation.

What other pro-angiogenic factors are essential in the modulation of angiogenesis in adipose tissues? Obese individuals usually have high circulating serum levels of VEGF, therefore administration of anti-angiogenic drugs such as anti-VEGF have been speculated to reduce hypertrophy and fat accumulation. Anti-angiogenic drugs have been used in mouse models to reduce adipose tissue mass however, administrating such drugs to human patients require more investigations. We speculate if it is possible to use pro-angiogenic factors such as VEGF to increase blood vasculature before subjecting obese individuals to the cold as a treatment against obesity. The pretreatment of VEGF would ensure the provision of sufficient nutrients and metabolites to support the WAT to brite transition.

In our cold acclimation mouse model, we showed that cold exposure transformed iWAT depot to brite adipose tissue that exhibits the high metabolic prolife usually seen in BAT. What about the intra-abdominal adipose tissues in cold exposed mice such as the gonadal, omental, and mesentery WAT? The ability to transform these "pure" intra-abdominal WAT to adopt a brite-like profile is more closely related to the clinical settings because it is the accumulation of visceral rather than subcutaneous fat that is more associated to obesity-related metabolic diseases. Independent groups have demonstrated that short-term exposure of adult individuals to the cold increases the FDG uptake in BAT (see 1.3.5). It will be of interest to investigate if the blood vasculatures in the WAT and BAT of these individuals are similar to that seen in our mouse model.

Our group has recently shown that with cold exposure, two of our genetic knockout mice, more specifically the apolipoprotein E knockout and low-density lipoprotein knockout strains, showed increased atherosclerotic plaque growth and instability due to the activation of UCP1 dependent lipolysis[175]. Therefore, close monitoring is required for this method of using cold exposure to increase the metabolism in obese individuals especially those with underlying pre-atherosclerotic plaques as they risk suffering from atherosclerotic-associated myocardial infarction or even stroke.

6 CONCLUSION

- Paper I: Tumor-derived PDGF-BB promotes tumor growth, angiogenesis and extramedullary hematopoiesis. PDGF-BB binds to PDGFR-β on stromal and perivascular cells and induces Epo expression, which accelerates tumor growth by stimulating endothelial cell proliferation, migration, sprouting and, tube formation. Moreover, extramedullary hematopoiesis-induced oxygenation supports tumor growth and serves as a protection against tumor-associated anemia.
- Paper II: Chemotherapy delivery to tumor-bearing mice with high circulating VEGF levels further suppresses bone marrow hematopoiesis. However, chemotherapy-induced bone marrow suppression can be rescued by pretreatment with the anti-angiogenic drug, sunitinib, followed by sequential delivery of chemotherapy drugs CTX and carboplatin.
- Paper III: We developed novel methods to study angiogenesis in adipose tissue.
- Paper IV: Cold exposure leads to activation of angiogenesis in white and brown adipose tissues. White to brite-like adipose tissue transformation is accompanied by increased expression levels of the brown-fat associated proteins, UCP1 and PGC-1α. Cold-induced angiogenesis and metabolic change in white and brown adipose tissue can be inhibited by VEGFR-2 blockade.

7 FUTURE PERSPECTIVES

7.1 CANCER

"Seeking a cure in cancer in our time" – Barack Obama

Scientific knowledge is dynamic where scientific theories and even long-standing dogmas are constantly challenged, refined and modified as we gather new data. Cancer patients have benefited from the advancement in technology and understanding of the disease, which has resulted in more concise early diagnosis, better screening and therapies as compared to three decades ago. However, cancer remains to be an enigma as shown by the complex process of evading the hosts' defense mechanisms to best exploit, and eventually wear them down. This also means that finding the cure for cancer would require a much broader and more complex incorporation of all the knowledge that has been gathered over the years.

The WHO has projected that the number of newly diagnosed cancer cases worldwide will rise from 11.3 million in 2007 to 15.5 million in 2030, while the number of deaths from cancer will increase 45% (from 7.9 million to 11.5 million) over the same time period. One of the reasons for this jump in numbers is due to growing number of over-60s as compared to any other age group in almost every country. Since the risk of most types of cancer increases with age, this would translate to a higher economic burden on most developed countries in the world that are faced with a higher aging population.

"Disease is the biggest money maker in our economy." – John H. Tobe

It has been estimated that the total medical expenditure on cancer treatment in Western Europe would increase by 15% in 2014. Many pharmaceutical companies are investing more into research and development of oncology drugs. This increasing attention on cancer research has also proven to be beneficial with the exponential advancement in development of medical oncology techniques. It was predicted that by the year 2058, 95% of all cancers could be controllable[176].

Taking metastatic colorectal cancer (MCC) as an example, the overall survival of MCC has almost doubled in a span of 15 years compared to a few decades ago when such a diagnosis, even at stage one, would be equivalent to a death sentence[84]. The advancement in cancer research has allowed us to better understand the genetics and other carcinogenic factors. This has also aided the pharmaceutical companies in the development of new cancer drugs, biomarkers, sophisticated diagnostic and screening tools to facilitate early detection and screening that benefited the cancer patients.

However, cancer remains as a sensitive topic to discuss as some types of cancer remain difficult to detect and do not have any effective therapy.

