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MECHANISM OF PATHOLOGICAL ANGIOGENESIS IN ADIPOSE TISSUE AND TUMOR

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Stockholm 2009

Published by Karolinska Institutet. Printed by Larserics Digital Print AB.

ISBN 978-91-7409-588-3

献给我亲爱的爸爸和妈妈

ABSTRACT

Angiogenesis is critical for both malignant diseases, such as cancer, and non-malignant diseases, such as obesity and metabolic disorders. Majority of tissues and organs are highly vascularized. The malfunctions of tissue microenvironment are always accompanied with or dependent on the alterations of vasculature. Therefore, anti-angiogenic agents provide novel therapeutic targets for prevention and treatment of malignant and non-malignant diseases.

This thesis presents the identification and further investigation of mechanism of angiogenesis in adipose tissue metabolism and cancer development. Multiple factors spatiotemporally regulate adipogenesis and angiogenesis. In a transgenic mouse model described in the first constituent paper, FOXC2 in the adipose tissue affects angiogenesis, vascular patterning and functions. FOXC2 controls angiopoietin-2 (Ang-2) expression by direct activation of its promoter in adipocytes. Remarkably, an Ang-2 specific antagonist L1-10 reverses the vascular alterations. In another physiological model described in the second paper, exposure of mice to cold temperature leads to activation of angiogenesis in both white and brown adipose tissues. Proangiogenic factors, such as VEGF, are upregulated, and endogenous angiogenesis inhibitors, such as thrombospondin, are downregulated during the adipose tissue transformation. Intriguingly, the cold-induced angiogenesis is independent of hypoxia. VEGFR2 blockage abolishes the cold-induced angiogenesis and significantly impairs nonshivering thermogenesis capacity. Unexpectedly, VEGFR1 blockage results in the opposite effects. Therefore, the application of angiogenesis modulators can have conceptual implications for the treatment of obesity and disorders.

In the third paper, we show both in vitro and in vivo that PDGF-B markedly induces erythropoietin (EPO) mRNA and protein expression levels by targeting the PDGFR positive stromal compartment. Tumor-produced PDGF-B systemically affects spleen and liver by causing hepato-splenomegaly and extramedullary hematopoiesis. PDGF-B induces erythropoietin and promotes tumor growth by, 1) stimulating tumor angiogenesis and 2) stimulating extramedullary hematopoiesis leading to increased oxygen perfusion. Thus, the EPO signaling pathway may be crucial for the development of anti-PDGF cancer therapy.

LIST OF PUBLICATIONS

I. **Xue Y**, Cao R, Nilsson D, Chen S, Westergren R, Hedlund EM, Martijn C, Rondahl L, Krauli P, Walum E, Enerbäck S, Cao Y. FOXC2 controls Ang-2 expression and modulates angiogenesis, vascular patterning, remodeling, and functions in adipose tissue. Proc Natl Acad Sci U S A. 2008 Jul 22;105(29):10167-72.

II. **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 Metab. 2009 Jan 7;9(1):99-109.

III. Wang Z*, **Xue Y***, Jensen L, Lim S, Ye X, Hedlund EM, Zhu Z, Cao R, Galter D, Cao Y. PDGF-B modulates hematopoiesis and tumor angiogenesis by switching on hypoxia-independent erythropoietin production in stromal cells. Manuscript. *Co-first author.

RELATED PUBLICATIONS

IV. **Xue Y**, Religa P, Cao R, Hansen AJ, Lucchini F, Jones B, Wu Y, Zhu Z, Pytowski B, Liang Y, Zhong W, Vezzoni P, Rozell B, Cao Y. Anti-VEGF agents confer survival advantages to tumor-bearing mice by improving cancer-associated systemic syndrome. Proc Natl Acad Sci U S A. 2008 Nov 25;105(47):18513-8.

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VI. Cao Y, Cao R, **Xue Y**. Therapeutic potential of antiangiogenic agents for prevention and treatment of obesity. Future Lipidol. 2008 Apr 3(2):153-162.

VII. Cao R, **Xue Y**, Hedlund EM, Zhong Z, Tritsaris K, Tondelli B, Lucchini F, Zhu Z, Dissing S, Cao Y. VEGFR-1-mediated pericyte ablation impairs vascular integrity in the adult retina and links VEGF-A and PlGF to cancer-associated retinopathy in mice. Manuscript submitted.

VIII. **Xue Y**, Chen F, Zhang DF, Cao Y. Tumor-derived VEGF modulates hematopoiesis. Manuscript submitted.

CONTENTS

LIST OF ABBREVIATIONS

ANG	Angiopoietin
BAT	Brown adipose tissue
DNA	Deoxyribonucleic acid
EPO	Erythropoietin
FGF	Fibroblast growth factor
FOXC2	Forkhead helix box C2
HGF	Hepatocyte growth factor
HIF1α	Hypoxia-inducible transcription factor 1 alpha
IGF	Insulin-like growth factor
NST	Non-shivering thermogenesis
PDGF	Platelet derived growth factor
PGC1α	Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
PlGF	Placenta growth factor
PPARγ	Peroxisome proliferator-activated receptor gamma
RNA	Ribonucleic acid
UCP1	Uncoupling protein 1
WAT	White adipose tissue
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 INTRODUCTION

1.1 ANGIOGENESIS

Blood vessels carry oxygen and nutrients to distal organs, and transport metabolic waste away from body (1). Angiogenesis is the growth of new capillaries from pre-existing blood vessels, and is critical for tissue, organ growth and repair in both embryonic and adult stages at physiological conditions (2, 3). An imbalance in the growth of blood vessels contributes to pathological alterations in numerous diseases. These include both malignant diseases, for instance cancer, and non-malignant diseases, for instance obesity (1, 4).

The driven forces for angiogenesis includes: 1) Hypoxia. Inefficient vascular supply and resultant reduction in tissue oxygen tension often lead to neovascularization to satisfy the needs of the tissue (5, 6). 2) Acidosis. Low pH induces various angiogenic factors, for instance VEGF, PD-ECGF, therefore stimulates angiogenesis (7-9). 3) Inflammation or immune response. An inflammatory microenvironment can lead to active angiogenesis in both cancer and obesity (10, 11). 4) Cellular stress. The pressure generated by proliferating cells can trigger angiogenic signals (12). 5) Genetic mutations. Activation of oncogenes or inactivation of tumor suppressor genes can regulate production of angiogenesis modulators (13).

1.1.1 Angiogenic stimulators

Various angiogenic regulators have been identified since the introduction of angiogenesis in the scientific society. The stability of vasculature is regulated by the homeostasis between angiogenic stimulators and inhibitors. At the quiescent stage of vasculature, angiogenic inhibitors expressed locally or transported through circulation,

counteract the stimulation from angiogenic factors and stabilize vascular network. However, angiogenesis is initiated in the tissue in the response to the production of either increased levels of stimulators, decreased levels of inhibitors, or both. The best-characterized angiogenic regulators include: 1) stimulators: angiopoietin-, VEGF-, PDGF- and FGF-families (14-16); 2) inhibitors: thrombospondin-family and proteolytic fragments (17). Besides traditional angiogenic regulators like growth factors and inhibitors, microRNAs are also identified to regulate angiogenesis in vivo (18-20).

1.1.1.1 Angiopoietin

Angiopoietions (Ang-1 and Ang-2) and their Tie-2 receptor tyrosine kinase have wide-ranging effects on blood vascular development, remodeling and maturation, lymphatic vascular patterning and hematopoiesis (21-24). In embryonic stage, the absence of angiopoietins or its receptor Tie-2 shows an apparent defect in vascular remodeling (16, 22, 25). Ang-2 is a naturally occurring antagonist of Ang-1 that competes for binding to the Tie-2 receptor and blocks Ang-1 induced Tie-2 autophosphorylation during vasculogenesis (26). The regulatory roles of Ang-1 and Ang-2 remain controversial. Ang-1 functions only as a Tie-2 activator, while Ang-2 can be either a Tie-2 activator or inhibitor depending on the cell type. However, it is well-accepted that Ang-2 is a Tie-2 agonist in lymphatic vessel patterning and a Tie-2 antagonist during blood vessel remodeling (22).

The interplay of angiopoietins and VEGF in tumor angiogenesis has been identified. Angiopoietins, particularly Ang-2, are induced in co-opted tumor vessels, preceding vessel regression (24). Ang-1 is anti-apoptotic for endothelial cells and the destabilizing action of Ang-2 facilitates the angiogenic action of VEGF in tumor (24).

Except tumor angiogensis, Ang-2 is involved in the angiogenesis of proliferative diabetic retinopathy (27).

1.1.1.2 VEGF

Until recently, VEGF family contains five structurally related glycoproteins: VEGF-A, -B, -C, -D and PlGF. They bind to three transmembrane receptor tyrosine kinases, VEGFR-1, -2 and -3, which are mainly present on endothelial cells (28-32). In addition to receptor tyrosine kinases, certain VEGF isoforms interact with neuropilin-1 and -2. VEGFs and VEGF receptors signaling pathways are critical for vasculogenesis in embryonic development, and important for inducing angiogenesis and lymphangiogensis in physiological and pathological situations.

