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ANGIOGENESIS MODULATES OBESITY AND INSULIN SENSITIVITY

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Angiogenesis modulates obesity and insulin sensitivity

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Everything will be okay in the end.
If it's not okay, it's not the end.

(Fernando Sabino)

ABSTRACT

Both white and brown fat are highly vascularized tissues and vascular functions in adipose tissues remain largely unknown. In this thesis work, we aimed to study blood vessels in modulation of adipose tissue functions under physiological conditions. To achieve these goals, we have used genetic mouse models in combination with pharmacological approaches as powerful tools to investigate adipose vascularization in modulation of adipose tissue functions and metabolism. We have developed unique *in vitro* and *in vivo* methods and techniques to functionally and mechanistically address the interactions between endothelial cells (ECs) and adipocytes (ACs). These novel methods and findings may potentially pave new avenues for development of therapeutics for treatment of obesity and metabolic diseases by targeting the adipose vasculature. In paper I, we describe novel methods to induce browning and angiogenic phenotypes in white adipose tissues (WAT) by exposing mice to cold ambient temperature (4°C). We have also defined methodologies to measure basal and non-shivering thermogenesis-related metabolism in mice. Several immunohistological methods that are coupled to confocal microscopy analysis were established to accurately quantify expression of gene products that are associated with thermogenesis and angiogenesis. These model systems and methodologies have provided a fundamental basis for subsequent projects within and outside our laboratory to study adipogenesis and metabolism. With the available methods developed in paper I, in paper II we have studied the age-related vascular effects in modulation of fat mass, AC functions, blood lipid profiles and insulin sensitivity. Notably, *Vegf* expression levels in various WATs underwent continuous changes in different age populations. Anti-VEGF and anti-VEGFR2 treatment showed marked variations of vascular regression, with middle-aged mice exhibiting modest sensitivity. Interestingly, anti-VEGF treatment produced opposing effects on WAT AC sizes in different age populations and affected vascular density and AC sizes in brown adipose tissue (BAT). Consistent with changes of vasculatures and AC sizes, anti-VEGF treatment significantly increased insulin sensitivity in all groups, except for a rather modest improvement of insulin sensitivity in the middle-aged group. Similar to healthy mice, anti-VEGF treatment substantially improved insulin sensitivity in obese mice on a high fat diet. Our findings demonstrate that adipose vasculatures show differential responses to anti-VEGF treatment in various age populations and have therapeutic implications for treatment of obesity and diabetes with anti-VEGF-based drugs. In paper III, we studied the paracrine regulation of AC functions by angiogenic ECs following cold- or pharmacologically induced adrenergic activation. We have found that ECs play an essential role in modulating AC functions during WAT browning. This paracrine effect is mediated by EC-derived PDGF-CC, which acts on progenitor cells to induce differentiation into ACs. Deletion of the *Pdgfc* gene in mice or blocking of PDGFRα largely impairs the paracrine regulation of AC functions during WAT browning. In paper IV, we have developed an effective and reliable lymphangiogenesis assay that allows us to study the lymphangiogenic capacity of various factors in the absence of pre-existing lymphatics and other angiogenic stimuli. We took advantage of the avascular nature of the cornea to study the lymphangiogenic effect of any given factor or combination of factors. To this end, we have tested several angiogenic factors that are commonly present in adipose tissues and quantitatively studied corneal lymphangiogenesis.

LIST OF SCIENTIFIC PAPERS

I. Sharon Lim*, **Jennifer Honek***, Yuan Xue, Takahiro Seki, Ziquan Cao, Patrik Andersson, Xiaojuan Yang, Kayoko Hosaka and Yihai Cao. Cold-induced activation of brown adipose tissue and adipose angiogenesis in mice. Nature Protocols. 2012 Vol. 7:606-15.
*Equal contribution

II. **Jennifer Honek**, Takahiro Seki, Hideki Iwamoto, Carina Fischer, Jingrong Li, Sharon Lim, Nilesh Samani, Jingwu Zang and Yihai Cao. Modulation of age-related insulin sensitivity by VEGF-dependent vascular plasticity in adipose tissues. Proceedings of the National Academy of Science. 2014 Vol. 111:14906-11.

III. Takahiro Seki, Kayoko Hosaka, Sharon Lim, **Jennifer Honek**, Yunlong Yang, Carina Fischer, Patrik Andersson, Xuri Li, Yizhi Liu, Masaki Nakamura, Hideki Iwamoto and Yihai Cao. Endothelial PDGF-CC mediates VEGF-angiogenesis-dependent thermogenesis in browning fat. Manuscript, 2014.