7.2 ANTI-ANGIOGENIC THERAPY IN CANCER

"If you have cancer and you are a mouse, we can take good care of you." – Judah Folkman

Anti-angiogenesis therapy has become one of the pillars for cancer therapy following surgery, chemotherapy and radiotherapy. Anti-angiogenic drugs such as bevacizumab have better efficacy when used in combination or as sequential therapy. However, these drugs are expensive especially when they have to be used in a combination regime. This further implicates economic strains due to the healthcare in the society. Even with the vast advancement in research, most patients are still unable to benefit from the new drugs because pharmaceutical companies are selling the drugs at a high price.

Again, using MCC as an example, the standard treatment includes using a 5'Fluorouracil plus leucovorin regime. One could also choose a combination of FOLFOX/bevacizumab and Irinotecan, followed by Cetuximab or Irinotecan, which has shown to increase life expectancy from 54 weeks to 116 weeks[177,178]. However, the combination regime is also costly, where the total drug cost increases from $4,000 to $173,000. The survival advantage of one year would cost $169,000 more. This implies that such benefits received from the advancement in cancer drug development will only apply to cancer patients from wealthy families or those from countries with better healthcare systems.

Basic science research allows us to better understand the evolution of cancer development and eventually find a way to conquer them, or at least to slow down the rate of cancer progression. However, not all the cancer patients receive these benefits from the current newly developed drugs. One of the reasons is that pharmaceutical companies pose to be the limiting step that deters cancer patients from benefiting from the new cancer drugs due to their high cost. It is important that pharmaceutical companies aim to reduce the overall health costs and improve patients' health. Another limiting factor is that oncologists are unable to keep up with the fast pace of development of novel cancer therapies. New oncology drugs that enter the market fail to reach the oncologists or cancer patients fast enough.

It is also important to identify biomarkers to determine the different patient cohorts and administrate the correct combination of drugs to the individual group.

Taking the withdrawal of bevacizumab from metastatic HER2-negative breast cancer patients as an example, Genentech proposed a phase III clinical trial to include the use of a biomarker to better identify the cohort of patients that will have maximum benefit with bevacizumab treatment[179,180]. Most cancer patients in their late stages of malignancy die from metastasis instead of the primary tumor mass. This increases the urgency for the development of more sophisticated imaging machines to effectively detect metastatic cancer cells, as well as drugs that can inhibit different phases in the metastatic cascade. Another emerging problem faced in cancer therapy is drug resistance[79,80]. We need to better understand how cancer cells develop mechanisms of resistance to drug treatment.

Since blood vessels are involved in almost every stages of tumor progression including growth and subsequent invasive metastatic cascades, anti-angiogenic drugs that inhibit the growth these vessels should provide great therapeutic avenues for cancer treatments. Why are we not able to effectively treat all types of cancer with anti-angiogenic drugs?

7.3 CONCLUDING REMARKS

"We don't know a millionth of one percent about anything." – Thomas A. Edison

Cancer is the world's second leading cause of most deaths in people. Cancer drugs unfortunately remain to be expensive, resulting in an economic strain on the healthcare systems in most countries. Our knowledge and understanding of the functions and mechanisms of cancer progression has increased immensely over the last 30 years. Yet, new questions arise that pose as challenges for the future. Are we able to come up with new drugs? Can we revisit existing therapeutic regimes to outsmart these intelligent cancer cells? With all the existing constrains, will we be able to cure cancer in our time?

7.4 OBESITY

The current global problem in obesity and obesity-related metabolic disorders such as type II diabetes and cardiovascular disorders are alarming and require immediate attention. Last year, the Organization for Economic Co-operation and Development (OECD) estimated the health expenditure on obesity to account for 1-3% of total health expenditure in most countries. The OECD also projected that two out of three people would be overweight or obese in some countries by 2020. In the last five years, the OECD has proposed affordable and cost-effective programs that governments can

adopt to prevent obesity and overweight. This includes implementing taxes on unhealthy food and beverages, as well as increasing health promotion messages through media to educate consumers on making healthier food options and also to encourage the increase of physical activity. Since childhood obesity is also on the rise, the OECD encourages governments to limit unhealthy food advertising to children. Would tax implementation on unhealthy food help to curb obesity or would it backfire and result in the reduction of healthier options in order to then pay for more expensive unhealthy food?

7.5 THERAPEUTIC INTERVENTION FOR OBESITY

Based on the current insights, the thermogenic activity of BAT is currently regarded as the 'body's best weapon' against obesity. As 40-50 g of fully activated BAT can burn up to 4 kg of WAT in a year, it highly suggests that increasing BAT activity could be used as an intervention for obesity[118,137]. Emerging evidence indicates the plausibility of using cold exposure to stimulate BAT activity and activate brite transition in adult humans[136,139,140]. In summary, these observations led to the conclusion that BAT activity negatively correlates with age and BMI, suggesting that obese individuals probably have low BAT activity, cold-induced BAT activation is present in higher amount in younger individuals, and BAT is more readily detectable in female individuals than in male. Therefore, the identification of novel methods to activate BAT and to stimulate browning in WAT will also become more critical, especially in clinical trials.