Vascular endothelial growth factor-A (VEGF-A, VEGF) is a mitogen primarily for vascular endothelial cells. It induces vascular leakage and permeability, as well as vascular endothelial cell proliferation (33, 34). VEGF have different isoforms including $VEGF_{206}$, $VEGF_{189}$, $VEGF_{183}$, $VEGF_{165}$, $VEGF_{148}$, $VEGF_{145}$ and $VEGF_{121}$, which display differential biological activities due to various heparin-binding affinities as well as tissue distribution patterns (35-37). The production level of VEGF protein is regulated by transcriptional, translational and post-translational mechanisms. At transcriptional level, hypoxia-induced factor-1alpha (HIF-1α) and peroxisome-proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), for instance, can directly upregulate VEGF mRNA in a hypoxia-dependent or -independent manner (38, 39). Further, VEGF mRNA and protein stabilities react to the tissue microenvironment, which make rapid responses to inadequate blood flow and falling oxygen partial pressure in the tissue (40).

Among all those isoforms, $VEGF_{165}$ has been extensively investigated for its function, signaling, expression and roles in various physiological and pathological conditions (41, 42). Genetic studies have shown that, the formation of blood vessels was abnormal but not abolished in heterozygous *Vegf*-deficient embryos and even more impaired in homozygous *Vegf*-deficient embryos, resulting in death at mid-gestation (43, 44). On cellular level, VEGF stimulates endothelial cell proliferation, migration and tube formation, as well as secretion of proteases which are responsible for the degradation of the extracellular matrix (45). On molecular level, VEGF activates different signaling molecules in endothelial cells, for instance, PLC, PKC, Akt and MAPK (46). Accumulating evidence shows that VEGF/VEGFR-2 signaling is the crucial pathway mediating angiogenic activity and vascular permeability. The functions mediated by VEGF/VEGFR-1 are less understood, although several vascular-related and nonvascular-related functions have been suggested (47-49).

1.1.1.3 PDGF

PDGF family contains PDGF-A, -B, -C and –D (50-53). Their corresponding receptors include PDGFRα, PDGFRβ, which can form either homodimers or heterodimers (54). PDGFs have crucial roles during embryonic development, but relative limited evidence for performing physiological functions in adult. The prototype member in the family is PDGF-B, which binds to all types of PDGF receptors. Disruption of *Pdgf-b* gene or the genes for its receptor *Pdgfr* in mice leads to the development of lethal hemorrhage and edema in late embryogenesis and absence of kidney glomerular mesangial cells (55). PDGF-B can directly induce angiogenesis, lymphangiogenesis and lymphatic metastasis in mouse corneal and tumor models (56, 57). In a combination with FGF-2, PDGF-B synergistically induces vascular networks *in vivo*, which can stabilize for more than a year even after withdrawing of angiogenic factors (57). PDGF-B activates

Ras, PIK3, Akt, IKK, NF-kappa-B, and promotes cellular proliferation and inhibits apoptosis (58).

1.1.1.4 EPO

Human erythropoietin (EPO) is an acidic glycoprotein hormone with a molecular weight of 34 kDa. EPO stimulates hematopoiesis and angiogenesis, provides protection against apoptosis of erythroid progenitors in bone marrow and brain neurons (59-62). EPO, primarily produced as a kidney cytokine regulating hematopoiesis in human, is also produced in other organs and tissues after oxidative or nitrosative stress, such as hypoxia (61, 63). Genetic approach shows that the expressions of EPO and its receptor EPOR are temporally and spatially segregated, which causes two distinct waves of erythrogenesis: 1) the primitive wave in the extraembryonic yolk sac; 2) the definitive wave in the fetal liver and spleen (64). The best-known function of recombinant human EPO is its effectiveness in treating the anemia of end-stage renal disease in clinic. However, the application of EPO is still under debate in different clinical settings (59, 65, 66).

1.1.2 Angiogenic inhibitors

1.1.2.1 Thrombospondin

Thrombospondin family of matricellular proteins includes five members, which are involved in the tissue remodeling at embryonic development, wound healing, synaptogenesis, angiogensis and neoplasia (67). They interact with extracellular matrix components, for instance collagen and fibronectin, as well as cell adhesion molecules, for instance integrins. The best-characterized member is thrombospodin-1 (TSP-1), which has been shown to suppress tumor growth by inhibiting angiogenesis and by activating transforming growth factor-β. TSP-1 inhibits angiogenesis through direct

effects on endothelial cell migration and survival, and through VEGF bioavailability. In genetic model, mice lacking *Tsp-1* have elevated vascular density in many tissues, and aberrant wound healing is observed in those mice (68, 69). TSP-1 binds to endothelial CD36 receptor, activating Src kinase and caspases (68).

1.1.2.2 Proteolytic fragments

Of all known angiogenesis inhibitors so far, nearly half of them are proteolytic fragments (70). Among them, angiostatin and endostatin have been reported to be the most specific inhibitors of angiogenesis (71, 72). These fragmental inhibitors are not directly produced by host or tumor cells, but converted by proteolytic activities. For instance, angiostatin, a potent angiogenesis inhibitor, is a 38 kDa internal fragment of plasminogen (73). Although those endogenous inhibitors have been shown to exhibit potent anti-angiogenesis and anti-tumor activities, their clinical applications are not without obstacles and require further investigation (71, 72, 74).

1.1.3 Pathological angiogenesis

Angiogenesis is of great importance in the progression of many diseases. To our knowledge, angiogenesis is known to be switched on in malignant diseases, such as cancer and inflammatory disorders. Many other diseases can be affected through the alteration of angiogenesis, including obesity, diabetes, diabetic retinopathy, bacterial infections, ischemic heart disease, stroke and preeclampsia (2).

The translational angiogenesis research includes two aspects: 1) the stimulation of angiogenesis in myocardial infarction, ischemia and stroke. For instance, angiogenesis is critically required for the recovery of heart and brain ischemia; 2) the inhibition of angiogenesis, alternatively anti-angiogenesis, in the inhibition of cancer development,

retinopathy, age-related macular disease, obesity and diabetes. Therefore, the understanding of pathological angiogenesis provides new therapeutics and strategies for fighting against various diseases.

1.2 ADIPOSE TISSUE

Adipose tissues containing adipocytes, vascular cells, fibroblasts and many other cell types are considered to be one of the largest organs in body. They can be categorized into two groups: 1) white adipose tissue (WAT), responsible for the storage of energy in the form of lipids; 2) brown adipose tissue (BAT), responsible for the expenditure of energy by thermogenesis. Adipose tissues are not only metabolic, but also endocrine organs. Many cytokines, adipokines and growth factors modulating the endocrine homeostasis in the body, are secreted by adipose tissues. The pathological alterations of adipose tissue can lead to both malformation of adipose tissue itself, such as obesity, and endocrine disorders, such as diabetes and cardiovascular diseases. Thus, great efforts are needed to expand our knowledge in the biology of adipose tissues and relevant diseases.

1.2.1 Adipose tissue transformation

The plasticity of adipose tissues defines their abilities for growth/regression throughout adult life and for transformation under certain physiological circumstance, which requires the alteration and cooperation of blood vessels (Figure 1). For instance, cold exposure can convert WAT into BAT, while warm exposure can transform BAT into WAT. Another example is pregnancy-lactation can transform WAT to milk-secreting glands, which will be reversed after the lactation. The biological significance for tissue transformation is that, a differentiated cell turns pheno-typically and functionally into a

different one of another type without undergoing dedifferentiation (75). This process can help the body to make rapid response to biological or environmental changes.