IV. Renhai Cao, Sharon Lim, Hong Ji, Yin Zhang, Yunlong Yang, **Jennifer Honek**, Eva-Maria Hedlund and Yihai Cao. Mouse corneal lymphangio-genesis model. Nature Protocols 2011. Vol. 6:817-26

Related publications that were not included in this thesis:

I. **Jennifer Honek***, Sharon Lim*, Carina Fischer*, Hideki Iwamoto, Takahiro Seki and Yihai Cao. Brown adipose tissue, thermogenesis, angiogenesis: pathophysiological aspects. Hormone Molecular Biology and Clinical Investigation. 2014 Vol. 19:5-11.
*Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

AC	Adipocyte
Adrb3	Beta-3 adrenergic receptor
Akt	Protein kinase B
ANP	Atrial natriuretic peptide
Ap2	Adipocyte protein 2
AS160	Akt substrate of 160 kDa, regulates GLUT4 translocation
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
C/EBP	CCAAT/enhancer-binding protein
CL	CL316,243; Disodium 5-[(2R)-2-[[(2R)-2-(3-Chlorophenyl)-2-hydroxyethyl] amino]propyl]-1,3-benzodioxole-2,2-dicarboxy-late; small molecule; specific beta-3 adrenoreceptor agonist
CT	Computed tomography
DIO	Diet-induced obesity
DIT	Diet-induced thermogenesis
EC	Endothelial cell
ECM	Extracellular matrix
epiWAT	Epididymal white adipose tissue, represents visceral adipose tissue (epiWAT and gWAT are used synonymously throughout this thesis)
FA	Fatty acid
FADH	Flavine adenine dinucleotide
FDG	Fluorodeoxyglucose

FFA	Free fatty acid
FNDC5	Fibronectin type III domain-containing protein 5
gWAT	gonadal WAT (gWAT and epiWAT are used synonymously throughout this thesis)
GLUT	Glucose transporter
Grb 2	Growth factor receptor-bound protein 2
GSV	GLUT4 storage vesicle
GTP	Guanosine triphosphate
H^+	Proton
HIF	Hypoxia inducible factor
HRE	Hypoxia response element
HSPG	Heparan sulfate proteoglycan
iWAT	Inguinal white adipose tissue (iWAT and scWAT are used synonymously throughout this thesis)
intBAT	Interscapular brown adipose tissue
IPITT	Intraperitoneal insulin tolerance test
IRS	Insulin receptor substrate
JNK	Jun N-terminal kinases
LR	Leptin receptor
MAPK	Mitogen-activated phosphokinase
Myf5	Myogenic factor 5
NADH	Nicotinamide adenine dinucleotide
NE	Norepinephrine
NEFA	Non-esterified free fatty acid
NP	Natriuretic peptide
NRP	Neuropilin
NST	Non-shivering thermogenesis

OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PBST	PBS with Triton X-100 (0.3% vol/vol)
PC	Pericyte
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PET	Positron emission tomography
PFA	Paraformaldehyde
PGC1α	PPARγ coactivator 1α, transcriptional coactivator
PHD	Prolyl hydroxylase domain-containing enzyme
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PLC-γ	Phospholipase C gamma
PlGF	Placenta-derived growth factor
PPAR γ	Peroxisome proliferator-activated receptor-gamma
PRDM16	PRD1-BF1-RIZ1 homologous domain containing 16
Pref-1	Preadipocyte factor 1
PTB domain	Phosphotyrosine binding domain
Rab	Member of the Ras superfamily of guanine nucleotide-binding proteins
ROS	Reactive oxygen species
RRP	Readily released pool
RTK	Receptor tyrosine kinase
scWAT	Subcutaneous white adipose tissue (scWAT and iWAT are used synonymously throughout this thesis)
SH2	Src homology-2
SH2P	Src-homology domain 2-containig phosphatase

SMC	Smooth muscle cell
STAT	Signal transducer and activator of transcription
T2D	Type 2 diabetes mellitus
TG	Triglyceride
TRP	Thermo transient receptor potential
TZDs	Thiazolidinediones
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WAT	White adipose tissue
Zfp423	Zinc finger protein 423, transcription factor

1 INTRODUCTION

1.1 OBESITY, METABOLIC DISORDERS AND THE ADIPOSE TISSUE

During the past decades, obesity has developed into an epidemic affecting millions of people worldwide. The number of obese individuals has nearly doubled since 1980. According to the World Health Organization, more than 1.4 billion adults are currently overweight and 500 million are clinically obese[1]. Obesity is diagnosed at a body mass index (BMI) ≥ 30 and it is characterized by increased lipid storage and expanded adipose tissue mass[1,2]. This condition affects multiple systems in the human body and constitutes a considerable risk factor for the development of severe and often fatal complications such as type 2 diabetes (T2D), coronary heart disease, hypertension, fatty liver disease, stroke, obstructive sleep apnea, dementia as well as certain types of cancer[2–4]. Obesity-related comorbidities globally account for the majority of deaths. In fact, obesity-related disorders are expected to dramatically affect longevity and a study has projected a potential decline in human life expectancy by the year 2020 for the first time following a steady increase[2–4].