It is now apparent that cold exposure is capable of inducing BAT activity in individuals, resulting in increased metabolism and energy expenditure[110,136,139,140,142,145]. However, it is noteworthy to mention that precautions should be taken to expose individuals with pre-atherosclerotic plaques to cold. It has been recently shown that cold exposure led to an increased UCP1 dependent lipolysis leading to instability of atherosclerotic plaques[175]. Apart from cold exposure, there are several potential methods that can activate the sympathetic nervous systems and mediate BAT activity, such as adrenergic agonists (ephedrine, isoprenaline), capsinoids and insulin[143,146]. These methods seem to be promising and further investigations will be needed to reveal their full potential.

Since adipocytes produce a myriad of adipokines, further elucidation of their functions is required to allow us to select better targets to combat obesity and prevent obesity-related metabolic diseases. The administration of anti-angiogenic drugs to

obese individuals remains controversial due to the differences in metabolic functions of WAT and BAT. Anti-angiogenic drugs targeted to WAT should inhibit blood vessels growth, thereby restricting their growth and expansion. On the other hand, pro-angiogenic drugs should be given to obese individuals seeking to increase their BAT activity since the increase in angiogenic vessels would translate to more oxygen and nutrients to meet the higher metabolic demands during BAT activation. In conclusion, the potential of anti-angiogenic drugs against obesity remains controversial and requires further investigations.

7.6 CONCLUDING REMARKS

Obesity and overweight epidemic is escalating, which puts individuals at a greater risk for life-threatening diseases, including type II diabetes, and cardiovascular disorders. The cause of obesity and overweight can be due to factors including genetics, sedentary lifestyle and unhealthy eating habits. Fortunately, adopting a healthier lifestyle such as eating better and increasing physical activity can prevent overweight and obesity, even for someone who is genetically predisposed to weight gain. Until we uncover the full potential of using anti-angiogenic drugs against obesity, adopting a healthier lifestyle habits seem to be the most economic and non-invasive approach to curb obesity.

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9 REFERENCES

1. Folkman, J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285, 1182-1186 (1971).
2. Poole, T.J. & Coffin, J.D. Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern. *The Journal of experimental zoology* 251, 224-231 (1989).
3. Burri, P.H. & Djonov, V. Intussusceptive angiogenesis--the alternative to capillary sprouting. *Molecular aspects of medicine* 23, S1-27 (2002).
4. Djonov, V., Baum, O. & Burri, P.H. Vascular remodeling by intussusceptive angiogenesis. *Cell and tissue research* 314, 107-117 (2003).
5. Carmeliet, P. Angiogenesis in health and disease. *Nat Med* 9, 653-660 (2003).
6. Ferrara, N. & Alitalo, K. Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med* 5, 1359-1364 (1999).
7. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1, 27-31 (1995).
8. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353-364 (1996).
9. Patarroyo, M., Tryggvason, K. & Virtanen, I. Laminin isoforms in tumor invasion, angiogenesis and metastasis. *Seminars in cancer biology* 12, 197-207 (2002).
10. Kapp, T.G., Rechenmacher, F., Sobahi, T.R. & Kessler, H. Integrin modulators: a patent review. *Expert opinion on therapeutic patents* 23, 1273-1295 (2013).
11. Hadler-Olsen, E., Winberg, J.O. & Uhlin-Hansen, L. Matrix metalloproteinases in cancer: their value as diagnostic and prognostic markers and therapeutic targets. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 34, 2041-2051 (2013).
12. Pennacchietti, S.*, et al.* Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer cell* 3, 347-361 (2003).
13. Carmeliet, P.*, et al.* Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394, 485-490 (1998).
14. Gerhardt, H.*, et al.* VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of cell biology* 161, 1163-1177 (2003).
15. Hellstrom, M., Phng, L.K. & Gerhardt, H. VEGF and Notch signaling: the yin and yang of angiogenic sprouting. *Cell adhesion & migration* 1, 133-136 (2007).
16. Hellstrom, M.*, et al.* Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* 445, 776-780 (2007).
17. Dimova, I.*, et al.* Inhibition of Notch signaling induces extensive intussusceptive neo-angiogenesis by recruitment of mononuclear cells. *Angiogenesis* 16, 921-937 (2013).
18. Stenina-Adognravi, O. Thrombospondins: old players, new games. *Current opinion in lipidology* 24, 401-409 (2013).
19. Cao, Y. Molecular mechanisms and therapeutic development of angiogenesis inhibitors. *Advances in cancer research* 100, 113-131 (2008).
20. O'Reilly, M.S.*, et al.* Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88, 277-285 (1997).
21. O'Reilly, M.S.*, et al.* Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79, 315-328 (1994).
22. Shchors, K.*, et al.* Increased invasiveness of MMP-9-deficient tumors in two mouse models of neuroendocrine tumorigenesis. *Oncogene* 32, 502-513 (2013).
23. Grunstein, J., Roberts, W.G., Mathieu-Costello, O., Hanahan, D. & Johnson, R.S. Tumor-derived expression of vascular endothelial growth factor is a critical factor in tumor expansion and vascular function. *Cancer Res* 59, 1592-1598 (1999).