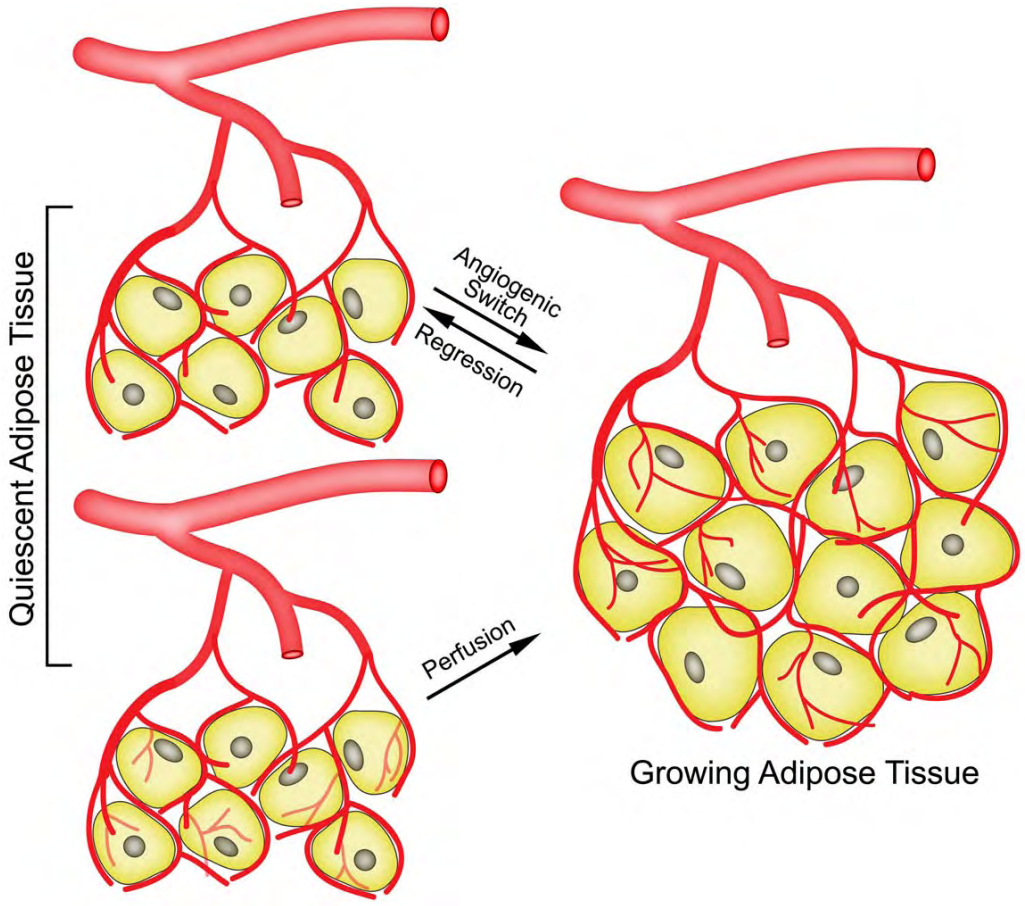

Figure 1. The expansion of adipose tissue requires cooperation from vasculature. When quiescent adipose tissue starts to grow, two possible processes might happen to blood vessels in order to support the adipose tissue expansion: 1) Angiogenesis is switched on by imbalance of angiogenic factors; 2) Previous non-functional blood vessels are perfused. Quiescent blood vessels are transformed into functional ones. On the contrary, angiogenic vessels might start to regress accompanied with the adipose tissue regression.

Previous studies have shown that after cold exposure brown adipocytes expressing uncoupling protein-1 (UCP1) arise in WAT areas of the adipose organ (76). A significant number of multilocular adipocytes arise within few days in visceral as well as subcutaneous WAT depots (77). This transition is accompanied by switching on an angiogenic phenotype (4). Conversely, the regression of capillary networks happens together with the transformation of BAT into WAT under certain circumstance.

1.2.2 Adipose tissue metabolism

White adipocytes display cell type-selective machinery required for triglyceride synthesis from fatty acid, hormone-stimulated glucose uptake and lipolysis (78). In addition, white adipocytes produce adipokines such as TNFα, leptin, resistin, adiponectin and RBP4 that modulate systemic metabolism though endocrine system (79). The inability to properly store triglycerides in adipose tissue results in adverse effects on glucose metabolism in the liver and skeletal muscle (80). Thus, WAT has been identified as an important therapeutic target for developing anti-diabetic drugs, such as the thiazolidinedione (81).

BAT shares similar function with WAT in terms of secreting many adipokines and regulating systemic metabolism. In contrast with WAT, the physiological role of BAT is to generate heat for maintaining body temperature (82, 83). Brown adipocytes perform this specialized function by utilizing their mitochondrial contents and uncoupling cellular respiration through the action of UCP1. Due to these functional differences, the balance between WAT and BAT affects systemic energy balance and may contribute to the development of obesity. Recent studies have shown that BAT exists in human throughout whole life, and are metabolically active (84-87). Understanding of the molecular mechanisms that control BAT development may therefore uncover new opportunities for intervention of metabolic diseases.

1.2.2.1 Thermogenesis

Thermogenesis is defined as a heat-producing process in organisms activated due to action of hormones and/or neurotransmitters (88). It occurs mostly in warm-blooded animals, but exists in a few species of thermogenic plants as well. Depending on whether it is initiated through the movement of muscles, thermogenesis can be

classified as shivering thermogenesis, non-shivering thermogenesis (NST) and diet-induced thermogenesis.

Mammals can maintain body temperature by shivering, known as shivering thermogenesis. Shivering is a body function in response to early hypothermia in warm-blooded animals. Muscle group around the vital organs shake in small movements in an attempt to create warmth by expending energy. But no mechanical action is produced because antagonistic muscle pairs are activated at the same time. NST is dependent on brown adipose tissue (82, 85, 88). In response to acute cold exposure, NST increases its capacity by expanding BAT mass and by elevating metabolic activity in brown adipocytes, in the presence of the protein known as UCP1, or thermogenin in the inner membrane of brown adipocyte mitochondria.

Thermogenesis is a plastic process, therefore, can be achieved by artificial methods. For instance, cold exposure is known to activate NST via sympathetic nerves. Another example, caffeine, a thermogenic substance, has been long time used to stimulate the metabolism in human body. Thus, developing novel thermogenesis regulators is of importance in controlling fluctuation in human body weight and treating metabolic disorders.

1.2.2.2 UCP1

The uncoupling proteins of mitochondria in BAT, including UCP1, UCP2 and UCP3, are unique components to mammalian cells. Compared with UCP2 and UCP3, UCP1 is present at much higher abundance. UCP1 transfers energy from ATP synthesis to thermogenesis in the mitochondria of brown adipocyte by catalyzing protons across the inner membrane. UCP1 knock-out mice are cold-sensitive but not obese (89).

However, UCP1 ablation in mice induces obesity and abolishes diet-induced thermogenesis exempt from thermal stress by living at the thermoneutral temperature (90).

UCP1 is regulated by various nuclear receptors and cofactors, such as peroxisome proliferator-activated receptor γ (PPARγ) and PPARγ coactivator 1 alpha (PGC1-α) (91). UCP1 mediates uncoupling of the mitochondrial respiratory chain and oxidative phosphorylation systems, thus provides heat production in brown adipocytes (92). However, the mechanisms of UCP1 mediating uncoupling are still unclear. Several models have been suggested regarding the UCP1 functions as a channel or as a carrier (82, 91).

UCP1 function is strictly regulated in order to effectively respond to acute thermoregulary needs of body. UCP1-dependent thermogenesis is modulated by the sympathetic nerve system, which is governed by selective hypothalamic regions. Sympathetic nerves anatomically reach adipocytes and functionally release noradrenaline to stimulate thermogenic activity of brown adipocytes by binding to α- and β-adrenergic receptors. Sympathetic nerve system also controls mitochondrio-genesis and brown adipocyte proliferation through β-adrenergic receptor-mediated pathway, and expression of UCP1 through PKA pathway (93-96).

1.2.3 Vascular and nerve networks in adipose tissue

Each adipose depot contains well-organized vascular and nerve networks. For instance, in anterior subcutaneous adipose tissue, there are dense vascularity and innervations (97). Blood vessels branch and reach almost every adipocyte, accompanied by nerves of different sizes. Thinner nerves contain a larger number of non-adrenergic fibers,

while thicker ones contain fibers immunoreactive for the neuropeptide Y (NPY) and other neuropeptide, such as substance P (98, 99). The unique morphology of nerve network in adipose tissue makes the sympathetic regulation of adipose metabolism to be possible and fast in response to any environmental changes.

Both WAT and BAT are highly vascularized (4, 100, 101). In addition to density, blood flow in adipose tissue is relatively high compared with other tissues, which supports the high metabolic activity (102). For instance, BAT is probably the densest vascularized tissues in the body (unpublished data). The high thermogenic activity of BAT requires a particularly high rate of blood perfusion to supply oxygen and substrates and to export heat and wastes. Another unique character of the adipose vasculature is that it appears to function as a progenitor niche and provide signals for adipocyte development. Most adipocytes descend from a pool of proliferating progenitors, which are already committed either in prenatal or in early postnatal life. These progenitors reside in the mural cell compartment of the adipose vasculature (103, 104).

1.2.4 Angiogenic factors in adipose tissue

Growing adipose tissue contains various cell types including adipocytes, adipose stromal cells, endothelial cells and inflammatory cells (4, 105). The heterogeneous cell populations determine a dozen growth factors and cytokines that either individually or synergistically regulate blood vessel growth, including leptin, VEGF, FGF-2, HGF, IGF, TNF-α, TGF-β, PlGF, VEGF-C, resistin, NPY, heparin-binding epidermal growth factor, angiopoietins, IL-6 and IL-8 (Figure 2) (4, 106-116).