1.1.1 Insulin sensitivity, hyperglycemia and Type 2 diabetes

The vast majority of diabetes cases worldwide can be attributed to non-insulin-dependent type 2 diabetes (T2D) mellitus[5]. This disease leads almost inevitably to macro- and microvascular complications often shortening the lifespan of the affected individual[5,6]. Symptoms of T2D include increased thirst, frequent urination, blurred vision, and impaired wound healing. Apart from these symptoms, the diagnostic criteria for T2D include a fasting blood glucose level of 126 mg/dl or higher. Additionally, an oral glucose tolerance test (OGTT) can be performed by administering a glucose load equivalent to 75 g anhydrous glucose. If the blood sugar level reaches 200 mg/dl or more two hours after this glucose challenge, T2D is diagnosed[5,7,8]. To evaluate chronic blood sugar levels (glycemia), a so-called A1C test can be performed. This test is named after hemoglobin A1C, a glycosylated form of hemoglobin. As hemoglobin is the oxygen-transport protein in erythrocytes, which typically have a turnover rate of 3 months, this blood test is used to assess the average blood glucose level over a longer period of time. This test can also be employed to identify individuals that are at high risk for developing T2D but do not yet meet the diagnostic criteria[5,9,10]. While genetic predisposition is involved in the development of T2D, environmental factors such as lifestyle and overeating play a pivotal role in the pathogenesis of this disease[5,11].

In the healthy individual, glycemia is tightly controlled via insulin production and secretion by pancreatic beta (β) cells in the Islets of Langerhans[12]. Insulin is synthesized as a pro-hormone in the endoplasmic reticulum and undergoes extensive posttranslational modifications as it is processed by proteases in the Golgi apparatus. The biologically active form of the insulin hormone is then packed into secretory vesicles[13–15]. Postprandial elevations of blood glucose promote release of insulin from the pancreas. The secretion of insulin is a biphasic process: In the early phase, preformed, stored insulin from the so-called

"readily-released pool (RRP)" is rapidly secreted. Once the RRP is depleted, in a second phase, a slower release of insulin which has been stored in secretory granules mobilized from the "reserve pool" is observed[13,14,16]. As a consequence of insulin secretion, glucose is taken up by peripheral insulin-sensitive organs such as liver, skeletal muscle and adipose tissue to restore normal blood glucose levels (normoglycemia). Apart from this direct mechanism, insulin promotes hepatic glycogenesis while inhibiting pancreatic secretion of the catabolic enzyme glucagon thereby preventing glycogenolysis and gluconeogenesis in the liver[15]. Together, these effects of insulin counteract postprandial hyperglycemia by regulating glucose homoeostasis.

Insulin-stimulated glucose uptake into peripheral tissues is mediated by glucose transporters (GLUT). Among these transporters, GLUT4 is responsible for glucose uptake in skeletal muscle and adipose tissue[12,17,18]. Under basal, unstimulated conditions, GLUT4 is sequestered in vesicles in an intracellular pool (Fig. 1).