24. Cao, Y. Multifarious functions of PDGFs and PDGFRs in tumor growth and metastasis. *Trends in molecular medicine* 19, 460-473 (2013).
25. Heldin, C.H. & Westermark, B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiological reviews* 79, 1283-1316 (1999).
26. Senger, D.R.*, et al.* Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219, 983-985 (1983).
27. Ferrara, N. & Henzel, W.J. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161, 851-858 (1989).
28. Cao, Y. Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Science signaling* 2, re1 (2009).
29. Matsumoto, T. & Claesson-Welsh, L. VEGF receptor signal transduction. *Science's STKE : signal transduction knowledge environment* 2001, re21 (2001).
30. Shibuya, M., Ito, N. & Claesson-Welsh, L. Structure and function of vascular endothelial growth factor receptor-1 and -2. *Current topics in microbiology and immunology* 237, 59-83 (1999).
31. Ferrara, N.*, et al.* Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380, 439-442 (1996).
32. Shalaby, F.*, et al.* Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376, 62-66 (1995).
33. Shibuya, M. VEGF-receptor inhibitors for anti-angiogenesis. *Nihon yakurigaku zasshi. Folia pharmacologica Japonica* 122, 498-503 (2003).
34. Mick, G.J., Wang, X. & McCormick, K. White adipocyte vascular endothelial growth factor: regulation by insulin. *Endocrinology* 143, 948-953 (2002).
35. Cao, Y. Angiogenesis and vascular functions in modulation of obesity, adipose metabolism, and insulin sensitivity. *Cell Metab* 18, 478-489 (2013).
36. Heldin, C.H. & Westermark, B. Platelet-derived growth factor: three isoforms and two receptor types. *Trends in genetics : TIG* 5, 108-111 (1989).
37. Abramsson, A.*, et al.* Analysis of mural cell recruitment to tumor vessels. *Circulation* 105, 112-117 (2002).
38. Hellstrom, M.*, et al.* Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *The Journal of cell biology* 153, 543-553 (2001).
39. Kaminski, W.E.*, et al.* Basis of hematopoietic defects in platelet-derived growth factor (PDGF)-B and PDGF beta-receptor null mice. *Blood* 97, 1990-1998 (2001).
40. McCarty, M.F.*, et al.* Overexpression of PDGF-BB decreases colorectal and pancreatic cancer growth by increasing tumor pericyte content. *J Clin Invest* 117, 2114-2122 (2007).
41. Hermanson, M.*, et al.* Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res* 52, 3213-3219 (1992).
42. Steller, E.J.*, et al.* PDGFRB promotes liver metastasis formation of mesenchymal-like colorectal tumor cells. *Neoplasia* 15, 204-217 (2013).
43. Jechlinger, M.*, et al.* Autocrine PDGFR signaling promotes mammary cancer metastasis. *J Clin Invest* 116, 1561-1570 (2006).
44. Nissen, L.J.*, et al.* Angiogenic factors FGF2 and PDGF-BB synergistically promote murine tumor neovascularization and metastasis. *J Clin Invest* 117, 2766-2777 (2007).
45. Terpos, E., Apperley, J. & Milojkovic, D. Imatinib and chronic myeloid leukemia: close to the bone. *Leukemia & lymphoma* 54, 1581-1582 (2013).
46. Vannorsdall, E.J., Collins, J.A., Chen, Q.C., Sarai, G. & Baer, M.R. Symptomatic response to imatinib mesylate in cutaneous mastocytosis associated with chronic myelomonocytic leukemia. *Current oncology* 20, e349-353 (2013).
47. Hosaka, K.*, et al.* Tumour PDGF-BB expression levels determine dual effects of anti-PDGF drugs on vascular remodelling and metastasis. *Nature communications* 4, 2129 (2013).
48. Bunn, H.F. Erythropoietin. *Cold Spring Harbor perspectives in medicine* 3, a011619 (2013).