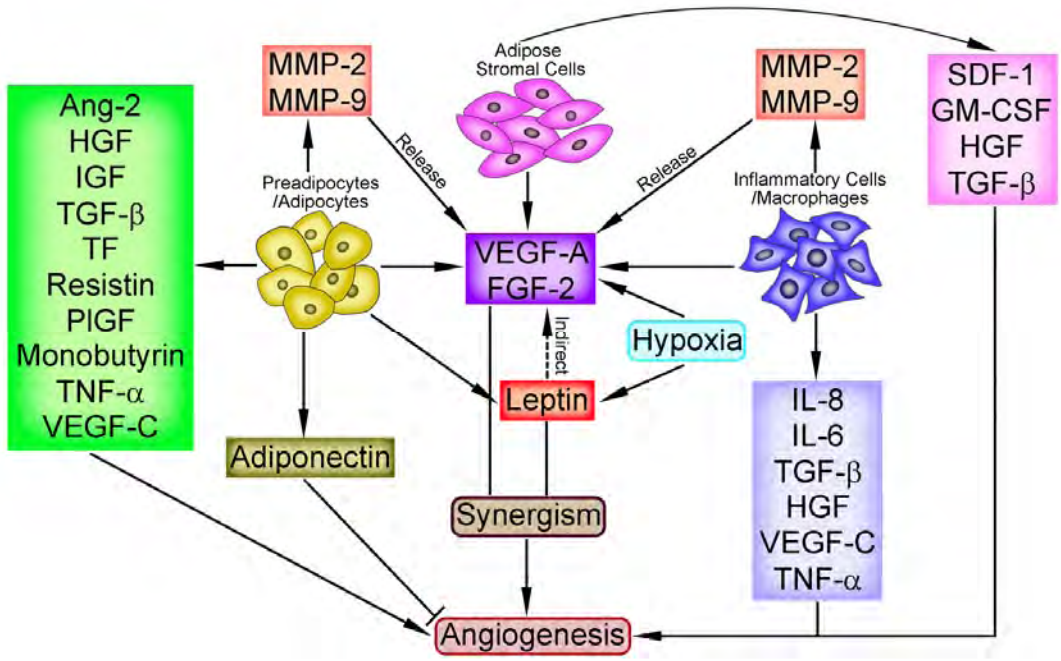

Figure 2. Adipocytes, stromal cells and inflammatory cells in adipose tissues can secret multiple factors, including VEGFs, FGF, MMPs, IGF, leptin, adiponectin, etc. Those factors can directly or indirectly induce adipose angiogenesis, either alone or in a synergistic manner.

1.2.4.1 VEGF

VEGF plays an important role in many growing and developing tissues either at healthy or pathological stages. The highest level of VEGF was produced in omentum fat pads compared with all the other tissues (117). Adipocytes, adipose-infiltrated inflammatory cells and stromal cells are all contributing to VEGF production (105, 118, 119). In human studies, VEGF is negatively correlated with the degree of obese (120). However, bearing the fact that adipocytes do not express VEGF receptors (121), the role of VEGF in adipose tissue transformation and regulating adipose metabolism requires further studies.

1.2.4.2 Angiopoietins

Angiopoietins bind to Tie-2 receptor to regulate vessel maintenance, growth and stabilization. In both high-fat-fed and genetic obesity models, mRNA and protein levels of Ang-1 were down-regulated in developing adipose tissue (116). Remarkably,

Ang-2 as a vascular remodeling factor is consistently upregulated during adipose tissue growth (116, 122). These findings support the notion that adipose tissue development requires continuous remodeling, maturation and patterning of vasculature.

1.2.5 Anti-angiogenesis in obesity

Obesity is related to increased food-intake, reduced metabolic and physical activities, dysfunction in endocrine system, as well as genetic inheritage (107, 123). Thus, the best way to treat obesity is generally by increasing energy expenditure and by decreasing food intake (124). The tight links between adipose tissue and blood vessels provide the novel therapeutics of treating metabolic diseases, *e.g.* obesity, by targeting the vasculature. Few angiogenic inhibitors, including small molecule TNP-470, and endogenous protein inhibitors angiostatin and endostatin, show promising effects in both high-fat-fed obese and genetically obese models (125, 126). The novelties of the application of anti-angiogenic targeting in treating obesity lie in two aspects: 1) these angiogenic inhibitors reduce the fat mass and body weight in mice without significantly affecting food intake; 2) the inhibitors achieve the anti-obesity activity via their anti-angiogenic effects, since they specifically target endothelial cells other than other cell types. After the treatment with angiogenesis inhibitors, adipose tissue undergoes vascular remodeling, contains more apoptotic cells and less proliferating endothelial cells compared with non-treated one (126). The angiogenesis inhibitor TNP-470 increases the insulin sensitivity in obese animal. This suggests that the anti-angiogenic agents may have effect on the development of type II diabetes caused by overweight (125).

1.3 CANCER

Cancer is a class of malignant diseases in which a group of various types of cells display uncontrolled growth, invasion, and sometimes metastasis. These three malignant properties of cancer (malignant tumor) differentiate it from benign tumor, which is self-limited and less invasive or less metastasis. Tumorigenesis is a multi-step process, involving numerous genetic alterations. There are six essential alterations in cell physiology that collectively determine malignant growth: 1) self-sufficiency in growth signals; 2) insensitivity to growth-inhibitory (anti-growth) signals; 3) evasion of programmed cell death (apoptosis); 4) limitless replicative potential; 5) sustained angiogenesis; 6) tissue invasion and metastasis (127). When each of these physiological changes is acquired during tumor development, the corresponding anti-cancer defense mechanism hardwired into cells and tissues is breached (127). The multiplicity of defenses mechanisms explains why cancer is relatively rare during an average human lifetime.

Tumor microenvironment has some unique features, such as hypoxia and other environmental stresses (*e.g.* acidosis, excessive growth factors or nutrient deprivation), which make an altered metabolic situation in tumor compared with other organs and tissues. Hypoxia and other stresses redirect intermediate metabolites, sustaining bioenergetics and cell survivals (128, 129). For instance, tumor signaling pathways regulate energy production and macromolecular synthesis. Proto-oncogenes, such as c-Myc, directly change the tumor metabolism and protein synthesis to order to support enhanced proliferation rates (130, 131). The crosstalk between the c-Myc and HIF pathways demonstrate an interplay between responses to oxygen deprivation and a key transcription factor regulating growth (129, 132, 133).

1.3.1 Causes and mechanism of cancer

Cancer is a diverse class of diseases which differ widely in their causes and biology and progressively develop as biological 'errors' accumulate. There are few causes of cancer: 1) Mutation, from chemical carcinogens or ionizing radiation. Certain carcinogens, such as tobacco and alcohol, or radiation, such as UV light and radon gas, can induce DNA mutations that lead to cell growth and metastasis. 2) Viral or bacterial infection (134). 15% of human cancers worldwide are associated with viruses or bacteria, such as human paplillomavirus, hepatitis B virus and Epstein-Barr virus. Viral or bacterial infection usually activates proto- or oncogenes, which induces uncontrolled cell division. 3) Hormonal imbalances. Some hormones can stimulate excessive cell growth. For instance, hyperestrogenic state promotes endometrial cancer (135). 4) Immune system dysfunction. The breakdown of immune surveillance, by viral infection such as HIV and HPV, is a possible etiology of cancer (136). 5) Heredity. A number of cancers are inherited due to defects in genes, such as *BRCA1* and *BRCA2* (137, 138). These causes of cancer are not mutually exclusive, which can exist in a certain type of cancer simultaneously.

The molecular mechanism for initiation of cancer can be generally categorized into three aspects: epigenetics, oncogenes and tumor suppressor genes. First, the alteration of gene expression is not necessarily associated with the changes in DNA sequence. Tumorigenesis can be induced by non-mutational changes in DNA, such as DNA methylation and acetylation of histone proteins bound to chromosomal DNA (139). Secondly, oncogenes, such as *Ras*, promote cell growth and produce mitogens further supporting tumor expansion (140). Mutations in the quiescent counterparts of oncogenes and proto-oncogenes, often produce the imbalanced amount and activity of product protein, which leads to the excessive cell growth (141). Thirdly, tumor

suppressors are, in general, transcription factors which can be activated by cellular stress or DNA damage. One of the best-known genes is p53, which is involved in nearly half of all types of cancers (142, 143). The mutations and consequent functional loss of tumor suppressor genes increase the likelihood for cancer occurrence and diagnosis (143). Major changes in cellular metabolism lead to the malfunction of cell growth, further causing cell death. However, once the microenvironment is tolerant to those molecular and cellular alterations, new genetic modification resulted from the 'evil' initiation will accelerate cancer progression.

1.3.2 Tumor angiogenesis

A small malignant cell population can obtain oxygen and nutrients from pre-existing and neighboring blood vessels (144-146). However, further tumor expansion requires accompanying vasculature, thus stimulates new blood vessel formation. More than 30 years ago, Dr. Judah Folkman raised the hypothesis that tumor growth is dependent on new blood vessel growth or angiogenesis (3). Studies have shown that mutations of oncogenes and tumor suppressor genes can lead to the switch of angiogenic phenotype in tumor (147). For instance, loss of p53 function leads to not only elevated levels of angiogenic factors such as VEGF expression, but also decreased levels of endogenous angiogenesis inhibitor TSP-1 (39, 148-150). Thus p53 may control the balance of angiogenic and anti-angiogenic factors toward neovascularizton.

Tumor starts to grow at an exponential rate as soon as the angiogenic phenotype is switched on. The growth rates between tumor cells and endothelial cells are unsynchronized, which lead to certain features of tumor vasculature (Figure 3, left panel) (151). First, tumor vasculature is a disorganized network. Tumor blood vessels are often irregular branched and patterned, represented by hemorrhagic, torturous and

leaky features (152). Secondly, the disorganized tumor vasculature results in chaotic blood flow in tumors. The poorly oxygenated blood cannot be effectively delivered to tumor cells (153). Thirdly, tumor blood vessels sometimes incorporate tumor cells onto vessel walls, a process named vascular mimicry, which significantly increases the chance of tumor cell dissemination to distal organs (154).