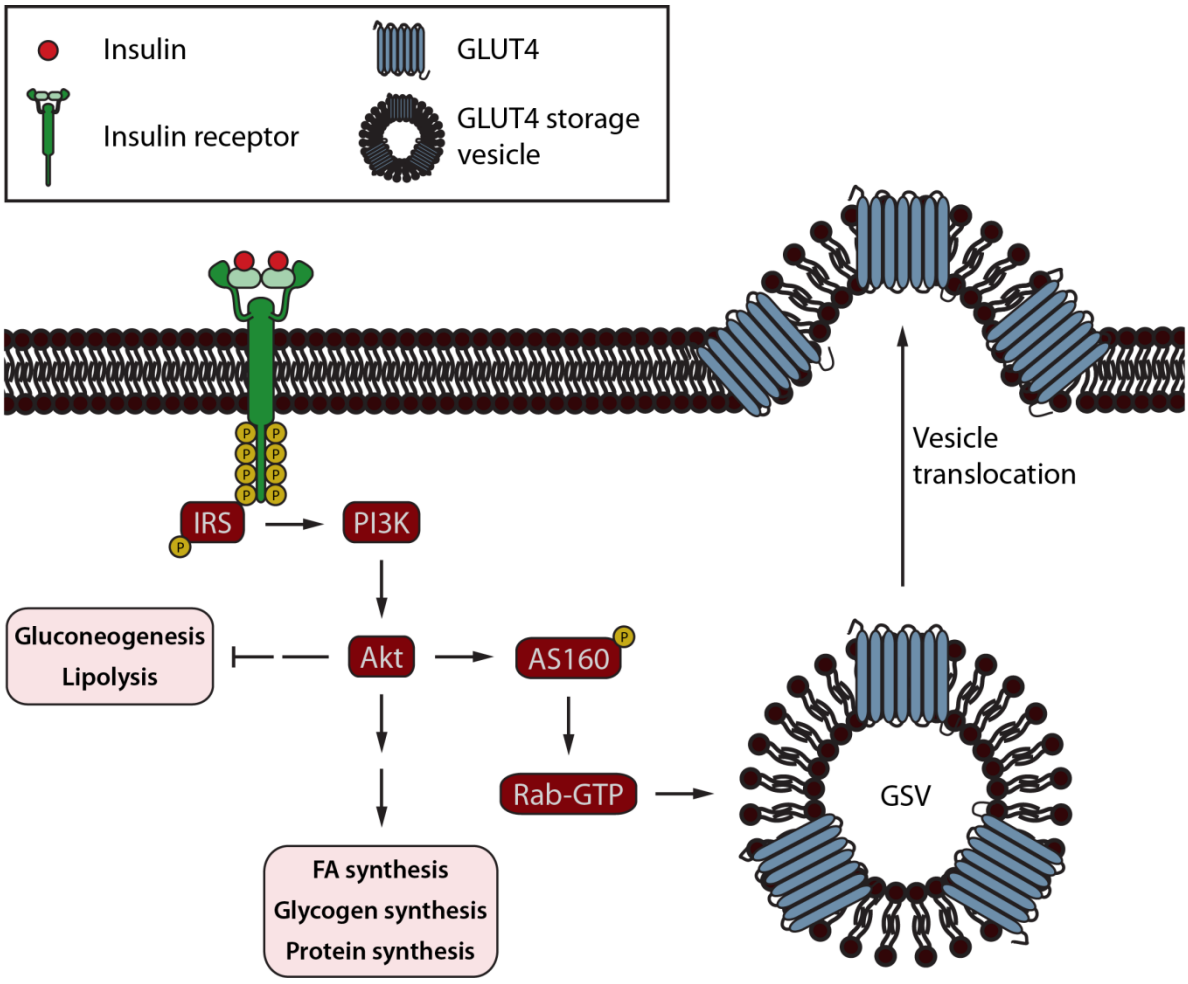

Figure 1: Effects of postprandial insulin secretion. Secreted insulin binds to the insulin receptor expressed on ACs, skeletal muscle cells and hepatocytes and triggers an intracellular signaling cascade. This leads to PI3K/Akt-dependent phosphorylation of target molecules resulting in trafficking of GSV and insertion of the glucose transporter GLUT4 into the plasma membrane enabling glucose uptake from the bloodstream into the cell. Furthermore, insulin signaling results in the inhibition of gluconeogenesis and lipolysis whereas synthesis of FA, glycogen and proteins is stimulated.

Insulin secretion and binding to its receptor triggers vesicle trafficking via the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway causing GLUT storage vesicles (GSV) to fuse with the plasma membrane resulting in relocation of GLUT4 to the cell surface[19–21]. Glucose molecules can then enter the cell by facilitated diffusion along the concentration gradient and are removed from the bloodstream thereby restoring normoglycemia[19,21,22].

Insulin action is modulated by a number of hormones, inflammatory cytokines and metabolites such as free fatty acids (FFAs) originating from the adipose tissue. Hypertrophic adipocytes (ACs) exhibit lower sensitivity to the lipolysis-suppressing effect of insulin thereby contributing to increases in circulating FFA and glycerol levels. Circulating FFAs and glycerol confer hepatic as well as skeletal muscle insulin resistance[23,24]. Therefore, obesity is often associated with insulin resistance. In insulin resistant individuals, liver, skeletal muscle and adipose tissue lose their ability to respond efficiently to insulin leading to reduced uptake of glucose from the bloodstream compared to healthy individuals. Additionally, postprandial insulin secretion fails to suppress hepatic gluconeogenesis and glycogenolysis thus further aggravating hyperglycemia[5,7,12]. Insulin resistance is often seen in obese patients that do not yet meet the diagnostic criteria for T2D and can usually be improved by lifestyle interventions such as exercise and weight loss[5]. Persistent insulin resistance will eventually cause pancreatic β cells to increase insulin production and secretion to compensate for the diminished responsiveness of target cells and organs to the hormone. Hyperinsulinemia, excess levels of circulating insulin, is therefore frequently seen at early stages of T2D[8,25,26]. Recently, this classical view of insulin resistance preceding hyperinsulinemia has been challenged by several studies indicating that insulin resistance may result from increased insulin levels. It remains to be elucidated in which order these conditions occur and it is possible that the pathogenesis of these events may vary between different individuals[26,27]. Nevertheless, untreated insulin resistance results in chronic hyperglycemia. Chronically elevated blood glucose levels can lower insulin secretion as glucose metabolism leads to the production of reactive oxygen species (ROS). There is evidence that large amounts of ROS in pancreatic β cells may cause damage to cellular components. Consequently, insulin production may be decreased due to β cell dysfunction and apoptosis[5,28–30].