49. Cao, Y. Erythropoietin in cancer: a dilemma in risk therapy. *Trends in endocrinology and metabolism: TEM* 24, 190-199 (2013).
50. Del Mastro, L., Gennari, A. & Donati, S. Chemotherapy of non-small-cell lung cancer: role of erythropoietin in the management of anemia. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 10 Suppl 5, S91-94 (1999).
51. Lipkin, G.W., Kendall, R., Haggett, P., Turney, J.H. & Brownjohn, A.M. Erythropoietin in acute renal failure. *Lancet* 1, 1029 (1989).
52. Macdougall, I.C.*, et al.* A peptide-based erythropoietin-receptor agonist for pure red-cell aplasia. *N Engl J Med* 361, 1848-1855 (2009).
53. Nayak-Rao, S. & McCormick, B. Erythropoietin use in CKD patients with cancer: to tread with caution? *Journal of nephrology* 26, 829-835 (2013).
54. Wang, L., Li, H.G., Xia, Z.S., Wen, J.M. & Lv, J. Prognostic significance of erythropoietin and erythropoietin receptor in gastric adenocarcinoma. *World J Gastroenterol* 17, 3933-3940 (2011).
55. Fagiani, E., Lorentz, P., Kopfstein, L. & Christofori, G. Angiopoietin-1 and -2 exert antagonistic functions in tumor angiogenesis, yet both induce lymphangiogenesis. *Cancer Res* 71, 5717-5727 (2011).
56. Maisonpierre, P.C.*, et al.* Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277, 55-60 (1997).
57. Suri, C.*, et al.* Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87, 1171-1180 (1996).
58. O'Reilly, M.S. Angiostatin: an endogenous inhibitor of angiogenesis and of tumor growth. *EXS* 79, 273-294 (1997).
59. Eder, J.P., Jr.*, et al.* Phase I clinical trial of recombinant human endostatin administered as a short intravenous infusion repeated daily. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 20, 3772-3784 (2002).
60. Good, D.J.*, et al.* A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci U S A* 87, 6624-6628 (1990).
61. Ikushima, H. & Miyazono, K. TGFbeta signalling: a complex web in cancer progression. *Nature reviews. Cancer* 10, 415-424 (2010).
62. Cursiefen, C.*, et al.* Roles of thrombospondin-1 and -2 in regulating corneal and iris angiogenesis. *Investigative ophthalmology & visual science* 45, 1117-1124 (2004).
63. Kulke, M.H.*, et al.* Phase II study of recombinant human endostatin in patients with advanced neuroendocrine tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 24, 3555-3561 (2006).
64. Folkman, J. Anti-angiogenesis: new concept for therapy of solid tumors. *Annals of surgery* 175, 409-416 (1972).
65. Cao, Y.*, et al.* Forty-year journey of angiogenesis translational research. *Science translational medicine* 3, 114rv113 (2011).
66. Cao, Y. Angiogenesis: What can it offer for future medicine? *Exp Cell Res* 316, 1304-1308 (2010).
67. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* 100, 57-70 (2000).
68. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* 144, 646-674 (2011).
69. Folkman, J., Merler, E., Abernathy, C. & Williams, G. Isolation of a tumor factor responsible for angiogenesis. *The Journal of experimental medicine* 133, 275-288 (1971).
70. Hillen, F. & Griffioen, A.W. Tumour vascularization: sprouting angiogenesis and beyond. *Cancer Metastasis Rev* 26, 489-502 (2007).
71. Ribatti, D. & Djonov, V. Intussusceptive microvascular growth in tumors. *Cancer letters* 316, 126-131 (2012).
72. Fukumura, D. & Jain, R.K. Imaging angiogenesis and the microenvironment. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* 116, 695-715 (2008).

73. Valastyan, S. & Weinberg, R.A. Tumor metastasis: molecular insights and evolving paradigms. *Cell* 147, 275-292 (2011).
74. Koontongkaew, S. The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas. *Journal of Cancer* 4, 66-83 (2013).
75. Bernstein, S.G.*, et al.* Prevalence of papillomarvirus infection in colposcopically directed cervical biopsy specimens in 1972 and 1982. *American journal of obstetrics and gynecology* 151, 577-581 (1985).
76. Hsu, I.C.*, et al.* Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* 350, 427-428 (1991).
77. Kekule, A.S.*, et al.* The preS2/S region of integrated hepatitis B virus DNA encodes a transcriptional transactivator. *Nature* 343, 457-461 (1990).
78. Fu, Z.*, et al.* The crosstalk: Tumor-infiltrating lymphocytes rich in regulatory T cells suppressed cancer-associated fibroblasts. *Acta oncologica* 52, 1760-1770 (2013).
79. Kerbel, R.S.*, et al.* Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. *Cancer Metastasis Rev* 20, 79-86 (2001).
80. Bracci, R., Maccaroni, E. & Cascinu, S. Transient sunitinib resistance in gastrointestinal stromal tumors. *N Engl J Med* 368, 2042-2043 (2013).
81. Weigelt, B., Peterse, J.L. & van 't Veer, L.J. Breast cancer metastasis: markers and models. *Nature reviews. Cancer* 5, 591-602 (2005).
82. Dimitriu, C.*, et al.* Clinical impact of cachexia on survival and outcome of cancer patients. *Romanian journal of internal medicine = Revue roumaine de medecine interne* 43, 173-185 (2005).
83. Cao, Y. Off-tumor target--beneficial site for antiangiogenic cancer therapy? *Nature reviews. Clinical oncology* 7, 604-608 (2010).
84. Hurwitz, H.*, et al.* Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 350, 2335-2342 (2004).
85. Jain, R.K. Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 31, 2205-2218 (2013).
86. Ferrara, N., Hillan, K.J., Gerber, H.P. & Novotny, W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature reviews. Drug discovery* 3, 391-400 (2004).
87. Mayer, R.J. Two steps forward in the treatment of colorectal cancer. *N Engl J Med* 350, 2406-2408 (2004).
88. Sandler, A.*, et al.* Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 355, 2542-2550 (2006).
89. Miller, K.*, et al.* Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* 357, 2666-2676 (2007).
90. Summers, J., Cohen, M.H., Keegan, P. & Pazdur, R. FDA drug approval summary: bevacizumab plus interferon for advanced renal cell carcinoma. *The oncologist* 15, 104-111 (2010).
91. Dai, F.*, et al.* Safety of Bevacizumab in Treating Metastatic Colorectal Cancer: A Systematic Review and Meta-analysis of All Randomized Clinical Trials. *Clinical drug investigation* 33, 779-788 (2013).
92. Kerbel, R.S. Reappraising antiangiogenic therapy for breast cancer. *Breast* 20 Suppl 3, S56-60 (2011).
93. Makita, N. & Iiri, T. Tyrosine kinase inhibitor-induced thyroid disorders: a review and hypothesis. *Thyroid : official journal of the American Thyroid Association* 23, 151-159 (2013).
94. Demetri, G.D. Identification and treatment of chemoresistant inoperable or metastatic GIST: experience with the selective tyrosine kinase inhibitor imatinib mesylate (STI571). *European journal of cancer* 38 Suppl 5, S52-59 (2002).
95. Barbany, G., Hoglund, M. & Simonsson, B. Complete molecular remission in chronic myelogenous leukemia after imatinib therapy. *N Engl J Med* 347, 539-540 (2002).