The molecular mechanisms regulating angiogenic switch usually involve upregulation of multiple transcrpitional and growth factors. Rapid expansion of tumor mass generates hypoxic condition in tumor. HIF-1α is commonly upregulated in tumor microenvironment. In addition, oncogenic regulation works either alone or in combination with hypoxia, to stimulate angiogenesis. For instance, upregulation of epidermal growth factor receptor (EGFR) and inactivation of tumor suppressor p53, can increase the synthesis rates or reduce the degradation of HIF1-α (74, 155). HIF1-α further upregulates expression levels of other growth factors, such as VEGF, EPO and Ang-2 (156, 157). In the early stage of tumor growth, only a few angiogenic factors, such as VEGF, are produced by tumor cells. However, during the tumor progression, genomic instability of tumor cells often leads to the expression of multiple growth factors, such as VEGF-C, FGFs, IGFs and PDGFs (Figure 3, right panel) (158).

1.3.3 Systemic effect of tumor-produced growth factors

Cancer is not a local disease. Cancer cachexia and paraneoplastic syndromes are the primary causes of mortality in cancer patients. Tumor produces cytokines and growth factors, such as TNF-α, IL-6 and VEGF, contribute to the development of systemic syndromes at advanced stage of malignancy (159, 160). For instance, VEGF systemically impairs functions of multiple organs including those in the hematopoietic

and endocrine systems, leading to early death of mice (161, 162). Anti-VEGF blockage significantly improves the function of organs and mice survivals without

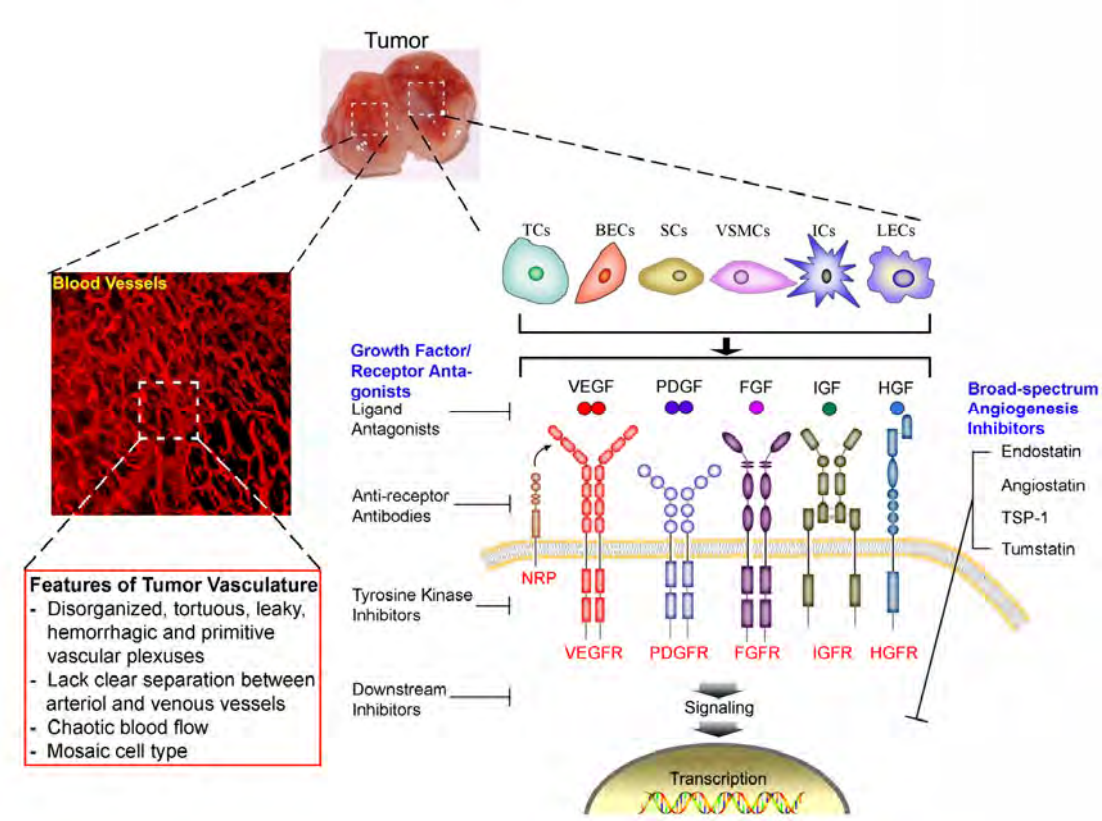

Figure 3. Tumor vasculature, angiogenic factors and anti-angiogenic strategy. Tumor vasculature displays an unorganized structure, including tortuous, leaky and hemorrhagic features (left panel). Heterogeneity of multiple cell types in tumor produces various angiogenic factors, including VEGFs, FGFs, PDGFs, IGFs and HGFs. TCs: tumor cell; BECs: blood endothelial cells; SCs: stromal cells; VSMCs: vascular smooth muscle cells; ICs: inflammatory cells; LECs: lymphatic endothelial cells. Anti-angiogenesis strategies include applications of endogenous angiogenesis inhibitors and anti-growth factors/receptors antagonists.

affecting tumor growth (161, 162). Treatments against cancer-associated systemic syndrome by inhibiting tumor-produced factors define novel targets for cancer therapy.

1.3.4 Anti-angiognesis in cancer therapy

The well-used strategies for cancer therapy include chemotherapy, radiotherapy and surgery, depending on types and classification of tumors and individual health condition. After the scientific community began to accept the principle of tumor

angiogenesis, anti-angiogenesis have experienced intensive investigation and anti-angiogenic therapy became the fourth modality of cancer treatment.

In general, there are four possible mechanisms by which anti-angiogenic agents produce beneficial efficacies in cancer patients: 1) Anti-angiogenesis, which directly decrease nutrients and oxygen supply through blood vessels. 2) Anti-vasculogenesis, which inhibits bone marrow-derived circulating endothelial cells in order to prevent tumor neovascularization. 3) Normalization of tumor vasculature, which improves the efficiency for chemo-drug delivery. 4) Vascular destruction and remodeling, which destroys the tumor vasculature by modifying the vascular pattern. According to those mechanisms described above, anti-angiogenic drugs can be designed to target angiogenic ligands, receptors and tyrosine kinases (Figure 3, right panel). Endogenous angiogenenic inhibitors, including TSP-1, endostatin and angiostatin, can also be used as the broad-spectrum angiogenesis drugs (Figure 3, right panel).

Since accumulative researches on pathological angiogenesis have been conducted, the complexity of mechanisms underlying anti-angiogenic strategy increases. Due to the heterogeneity character, multiple angiogenic factors are utilized by tumor to escape from the treatments. For instance, anti-VEGF therapy often encounter drug resistant problem (163). Thus, understanding of interplay between tumor angiogenic factors is critical for developing new therapeutics.

2 AIMS

The overall aims of this thesis were to study the role of multiple angiogenic factors in adipose tissue metabolism and tumor development.

The specific aims were:

To study how angiogenic switches were turned on in the adipose tissue transformation, and how anti-angiogenesis regulates adipose metabolism.

To study the systemic effect of angiogenic factors and the interplay of different angiogenic factors.

3 METHODS

To conduct experiments stated in this thesis, following methods have been employed:

In vivo models:
Mouse tumor model
Mouse wound healing model
Mouse cold adaptation model
Vascular permeability assay

In vitro methods:
Immunohistochemical methods
Visualization of immunostaining by various microscopes

The detailed procedures and protocols of these methods are described in papers respectively. In current section, I would like to discuss the background, key parameters and applications of these methods in angiogenesis and relevant research.

Tumor models
The experimental tumor models in laboratory animal include xenograft/transplant model, spontaneous model, orthotropic model, and so on. All these models try to mimic the human autochthonous cancer at different levels, and with different advantages. In current study, the xenograft/transplant tumor model is used by injecting tumor cells into recipient animals. Tumor cells can be injected intravenous, intraperitoneal, or subcutaneous, enabling the accurate monitoring of tumor localization. Tumor cells can be integrated with research aiming genes, such as VEGF and PDGF, or reporter genes, such as GFP. Cell numbers/volumes to be injected should be determined by the characters of tumor growth rate, which mean the faster growth rate is, the fewer cells ought to be injected. 1 million tumor cells were injected into each mouse in studies discussed in the current thesis. For evaluating the vasculature in tumor, tumor size can be critical because fast growing tumor with big size (~1.0 cm^3) usually have necrosis in the center which significantly affects the vascular network. Thus, tumor with proper size (0.5-1.0 cm^3) should be controlled and used for evaluation of tumor angiogenesis.

Wound healing model
Wound healing process is divided into few sequential, yet overlapping, phases, including homeostasis, inflammatory, proliferation and remodeling stages (164). Angiogenesis is one critical component in proliferation stage. Therefore, wound healing assay can be used to evaluate the function of angiogenic vessels, although it is a much more complex process. To investigate the angiogenic activity induced by certain molecules or genes/proteins, two methods can be used: applying the angiogenic factors or inhibitors locally to wound, and utilizing genetic modified animal as the model. To perform the wound healing assay, a pair of small circle incision (5 mm in diameter) is created on dorsal back of each mouse. The healing of wound is followed by daily

inspection of wound size, scar formation and re-growth of epidermal layers. Local infection should be closely inspected to ensure it does not interfere wound healing.