Overall, T2D has devastating effects on the patient's health. Acute complications include the life-threatening conditions ketoacidosis and coma. Long-term consequences of diabetes can be almost exclusively traced back to micro- and macrovascular complications as chronically elevated blood glucose levels eventually cause damage to blood vessels.[5,8,30]. Microvascular diseases arise due to damage to small blood vessels resulting in retinopathy, nephropathy and neuropathy. Macrovascular damage affects large vessels and arteries leading to cardiovascular complications including myocardial infarction and stroke[7,8,31,32].

It is noteworthy that excess adipose tissue mass itself does not automatically increase the risk for obesity-related co-morbidities. In fact, around 15 % of obese individuals do not develop

metabolic disorders[4,33]. Furthermore, recent studies indicate that fat distribution rather than fat mass *per se* determines the predisposition for developing obesity-related illnesses[4,34,35]. According to these studies, visceral fat mass reduction could markedly improve metabolic parameters such as glucose metabolism and insulin sensitivity while removal of subcutaneous (sc) adipose tissue was not associated with significant benefits[34–36]. Further indications that adipose mass cannot always be correlated with metabolic health are derived from lipodystrophy studies[4,23,37]. Lipodystrophy is a rare condition which manifests in partial or generalized absence of adipose tissues. This results in ectopic lipid deposition in non-adipose organs such as liver, skeletal muscle or pancreas causing dysfunction or apoptosis of cells in these organs[38]. As a result, patients suffer from metabolic dysfunctions such as insulin resistance, dyslipidemia and non-alcoholic fatty liver[23,37,39,40]. In affected individuals, absence of functional adipose tissue mass is associated with health risks similar to those seen in obese patients indicating that adipose tissue dysfunction is the major cause for the development of metabolic disorders[2–4,41].

1.1.2 The role of ageing in obesity and metabolic disorders

Insulin sensitivity is frequently decreased in older individuals. Normal weight adults aged 55-64 years display the same relative risk of developing T2D as younger, obese patients thus identifying ageing as an independent risk factor to develop insulin resistance[42–45]. Although the underlying mechanisms have not been fully elucidated yet, it is known that increasing visceral adiposity as well as accumulating senescent cells contribute to a pro-inflammatory cytokine profile in the blood. Elevated levels of inflammatory cytokines might hamper insulin signaling leading to insulin resistance[46]. Studies have also shown that ageing increases inducible nitric oxide synthase. This enzyme interferes with skeletal muscle insulin sensitivity through nitrosation of insulin receptor substrate (IRS)1 and other proteins involved in insulin signaling[47]. During the ageing process, considerable alterations of total fat mass as well as adipose tissue distribution occur. Several human and rodent studies indicate an increase in adipose tissue mass in middle-aged individuals compared to younger subjects. At old age however, stagnation and even decline of body weight, BMI and total fat mass have been described[48–53]. Moreover, with progressing age, adipose tissue is redistributed from subcutaneous locations to visceral depots and ectopic fat storage sites such as liver and muscle due a reduced lipid-storage capacity of ACs[50,54,55]. Both visceral adiposity and ectopic lipid deposition are correlated with metabolic disorders[39,56]. Obesity has been reported to accelerate ageing of adipose tissue and thereby promotes a rise in age-related diseases such as cardiovascular disease, cancer and neurodegenerative disorders in younger, obese subjects[57]. Senescence-like alterations have been identified in the adipose tissue of obese individuals including telomere dysfunction, increased oxidative stress and DNA damage[57,58].

In summary, obesity-related metabolic disorders primarily result from a chronic positive energy balance. Per definition, energy balance is composed of two components, namely energy intake and energy expenditure. In homeothermic animals that maintain a constant core body temperature independent from ambient temperature changes, energy expenditure is

determined by the basal metabolic rate, physical activity as well as facultative thermogenesis[59]. Energy intake and expenditure are at least in part regulated by the two distinct types of adipose tissues present in the body: white and brown adipose tissue.