96. Elliott, M.A., Mesa, R.A. & Tefferi, A. Adverse events after imatinib mesylate therapy. *N Engl J Med* 346, 712-713 (2002).
97. Raymond, E.*, et al.* Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. *N Engl J Med* 364, 501-513 (2011).
98. Motzer, R.J.*, et al.* Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. *N Engl J Med* 356, 115-124 (2007).
99. Rock, E.P.*, et al.* Food and Drug Administration drug approval summary: Sunitinib malate for the treatment of gastrointestinal stromal tumor and advanced renal cell carcinoma. *The oncologist* 12, 107-113 (2007).
100. Gan, H.K., Seruga, B. & Knox, J.J. Sunitinib in solid tumors. *Expert opinion on investigational drugs* 18, 821-834 (2009).
101. Unger, F.T., Klasen, H.A., Tchartchian, G., de Wilde, R.L. & Witte, I. DNA damage induced by cis- and carboplatin as indicator for in vitro sensitivity of ovarian carcinoma cells. *BMC cancer* 9, 359 (2009).
102. Malhotra, V. & Perry, M.C. Classical chemotherapy: mechanisms, toxicities and the therapeutic window. *Cancer biology & therapy* 2, S2-4 (2003).
103. Gencheva, M.*, et al.* Bone marrow osteoblast vulnerability to chemotherapy. *European journal of haematology* 90, 469-478 (2013).
104. Heidary, N., Naik, H. & Burgin, S. Chemotherapeutic agents and the skin: An update. *Journal of the American Academy of Dermatology* 58, 545-570 (2008).
105. Ramalingam, S. & Belani, C.P. Carboplatin/gemcitabine combination in advanced NSCLC. *Oncology* 18, 21-26 (2004).
106. Stuckey, A. & Dizon, D.S. Novel antiangiogenic therapies in ovarian cancer. *Women's health* 8, 447-453 (2012).
107. Strati, P.*, et al.* Myelosuppression after frontline fludarabine, cyclophosphamide, and rituximab in patients with chronic lymphocytic leukemia: Analysis of persistent and new-onset cytopenia. *Cancer* 119, 3805-3811 (2013).
108. Smorlesi, A., Frontini, A., Giordano, A. & Cinti, S. The adipose organ: white-brown adipocyte plasticity and metabolic inflammation. *Obesity reviews : an official journal of the International Association for the Study of Obesity* 13 Suppl 2, 83-96 (2012).
109. Cinti, S. The adipose organ at a glance. *Disease models & mechanisms* 5, 588-594 (2012).
110. Yoneshiro, T.*, et al.* Recruited brown adipose tissue as an antiobesity agent in humans. *J Clin Invest* 123, 3404-3408 (2013).
111. Harms, M. & Seale, P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* 19, 1252-1263 (2013).
112. Giralt, M. & Villarroya, F. White, brown, beige/brite: different adipose cells for different functions? *Endocrinology* 154, 2992-3000 (2013).
113. Cao, Y. Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. *Nature reviews. Drug discovery* 9, 107-115 (2010).
114. Cao, Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest* 117, 2362-2368 (2007).
115. Hausman, G.J. & Richardson, R.L. Adipose tissue angiogenesis. *J Anim Sci* 82, 925-934 (2004).
116. Brakenhielm, E. & Cao, Y. Angiogenesis in adipose tissue. *Methods in molecular biology* 456, 65-81 (2008).
117. Himms-Hagen, J. Obesity may be due to a malfunctioning of brown fat. *Canadian Medical Association journal* 121, 1361-1364 (1979).
118. Rothwell, N.J. & Stock, M.J. A role for brown adipose tissue in diet-induced thermogenesis. *Nature* 281, 31-35 (1979).
119. Cannon, B. & Nedergaard, J. Metabolic consequences of the presence or absence of the thermogenic capacity of brown adipose tissue in mice (and probably in humans). *Int J Obes (Lond)* 34 Suppl 1, S7-16 (2010).
120. Ledoux, S.*, et al.* Angiogenesis associated with visceral and subcutaneous adipose tissue in severe human obesity. *Diabetes* 57, 3247-3257 (2008).
121. Neels, J.G., Thinnes, T. & Loskutoff, D.J. Angiogenesis in an in vivo model of adipose tissue development. *Faseb J* 18, 983-985 (2004).
122. Rupnick, M.A.*, et al.* Adipose tissue mass can be regulated through the vasculature. *Proc Natl Acad Sci U S A* 99, 10730-10735 (2002).