Cold adaptation model

The thermo-neutral environment temperature for mice is 30˚C. Thus, all mice should be acclimated at 30˚C before the start of experiments. Due to mice behavior and humanity reasons, mice should be adapted at 18˚C for either one week (wild-type mice) or three weeks (UCP1 knock-out mice), at least, before moved into 4˚C environment. Because UCP1 knock-out mice are thermogenic incompetence, they are sensitive to the environmental changes. At 4˚C environment, UCP1 knock-out mice should be closely checked to ensure their cold-tolerance.

Vascular permeability assays

Vascular permeability is to assess the function of blood vessels. In tissues, not all immuno-stained blood vessels are real functional ones with perfusion. Thus, the direct assessment of perfusion and permeability is important to evaluate the vascular function. There are few in vivo vascular permeability assays. Miles permeability assay is one of the most commonly used methods to investigate vascular permeability, which injects high-molecular-weight dye, such as Evans blue, into circulation of an animal. Extravasation of dye can be monitored over time, and quantified by extraction methods (162). However, this method is not accurate enough, and usually produces data with high variations. Another method is to explore the ultrastructure of endothelium with or without particles, such as ferritin and colloidal gold particles, under electronic microscope (165). This method is high-tech-oriented, accurate, informative, but rather expensive and long time to perform. The third method is to combine dye/molecule injection and microscope technique to illustrate the vascular morphology and leakage of dye on tissue sections. This method is easy to perform, yet produces accurate data and directly gives the morphology of vascular permeability in functional blood vessels. This method is applied in current studies (paper III).

Immunohistochemistry

Immunohistochemical methods are particularly useful for the studies of *in vivo* angiogenic and anti-angiogenic effects, which require both morphological and quantitative assessments. Quantification of blood vessel density requires the immuno-staining of endothelial specific antigens including vWF, vascular cell adhesion molecules (VE-Cadherin, CD34 and CD31/PECAM-1), receptors (VEGFRs, TIE-2 and Ephrins), and extracellular lamina components (collagen-IV). Since all these markers have their particular expression profiles, such as VEGFR1 mainly expressed on large vessels while VEGFR2 preferring small vessels, a proper selection of antibodies pefers to be determined before performing immuno-staining. Quantification of the proliferation of endothelial cells in tissues requires the triple staining of endothelial nuclei (DAPI or PI), endothelium (CD31, CD34 or isolectin-B4) and proliferative markers (Ki-67 or PCNA). For accurate quantification, sections of immuno-staining should be used to obtain the raw data.

Visualization with various microscopes

The application of various microscopes offers opportunities to access histological patterns, particularly vasculature, at cellular levels. The vascular network can be

illustrated by whole mount staining, followed by the confocal laser scanning with confocal microscope. A series of fine image layers can be obtained from laser scanning, which will be rendered into 3-dimension projection. With the 3-D images, the morphology of blood vasculature can be clearly evaluated. The quantification of density of blood vessels is often based on the single layer images. The co-localization of different proteins and/or cellular compartments should be based on single layer images with 180˚-angle view. However, if certain methods limit the application of confocal laser scan microscope, regular fluorescence microscope can be used on tissue sections (cryostat and paraffin section) to access the vascular network.

4 RESULTS AND DISCUSSION

4.1 FOXC2 REGULATES ANGIOGENESIS IN ADIPOSE TISSUE (PAPER I)

We found that, white and brown adipose tissues contained a considerably high density of microvessels appearing as vascular plexuses in FOXC2 transgenic mouse (FOXC2-TM), which showed redistribution of vascular smooth muscle cells and pericytes. The structure of blood vessels appeared to be a dense network with irregular and tangled vascularity. The total number of α-SMA-positive arterial vessels was significantly decreased in both WAT and BAT of FOXC2-TM as compared with wide-type mice. Surprisingly, a considerable number of α-SMA-positive VSMCs were associated with microvessels, including the dense vascular plexuses, suggesting redistribution of VSMCs. In contrast, total numbers of NG2-positive pericytes were significantly decreased in WAT and BAT of FOXC2-TM, but nearly all pericytes remained associated with microvessels.

We further found that the expression levels of several potent angiogenic factors were significantly increased in the abdominal WAT of FOXC2-TM by using an Affymetrix gene array and quantitative real-time PCR assays. Ang-2 was one of the most up-regulated gene products in the adipose tissue of FOXC2-TM. A nearly 6-fold increase of Ang-2 was detected in WAT of FOXC2-TM as compared with that of WT mice. Ang-2 promoter fused with the luciferase reporter was cloned and transfected into 3T3 differentiated preadipocytes in the presence and absence of FOXC2. Interestingly, Ang-2 promoter activity was increased in a dose-dependent fashion after the addition of FOXC2. Nearly an 8-fold increase of luciferase activity was observed at 200 ng of FOXC-2. Mutagenesis showed that Fkh4 region on FOXC2 promoter was the

responsible element for FOXC2-induced transcription activity. These findings show that FOXC-2 directly activates Ang-2 promoter activity and controls its expression. We next isolated preadipocytes from $FOXC2^{+/+}$, $FOXC2^{+/-}$ heterozygous, and $FOXC2^{-/-}$ knockout mice to quantitatively correlate expression levels of FOXC2 with those of Ang-2. Expectedly, an ideal correlation of expression levels between FOXC2 and Ang-2 existed in $FOXC2^{+/+}$ preadipocytes. Taken together, these data demonstrate that FOXC-2 transcriptionally up-regulates Ang-2 expression in adipocytes.

Interestingly, administration of L1-10 to FOXC2-TM at a dose of 4 mg/kg, a dose known to block Ang-2 function *in vivo*, virtually completely reversed the FOXC-2-induced angiogenic phenotype. Immunohistological analysis showed that the primitive vascular plexus-shaped vessels in inguinal WAT were normalized to well-structured vascular networks, which were indistinguishable from those in wide-type adipose tissues. Quantification showed that blockage of Ang-2 led to decreased total number of VSMC-coated vascular area and increased large arterial vessel association. These data provide compelling evidence that Ang-2 is responsible for the FOXC-2-induced vascular maturation and patterning in the adipose tissue.

To evaluate the function of blood vessels, we performed full skin wound-healing experiments. At day 11 after the creation of the wound, all WT mice exhibited complete healing of the wound beds (n = 10). In contrast, a significantly delayed wound healing was observed in FOXC2-TM. Notably, significantly larger diameter wounds already became obvious at day 4 after the creation of the wound and significant differences remained throughout the entire experiments. Immunohistochemical analysis showed that a significantly higher number of CD31-positive vessels were present in wound tissue of FOXC2-TM than in that of WT mice, suggesting that

impairment of wound healing was not due to defects of neovascularization but abnormality of vascular function. These results demonstrate that the vascular adaptation seen in FOXC2-TM results in remodeling of existing vessels and formation of premature new vessels that lead to delayed wound healing.

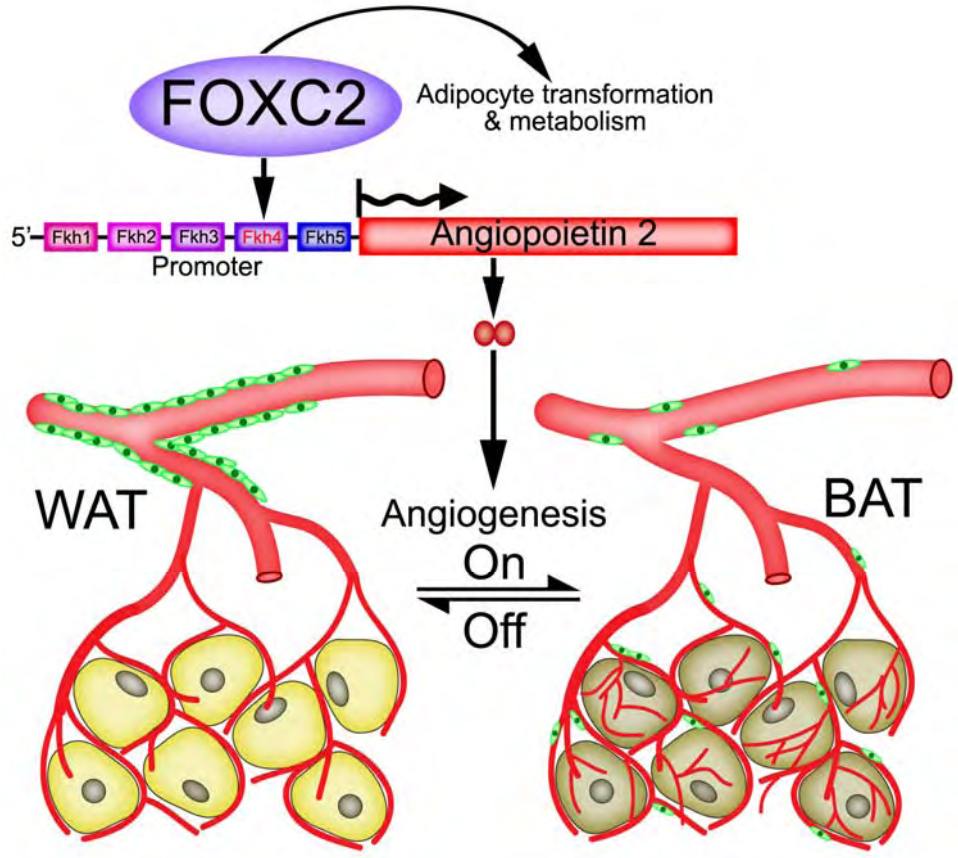

Figure 4. FOXC2 controls angiogenesis and mural cells redistribution by transcriptionally regulating Ang-2. FOXC2 targets on the Fkh4 domain of Ang-2 promoter and upregulates the Ang-2 expression level. Ang-2 further switches on the angiogenesis in accompanied with the adipose tissue transformation. Mural cells, particularly smooth muscle cells, are redistributed from large vessels into small ones. Anti-Ang-2 inhibition can block the angiogenesis process and smooth muscle cells redistribution.