1.1.3 White adipose tissue

White adipose tissue (WAT) is present in various species. However, its absolute mass greatly varies between different species but even within the same animal as seen in hibernating animals[60]. The major function of WAT is storage of excess energy in the form of triglycerides (TGs). In times of negative energy balance, e.g. during fasting or starvation, energy can be mobilized by lipolysis and becomes readily available to sustain bodily functions. Thereby, WAT is critical for maintaining whole-body energy homeostasis. In lean subjects, around 20% of the total body weight is WAT and in obese individuals this number can reach up to 50%[61]. This clearly illustrates that minor changes in AC functions can dramatically affect the whole body. WAT exists in multiple depots throughout the human body and there are marked morphological, functional and metabolic differences between subcutaneous and visceral white fat. WAT is characterized by structural heterogeneity and composed of a number of different cell types. While roughly 50% of cells in WAT are mature ACs, they are interspersed with ECs, macrophages, fibroblasts as well as preadipocytes forming the stromal vascular fraction[61–64]. White ACs typically contain a large, unilocular lipid droplet and have a low cytoplasmic content, the nucleus is located close to the plasma membrane and the periplasmic rim contains a few mitochondria[65,66]. The size of white ACs ranges from 25 – 200 µm and can be adjusted depending on nutrient availability[61,64]. In times of positive energy balance, TGs accumulate within white ACs resulting in an increase of cell diameter. This presence of large, lipid-laden ACs is termed hypertrophy and constitutes the major mechanism of adipose tissue expansion in adults[3,67,68]. While the number of ACs is largely determined during childhood and adolescence, there are now indications that fat cell number may be increased by differentiation of committed adipose precursor cells. Thereby, chronic overeating may result in AC hyperplasia after existing ACs reach a critical maximum size and cannot deposit more lipids[3,4,67–69]. Interestingly, AC hyperplasia may be beneficial and protective for metabolic health as compared to hypertrophy. Several studies indicate that enlarged ACs are correlated with altered gene expression profiles, a pro-inflammatory phenotype and adipokine secretion patterns that are associated with obesity-related disorders such as insulin resistance[3,70–73]. As ACs undergo hypertrophy, their oxidative capacity as well as mitochondrial content decrease. When reaching a critical size, hypoxia-related stress is induced leading to cell death. Adipocyte apoptosis recruits macrophages that remove AC remnants and form characteristic “crown-like” structures. Consequently, obesity is associated with macrophage infiltration and a state of chronic low-grade inflammation[4,39,66,69,71,74–77].

Apart from its significant role in energy-storage, adipose tissue has fundamental endocrine functions. Over 600 biologically active factors are produced and secreted in WAT[3,78]. Adipose tissue-derived adipokines modulate a number of physiological events via autocrine, paracrine or endocrine (systemic) signaling. Apart from local effects within the adipose

tissue, such as inflammation or angiogenesis, adipokines regulate various systemic processes including appetite and satiety, insulin sensitivity, energy expenditure, blood pressure as well as endothelial cell functions[3,4,78–86]. The adipokine secretion pattern reflects the functionality of the adipose tissue and a shift towards a pro-inflammatory, diabetogenic profile indicates adipose tissue dysfunction[70,71,80,87].

Among the large number of adipokines, leptin is one of the most studied factors secreted by the adipose tissue. The peptide hormone is the product of the *ob* gene and almost exclusively expressed by differentiated, mature white ACs[88–91]. Leptin actions are mediated via the leptin receptor (LR) which is present in several tissues with highest levels in the hypothalamus. The major function of leptin is signaling of nutritional status, regulation of appetite and energy homeostasis. Circulating leptin signals to the central nervous system that energy repletion is achieved, inducing satiety[89,92,93]. A state of chronically decreased leptin levels result in hyperphagia and is involved in development or progression of obesity. Leptin deficiency in rodent models due to mutations in the *ob* gene or in the gene encoding LR increases food intake while decreasing energy expenditure, mimicking a starvation response[89,92–96]. Notably, a positive correlation between BMI or total body fat weight with circulating leptin levels has been shown. These findings suggest a dysregulation of leptin signaling rather than leptin production in humans as well as in rodent models of diet-induced obesity (DIO)[89,96,97]. Whereas obesity can be reversed by exogenous leptin administration to animals deficient in this hormone, effects are only moderate or completely absent in hyperleptinaemic subjects indicating leptin resistance[92,98,99].

1.1.4 Brown adipose tissue and non-shivering thermogenesis

While WAT serves an energy repository, brown adipose tissue (BAT) is a thermoregulatory organ that dissipates stored energy to generate heat in a process termed non-shivering thermogenesis (NST). This process is only activated when the organism is in acute demand of heat (facultative thermogenesis). Increased NST capacity develops over time following chronic exposure to certain stimuli (adaptive thermogenesis)[100]. Adaptive thermogenesis consists of several steps including mitochondrial biogenesis, expression of mitochondrial and respiratory chain enzymes as well as expression of proteins that uncouple oxidative phosphorylation[101].