123. Komorowski, J.*, et al.* Systemic blood osteopontin, endostatin, and E-selectin concentrations after vertical banding surgery in severely obese adults. *Cytokine* 55, 56-61 (2011).
124. Kong, P.*, et al.* Thrombospondin-1 regulates adiposity and metabolic dysfunction in diet-induced obesity enhancing adipose inflammation and stimulating adipocyte proliferation. *American journal of physiology. Endocrinology and metabolism* 305, E439-450 (2013).
125. Cho, C.H.*, et al.* Angiogenic role of LYVE-1-positive macrophages in adipose tissue. *Circ Res* 100, e47-57 (2007).
126. Sung, H.K.*, et al.* Adipose vascular endothelial growth factor regulates metabolic homeostasis through angiogenesis. *Cell Metab* 17, 61-72 (2013).
127. Fukumura, D.*, et al.* Paracrine regulation of angiogenesis and adipocyte differentiation during in vivo adipogenesis. *Circ Res* 93, e88-97 (2003).
128. Tam, J.*, et al.* Blockade of VEGFR2 and not VEGFR1 can limit diet-induced fat tissue expansion: role of local versus bone marrow-derived endothelial cells. *PLoS One* 4, e4974 (2009).
129. Pasarica, M.*, et al.* Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes* 58, 718-725 (2009).
130. Brakenhielm, E.*, et al.* Angiogenesis inhibitor, TNP-470, prevents diet-induced and genetic obesity in mice. *Circ Res* 94, 1579-1588 (2004).
131. Petrovic, N., Shabalina, I.G., Timmons, J.A., Cannon, B. & Nedergaard, J. Thermogenically competent nonadrenergic recruitment in brown preadipocytes by a PPARgamma agonist. *American journal of physiology. Endocrinology and metabolism* 295, E287-296 (2008).
132. Lin, C.S. & Klingenberg, M. Characteristics of the isolated purine nucleotide binding protein from brown fat mitochondria. *Biochemistry* 21, 2950-2956 (1982).
133. Feldmann, H.M., Golozoubova, V., Cannon, B. & Nedergaard, J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metab* 9, 203-209 (2009).
134. Bartelt, A.*, et al.* Brown adipose tissue activity controls triglyceride clearance. *Nat Med* 17, 200-205 (2011).
135. Gunawardana, S.C. & Piston, D.W. Reversal of type 1 diabetes in mice by brown adipose tissue transplant. *Diabetes* 61, 674-682 (2012).
136. van Marken Lichtenbelt, W.D.*, et al.* Cold-activated brown adipose tissue in healthy men. *N Engl J Med* 360, 1500-1508 (2009).
137. Rothwell, N.J. & Stock, M.J. Acute effects of fat and carbohydrate on metabolic rate in normal, cold-acclimated and lean and obese (fa/fa) Zucker rats. *Metabolism: clinical and experimental* 32, 371-376 (1983).
138. Cameron, I.L. & Smith, R.E. Cytological Responses of Brown Fat Tissue in Cold-Exposed Rats. *The Journal of cell biology* 23, 89-100 (1964).
139. Virtanen, K.A.*, et al.* Functional brown adipose tissue in healthy adults. *N Engl J Med* 360, 1518-1525 (2009).
140. Cypess, A.M.*, et al.* Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* 360, 1509-1517 (2009).
141. Qiang, L.*, et al.* Brown remodeling of white adipose tissue by SirT1-dependent deacetylation of Ppargamma. *Cell* 150, 620-632 (2012).
142. Vosselman, M.J., van Marken Lichtenbelt, W.D. & Schrauwen, P. Energy dissipation in brown adipose tissue: From mice to men. *Molecular and cellular endocrinology* 379, 43-50 (2013).
143. Yoneshiro, T., Aita, S., Kawai, Y., Iwanaga, T. & Saito, M. Nonpungent capsaicin analogs (capsinoids) increase energy expenditure through the activation of brown adipose tissue in humans. *The American journal of clinical nutrition* 95, 845-850 (2012).
144. Cypess, A.M.*, et al.* Cold but not sympathomimetics activates human brown adipose tissue in vivo. *Proc Natl Acad Sci U S A* 109, 10001-10005 (2012).
145. van der Lans, A.A.*, et al.* Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. *J Clin Invest* 123, 3395-3403 (2013).