4.2 COLD-INDUCED ANGIOGENESIS IN ADIPOSE TISSUE (PAPER II)

We have found that a large increase in vascularization was observed macroscopically in both inguinal and subaxillary adipose tissues of mice exposed to 4°C, as compared to the situation in controls at 30°C. Immunohistochemical analysis of inguinal WAT with an anti-CD31 antibody revealed significant increases in vascularity after cold

acclimation. Similarly, a considerable increase in microvessel density was detected in BAT after 5 weeks of exposure to cold. In addition to vasculature, inguinal adipose tissue of the cold-acclimated mice demonstrated the development of a more BAT-like phenotype in the WAT depots, in that the tissue exhibited a reduction in the size of the lipid depots and the average size of the adipocytes and a more multilocular appearance of the cells. The prohibitin-positive mitochondria were significantly increased in inguinal adipocytes.

To study whether the adipose vasculature was actively proliferating during cold acclimation, inguinal adipose tissue sections were double stained with antibodies against the endothelial cell marker CD31 and against the marker of proliferating cells, Ki67. In sections from animals cold-exposed for 1 week, a significant number of proliferating endothelial cells were detected in angiogenic vessels. These findings demonstrate that endothelial cell proliferation contributes to the cold-induced angiogenic phenotype.

To further explore the molecular mechanisms inducing the angiogenic switch, we performed Affymetrix gene array analysis of inguinal WAT of mice exposed at 4°C for 1 week and 5 weeks. Four most upregulated genes were *Ucp1*, *Elovl3* (elongation of very long-chain fatty acids), *Fabp3* (muscle and heart fatty acid binding protein), and *Cpt1b* (muscle-type carnitine palmitoyl transferase 1), which are all strongly associated with BAT as compared to WAT. *Vegfa* was significantly upregulated after 1 week of exposure to cold, and the expression then returned to initial values by 5 weeks, validated and quantified by northern blot. A marked increase in *Pgc-1α* gene expression was detected after 1 week of exposure to cold and this expression was also transient, which was further validated by qRT-PCR. In contrast, *Tsp*, an endogenous

angiogenesis inhibitor, was significantly downregulated in cold exposure group. These findings demonstrate that the cold stimulus triggers an angiogenic switch by inducing upregulation of proangiogenic factors and simultaneous downregulation of endogenous angiogenesis inhibitors not only in conventional BAT depots but also in WAT depots.

The cold-stimulated increase in metabolism (nonshivering thermogenesis) that occurs in wild-type mice is fully dependent on the presence of the brown-fat-specific uncoupling protein UCP1. In UCP1 knockout (*UCP1*$^{-/-}$) mice, there should be minimal hypoxia in adipose tissues, even in the cold. If angiogenesis is a result of tissue hypoxia, it should be correspondingly diminished in the *UCP1*$^{-/-}$ mice. At the end of the cold acclimation period, *UCP1*$^{-/-}$ mice exhibited a vascular phenotype in both WAT and BAT similar to that seen in the tissues of wild-type mice. This strongly suggests that the cold-induced angiogenesis occurred through a hypoxia-independent mechanism.

VEGFR2 blockage with monoclonal VEGFR2 antibody, as well as sunitinib, virtually completely prevented the cold-induced angiogenesis in both inguinal WAT and interscapular BAT, and a VEGFR2-mediated angiogenic signal was, therefore, ubiquitously required for the cold-induced angiogenic switch in all adipose tissues studied. In contrast to the effect of VEGFR2 blockage, inhibition of VEGFR1 signaling by a neutralizing antibody significantly enhanced angiogenesis in both WAT and BAT, suggesting a negative role of VEGFR1 signaling in regulation of adipose angiogenesis.

During the treatments, we observed that the VEGFR2 antibody induced behavioral alterations in the mice. In cold, the VEGFR2-antibody-treated mice accumulated

significantly more nest materials than the controls and the VEGFR1-treated mice. These changes in response to cold suggested that inhibition of angiogenesis by VEGFR2 blockage might have influenced the ability of mice to satisfactorily develop the capacity for and to utilize NST. The anti-VEGFR2-treated group showed both a lower and a delayed response to the NE injection, establishing that inhibition of neovascularization leads to a diminished capacity for NST and can thus explain the altered behavior in the cold of this group of animals. Surprisingly, the magnitude of the response in anti-VEGFR1-treated mice was greater than that of the buffer-treated animals. The thermogenic response was limited by the magnitude of the angiogenic response to cold.

4.3 PDGF-B-INDUCED STROMAL EPO STIMULATES HEMATOPOIESIS AND TUMOR ANGIOGENESIS (PAPER III)

Histological analysis showed that the PDGF-B tumor contained a large proportion of stroma, which infiltrated between tumor cells. We estimated that greater than 50% of the PDGF-B tumor tissue consisted of stroma. In contrast, the vector control tumor lacked obvious infiltration of stromal tissue. In addition to stromal tissue expansion, the density of microvessels was significantly greater in the PDGF-B tumor relative to control, suggesting that PDGF-B contributes significantly to tumor angiogenesis.

PDGF-B tumor-bearing mice exhibited marked splenomegaly and hepatomegaly relative to control mice. Intriguingly, histological analysis showed marked expansion of the red pulp, which became indistinguishable from the white pulp. PDGF-B levels in the plasma of PDGF-B tumor-bearing mice were significantly greater than those in controls. These findings demonstrate that tumor-derived PDGF-B enters the circulation and triggers active extramedullary hematopoiesis in these organs.

Isolated stromal fibroblasts expressed high levels of PDGFR-α and PDGFR-β. *In vivo*, PDGFR-α and PDGFR-β were not expressed in hepatocytes or in splenocytes, rather they were exclusively expressed in the stromal compartment. Thus, it is reasonable to assume that PDGF-B targets the stromal compartment *in vivo*.

We found that the circulating levels of EPO in both vector and PDGF-B tumor-bearing mice. EPO protein was increased nearly 3-fold in the plasma of PDGF-B tumor-bearing mice compared with controls. This result suggested that PDGF-B may upregulate EPO expression. Considering PDGFRs expression restrictly on stromal tissue, both hepatocytes and splenocytes are not the sources for EPO. To further validate whether the stroma produces EPO, we used PDGF-B to stimulate stromal cell lines at different concentrations. Interestingly, PDGF-B induced EPO messenger RNA production in a dose-dependent manner. At 150 ng/ml, PDGF-B increased EPO production by 5-fold. Meanwhile, PDGFR-α or PDGFR-β antibodies could reduce the PDGF-B-induced EPO production, indicating that both receptors are involved *in vitro*. Thus, these data suggested that PDGF-B directly stimulated tissue and organ stroma to induce EPO production.

In situ hybridization on tumor, spleen, liver and kidney revealed that EPO production was significantly elevated in both liver and spleen, trendily elevated in kidney. On tumor tissue, there was no significant difference in terms of *Epo* mRNA level. Meanwhile, we determined the hypoxic situation in those tissues/organs by Hypoxyprobe. No significant differences of tissue hypoxia were determined in tumor, spleen and liver between control and PDGF-B tumor bearing mice. Therefore, these results showed that PDGF-B induced EPO expression in stromal compartment *in vivo*,

which was independent of hypoxia. In the tumor model, an anti-EPO antibody could partially inhibit PDGF-B induced tumor growth. Additionally, this antibody could prevent hepato-splenomegaly in PDGF-B tumor bearing mice. Thus, these data showed that stromal-derived EPO contributed to PDGF-B-induced tumor growth and extramedullary hematopoiesis.

We further tested our hypothesis in an *in vivo* adenovirus model. Delivery of PDGF-B over-expressing adenovirus (Ad-PDGF-B) in mice could induce splenomegaly and hepatomegaly. Consistent with hepato-splenomegaly, circulating level of EPO in mice was significantly increased. Furthermore, the high level of Ad-PDGF-B-induced EPO increased the hematological parameters, including hematocrit, hemoglobin and red blood cells. Both PDGFR-β blockage and administration of STI571 significantly inhibited splenomegaly, and decreased hemoglobin and red blood cells. These data validated that PDGF/PDGFR-mediated upregulation of EPO is responsible for hematopoiesis.