In mammals, BAT-related heat production is required and activated during the postnatal stage as well as in hibernating animals or during exposure to low ambient temperature. BAT-thermogenesis is centrally regulated via hypothalamic pathways. Not only functional but also morphological differences are evident between WAT and BAT. The darker color of this thermoregulatory tissue is in part due to the dense vascularization of BAT. The tissue is innervated by sympathetic nerves that are distributed abundantly in BAT[102,103]. Brown ACs typically reach maximum diameters of 60 µm and are thereby markedly smaller in size compared to white ACs[64,103]. Each BAT AC contains multiple smaller lipid droplets and a large number of mitochondria expressing the BAT-specific uncoupling protein 1 (UCP1), which is located in the inner mitochondrial membrane[102–104]. This protein, also known as

thermogenin, uncouples oxidative phosphorylation from ATP synthesis and generates heat instead. This is achieved by dissipating the intermembrane proton gradient as UCP1 causes protons (H^+) to leak into the mitochondrial matrix, thereby bypassing the ATP synthase[102–106].

BAT thermogenesis is activated by the release of the neurotransmitter norepinephrine (NE) from sympathetic nerve termini upon certain stimuli such as cold or food intake[107]. NE binds to β3 adrenergic receptors (Adrb3) expressed on brown ACs resulting in adenylate cyclase activation and subsequent cyclic AMP (cAMP) formation (Fig. 2).