146. Carey, A.L.*, et al.* Ephedrine activates brown adipose tissue in lean but not obese humans. *Diabetologia* 56, 147-155 (2013).
147. Wu, J.*, et al.* Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* 150, 366-376 (2012).
148. Sanchez-Gurmaches, J. & Guertin, D.A. Adipocyte lineages: Tracing back the origins of fat. *Biochimica et biophysica acta* (2013).
149. Rajakumari, S.*, et al.* EBF2 determines and maintains brown adipocyte identity. *Cell Metab* 17, 562-574 (2013).
150. Schulz, C., Paulus, K., Johren, O. & Lehnert, H. Intranasal leptin reduces appetite and induces weight loss in rats with diet-induced obesity (DIO). *Endocrinology* 153, 143-153 (2012).
151. Tang, B.L. & Chua, C.E. Is systemic activation of Sirt1 beneficial for ageing-associated metabolic disorders? *Biochem Biophys Res Commun* 391, 6-10 (2010).
152. Madsen, L.*, et al.* UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS One* 5, e11391 (2010).
153. Barneda, D., Frontini, A., Cinti, S. & Christian, M. Dynamic changes in lipid droplet-associated proteins in the "browning" of white adipose tissues. *Biochimica et biophysica acta* 1831, 924-933 (2013).
154. White, H.M., Acton, A.J. & Considine, R.V. The angiogenic inhibitor TNP-470 decreases caloric intake and weight gain in high-fat fed mice. *Obesity* 20, 2003-2009 (2012).
155. Cao, R.*, et al.* Suppression of angiogenesis and tumor growth by the inhibitor K1-5 generated by plasmin-mediated proteolysis. *Proc Natl Acad Sci U S A* 96, 5728-5733 (1999).
156. Paioli, A.*, et al.* Chemotherapy-related toxicity in patients with non-metastatic Ewing sarcoma: influence of sex and age. *Journal of chemotherapy* (2013).
157. Nathanson, L. & Hall, T.C. Introduction: paraneoplastic syndromes. *Seminars in oncology* 24, 265-268 (1997).
158. Xue, Y.*, et al.* Anti-VEGF agents confer survival advantages to tumor-bearing mice by improving cancer-associated systemic syndrome. *Proc Natl Acad Sci U S A* 105, 18513-18518 (2008).
159. Noriki, S.*, et al.* Multi-organ damage (MOD) induced by cancer cachexia and its pathogenesis. *Basic and applied histochemistry* 33, 337-346 (1989).
160. Tisdale, M.J. Cachexia in cancer patients. *Nature reviews. Cancer* 2, 862-871 (2002).
161. Bostrom, P.*, et al.* A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* 481, 463-468 (2012).
162. Cao, Y. Antiangiogenic cancer therapy: why do mouse and human patients respond in a different way to the same drug? *The International journal of developmental biology* 55, 557-562 (2011).
163. Leitzel, K.*, et al.* Elevated plasma platelet-derived growth factor B-chain levels in cancer patients. *Cancer Res* 51, 4149-4154 (1991).
164. Kurimoto, M., Nishijima, M., Hirashima, Y., Endo, S. & Takaku, A. Plasma platelet-derived growth factor-B chain is elevated in patients with extensively large brain tumour. *Acta neurochirurgica* 137, 182-187 (1995).
165. Oster, H.S., Neumann, D., Hoffman, M. & Mittelman, M. Erythropoietin: the swinging pendulum. *Leukemia research* 36, 939-944 (2012).
166. Milano, M. & Schneider, M. EPO in cancer anemia: benefits and potential risks. *Critical reviews in oncology/hematology* 62, 119-125 (2007).
167. Maiese, K., Chong, Z.Z. & Shang, Y.C. Raves and risks for erythropoietin. *Cytokine & growth factor reviews* 19, 145-155 (2008).
168. Al Diab, A.I. Cancer-related venous thromboembolism: insight into underestimated risk factors. *Hematology/oncology and stem cell therapy* 3, 191-195 (2010).
169. Yang, J., Xiao, Z., Li, T., Gu, X. & Fan, B. Erythropoietin promotes the growth of pituitary adenomas by enhancing angiogenesis. *Int J Oncol* 40, 1230-1237 (2012).

170. Morais, C., Johnson, D.W., Vesey, D.A. & Gobe, G.C. Functional significance of erythropoietin in renal cell carcinoma. *BMC cancer* 13, 14 (2013).
171. Chen, L.L.*, et al.* A missense mutation in KIT kinase domain 1 correlates with imatinib resistance in gastrointestinal stromal tumors. *Cancer Res* 64, 5913-5919 (2004).
172. Demetri, G.D.*, et al.* Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 347, 472-480 (2002).
173. Chen, J.*, et al.* Neoadjuvant rh-endostatin, docetaxel and epirubicin for breast cancer: efficacy and safety in a prospective, randomized, phase II study. *BMC cancer* 13, 248 (2013).
174. Lim, S.*, et al.* Cold-induced activation of brown adipose tissue and adipose angiogenesis in mice. *Nature protocols* 7, 606-615 (2012).
175. Dong, M.*, et al.* Cold Exposure Promotes Atherosclerotic Plaque Growth and Instability via UCP1-Dependent Lipolysis. *Cell Metab* 18, 118-129 (2013).
176. Bosanquet, N. & Sikora, K. The economics of cancer care in the UK. *The lancet oncology* 5, 568-574 (2004).
177. Carrato, A.*, et al.* Fluorouracil, leucovorin, and irinotecan plus either sunitinib or placebo in metastatic colorectal cancer: a randomized, phase III trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 31, 1341-1347 (2013).
178. Souglakos, J.*, et al.* FOLFOXIRI (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) vs FOLFIRI (folinic acid, 5-fluorouracil and irinotecan) as first-line treatment in metastatic colorectal cancer (MCC): a multicentre randomised phase III trial from the Hellenic Oncology Research Group (HORG). *British journal of cancer* 94, 798-805 (2006).
179. Dawood, S.*, et al.* The use of bevacizumab among women with metastatic breast cancer: a survey on clinical practice and the ongoing controversy. *Cancer* 118, 2780-2786 (2012).
180. Stevenson, C.E.*, et al.* Bevacizumab and breast cancer: what does the future hold? *Future oncology* 8, 403-414 (2012).