5 CONCLUSIONS AND PERSPECTIVES

Obesity and cancer are complex diseases that may not be cured by single drug or scheme. Conventional anti-obese drugs control body weight by altering appetite, metabolism, or calorie absorption. Conventional anti-cancer/tumor drugs (small molecules) inhibit tumor growth by affecting rapidly dividing cancer cells. Targeted therapy, by applying bio-active antibodies, has had significant impact in the treatment of certain types of diseases, and is an active research area nowadays. Since the first anti-VEGF drug Bevacizumab was approved for the treatment of human colorectal cancer in 2004, many drugs have been developed and used in fighting against cancer in clinic.

Can anti-angiogenic drugs apply to the treatment of obesity in clinic?

Translational research preciously showed that anti-angiogenic drug could inhibit obesity and increase insulin sensitivity in both high-fat-fed and genetic obese mouse models (125, 126). However, there are still little known about the interplay among multiple angiogenic factors expressed in adipose tissue, in regulating adipose tissue growth and metabolism.

With the knowledge we have so far, it is too early to say whether the anti-angiogenic drugs can be used for the treatment of obesity in human. More and more evidence, including the results presented in the current thesis, showed that interruption of angiogenesis in adipose tissue could modify vascular pattern, maturation and adipose tissue metabolism. We then ask how we should use anti-angiogenic drugs in clinical settings. Lessons can probably be learned from clinical cancer research. For instance, in treating cancer, anti-angiogenic agents have been used in the combination with

chemo-drugs, which offer great success to regress the tumor vasculature and tumor mass. Currently, some prescription weight loss drugs have been recommended for short-term use in clinic, including Xenical, Reductil, Rimanabant, etc. Can anti-angiogenic agents be used in the combination with those drugs? If so, how should these types of drug be used in order to achieve the best efficiency? Are there any drug resistant problems associated with these scenarios?

In adipose tissue, stimulate or inhibit?

Adipose tissue growth and expansion are dependent on angiogenesis. Consistent growth of blood vessels in WAT would probably provide sufficient nutrients and oxygen, thus better opportunities for expanding adipose tissue mass. In addition to angiogenesis-depended tissue expansion, adipocytes directly descend from a pool of proliferating mural cell compartment of the adipose vasculature (104). The adipose vasculature appears to be a progenitor niche for adipocyte development. Therefore, inhibition of angiogenesis in WAT would be critical for directly reducing the adipose tissue mass.

On the contrary, growing blood vessels in BAT would further accelerate adipose tissue metabolism, which lead to the great consumption of oxygen, fat and carbohydrates. Studies showed that 20 gram active BAT can consume energy equivalent to about 4 kg adipose tissue in human per year (87). Therefore, stimulation of angiogenesis in BAT would contribute to the energy expenditure for interfering obesity.

White and brown adipose tissues perform completely different function in the body. The biological significance of adipose vasculature varies. Thus, applications of

stimulation or inhibition of angiogenesis should be conducted in a tissue specific manner.

Cancer, local or systemic treatment?

Currently, most of strategies for anti-angiogenic cancer treatment focus on tumor itself. They target different compartments in the tumor local environment, such as cancer cells and endothelial cells. This usually leads to two consequences: 1) Drug resistance. Certain patients benefit from anti-angiogenic therapy in the beginning. However, when other cancer signaling pathways are switched on, tumors start resistant to previous treatments. 2) Inhibition of tumor growth does not always lead to improvement of survival. Tumor/cancer is not a local disease, which secrets multiple factors and proteins systemically affecting distal organs and tissues, known as cachexia or paraneoplastic syndrome. Many drugs have shown impressive inhibition of tumor growth at animal models, but have displayed little survival benefits for patients in clinical settings. Therefore, various strategies of combination therapy against molecular targets, together with the understanding of their systemic effects, would provide novel and effective therapeutic insights.

Angiogenesis, lying under many physiological and pathological situations, gives us the opportunity to investigate and understand diseases from a different perspective, and leads us to think from a systemic point of view. With understanding the mechanism of pathological angiogenesis and anti-angiogenesis in adipose tissue and tumor, various therapeutic scenarios will emerge for the treatment of obesity, metabolic disorders and cancer.

6 ACKNOWLEDGEMENTS

This thesis work was performed at the Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet. I would like to express my gratitude to the institution for providing excellent research environment. I would like to express my gratitude to those who helped me during past few years. Particularly:

Professor *Yihai Cao*, my supervisor. Thank you for recruiting me to your laboratory and introducing me to the angiogenesis research. Your intuition to science is so impressive. The projects you gave me are always interesting to work with.

Professor *Eva Severinson*. Thank you for giving me the opportunity to study at Biovetenskapliga Forskarskola. The offer letter you issued started my new era in Sweden. *Camilla Ahlqvist*, thank you for numerous help when I just started my life here.

Professor *Barbara Cannon* and Professer *Jan Nedergaard*. Thank you for the project collaboration, sharing your expertise of adipose tissue metabolism, and always willing to arrange another meeting to discuss complicated questions. Dr. *Natasha Petrovic*. Without your help, I couldn't complete the cold-induced angiogenesis project.

Professor *Sven Enerbäck*. Thank you for the project collaboration and offering so many essential materials and opportunities.

Professor *Franco Lucchini* and Professor *Paolo Vezzoni*. I appreciate for offering me the opportunity to work in your laboratory. *Barbara Tondelli, Michela Lizier, Andrea Perota* for your friendship and numerous help while my stay in Cremona and Milano.

Dr. *Björn Rozell*. Thank you for teaching me the pathological knowledge and performing analyses on numerous samples. I always dream of having the perfect memory as you have.

Dr. *Dagmar Galter*. Thank you for the project collaboration and the pretty *in situ* hybridization images.

I'd like to thank:
Dr. Renhai Cao for teaching me everything from beginning and sharing your research experiences with me. *Dr. Piotr Religa* for being good friend and collaborating on few projects. *Dr. Ebba Bråkenhelm* for being a true model for me and teaching me a lot of thing by emails. *Lasse Jensen* for being good friend and introducing your band Eterno to me. *Eva-Maria Hedlund* for helping with endless paper work in Swedish and sharing your girls' intuition on many things. *Samantha Lee* for valuable discussion on various topics, and teaching me many English words. *Sharon Lim* for being my good apprentice and assisting me with lots of lab work. The current and past members from Cao group: *Li Chen, Dr. Kayoko Hosaka, Meit Björndahl, Dr. Johan Nissen, Xing Zhao, Dr. Zongwei Wang, Anne Hennig, Pegah Rouhi, Danfang Zhang, Junwei Zhang, Hong Ji, Funeng Jiang, Dr. Mingyong Han, Ziquan Cao, Veronica Delogu.* Thank you

for sharing your reagents, your happiness and stories with me. More important, thank you for your friendship!

All friends at MTC: *Ying Zhao, Yao Shi, Wenjie Bao, Liying Chen, Hai Li, Chengxi Shi, Xingqi Chen, Fu Chen, Ziming Du, Qin Li, Jiezhi Zou, Dr.Lifu Hu, Qinzi Yan, Liang Wu, Xiangqun Ye, Jacob Lovén and Mo Lin*. The conversation topics during our lunch have been changing all the time, but not the laugh we have.

Previous MTCer or KIer: *Ying Huang, Di Sun, Xiaoda Wang, Hao Mo, Hao Wu, Ying Dou, Jun Ma*. Wherever you go, I will never forget you.

All friends at Karolinska: *Jinfeng Shen, Bin Zhao, Jingwen Shi, Anquan Liu, Ying Sun, Liqun He, Xiaofeng Zheng, Xin Wang, Wei Jiao, Jie Yan, Min Wan, Jiaqi Huang, Ci Song, Ning Wang, Yu Qian.* I am so lucky to have friends like you! Particularly, *Qing Cheng*, my great friend, we two came to Sweden on the same plane. Now I graduate, you need to hurry up a little bit☺. Dearest *Yu*, life is beautiful with you.

All friends outside KI: *Xi Zhang, Yi Liu, Maria Karlsson, Yuan Fang, Ruoyan Meng*. My life as a medical researcher is quite different from yours. I appreciate that you share your own stories with me. Particular to *Xi*, I always appreciate your valuable suggestion and help for my career development.

All friends from Biovetenskapliga Forskarskola 2004: *Peter Hammer, Hye-ryung Jung, Mikael Crona, Therese Eriksson, Malin Stoltz, Salah Mahmoudi .....* With your friendship, my first year in Sweden was so great!

Ambassador *Mingming Chen* and Counselor *Ning Zhang* from Chinese Embassy at Stockholm. Thanks for your kind support to me and KI Chinese student union while I served in.

To my family: my cousins *Yihao and Haoyang*, my uncles *Baoping* and *Huaqiang*, aunties *Baoli* and *Xiaoguang* and my grandmas. The happiest thing for me while in Sweden is to talk with you over the phone every week. My father *Yuqin* and mother *Baorong*. With all your love and encouragement during past twenty-seven years, I am now getting my PhD degree. I might not be a good son by Chinese culture and tradition. But I will let you proud of me, I am sure.

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