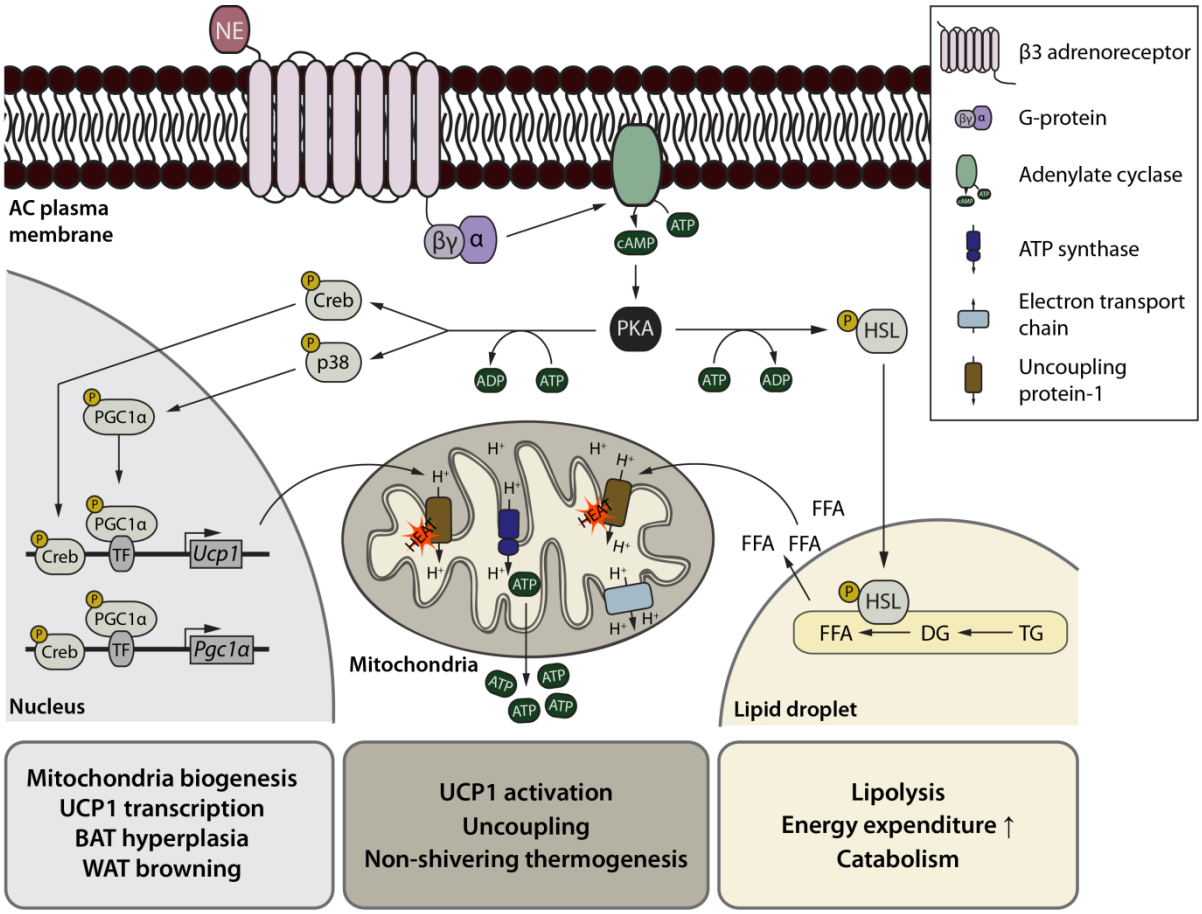

Figure 2: Adrenergic activation of beige and brown adipocytes. Activation of the β3 adrenergic receptor results in elevated intracellular cAMP levels leading to activation of protein kinase A (PKA). This kinase phosphorylates and activates the enzyme hormone sensitive lipase (HSL) catalyzing the last step in TG lipolysis. Free fatty acids (FFAs) are released and serve as a substrate for UCP1-dependent thermogenesis. TG-derived FFAs are further metabolized via β-oxidation and enter the tricarboxylic acid cycle in the mitochondria; thereby, protons (H^+) accumulate in the mitochondrial intermembrane space. UCP1 enables H^+ to translocate back into the mitochondrial matrix and dissipate oxidative phosphorylation from ATP synthesis in favor of heat generation. Chronic adrenergic activation results in PKA-dependent activation of PGC1α and subsequent upregulation of BAT-specific genes such as *Ucp1*, mitochondira biogenesis, BAT hyperplasia and browning of WAT.

The second messenger cAMP, in turn, activates protein kinase A (PKA) which further mediates adrenergic signaling via phosphorylation of various target proteins. It has been shown that prolonged adrenergic activation results in mitochondrial biogenesis, BAT hyperplasia and elevated levels of UCP1[103,105]. Activated PKA is thereby involved in transmitting both acute and chronic effects of adrenergic stimulation: PKA phosphorylates the transcription factor CREB which activates transcription of the *Ucp1* gene. Expression of

this gene is further stimulated by PKA-mediated activation of the p38 pathway[102,103,105]. The acute thermogenic effect however is mediated by PKA-induced lipolysis. Activated PKA phosphorylates perilipin, a protein covering TGs within the fat cell, which then dissociates from the lipids leaving them vulnerable for lipolytic attack. PKA further activates hormone-sensitive lipase (HSL) which catalyzes the rate-limiting step in lipolysis of TGs[103,108–111]. FFAs liberated from TGs serve two purposes in subsequent UCP1 activity: they are directly involved in UCP1 activation but also serve as substrates for thermogenesis[103,112,113].

While the mechanism of fatty acid (FA)-induced UCP1 activation remains to be elucidated, the catabolic pathway of FAs is well established. After entering the mitochondria via the carnitine shuttle system, they undergo β-oxidation and the liberated acetyl-CoA moieties are further oxidized in the tricarboxylic acid cycle. This leads to the production of NADH and FADH, conveying electrons to the electron transport chain. During this process, H^+ are moved across the inner mitochondrial membrane into the intermembrane space creating a membrane potential. Via ATP synthase, H^+ are channeled back into the mitochondrial matrix catalyzing ATP production. In brown fat cells however, this process is uncoupled by UCP1. This protein mediates a proton leak enabling H^+ to return to the mitochondrial matrix independent of ATP production and instead generates heat[114–116]. BAT activity results in combustion of large amounts of lipids and glucose and has therefore been implicated in therapeutic intervention to counteract obesity[102–104,117,118].

In rodents, BAT is found in distinct anatomical locations. The most studied rodent BAT depot is located subcutaneously in the interscapular region and consists of two lobes[106,119,120]. Until recently, it was believed that BAT in humans is only present in neonates and does not exist in significant amounts in adults. However, 18[F]-2-fluorodeoxyglucose (FDG) positron emission tomography (PET) combined with computed tomography (CT) has revealed that BAT is present and active in adult humans. Human BAT is primarily located in the supraclavicular area, the neck region, paravertebral as well as in para-aortic and suprarenal locations[105,121–124]. However, amount and activity of human BAT seem to vary greatly between individuals. Even within the same subject, exogenous factors such as seasonal changes in ambient temperature affect the BAT status due to the plasticity of this tissue[102,107,122–124]. Interestingly, several studies point towards an inverse correlation between prevalence or activity of human BAT and obesity-related factors such as body fat percentage or BMI[102,125–127]. These findings suggest, that targeting BAT may indeed offer an attractive therapeutic approach to counteract obesity and obesity-related metabolic disorders.

1.1.5 Adipogenesis and developmental origin of white and brown ACs

In higher mammals, adipogenesis, the formation of adipose tissue, occurs during mid-gestation. In two distinct phases, determination and differentiation, the adipogenic process gives rise to WAT and BAT (Fig. 3). While BAT is fully developed in the newborn in different species, substantial amounts of WAT are not detectable at birth in small rodents while it is present in human babies[60,128,129].

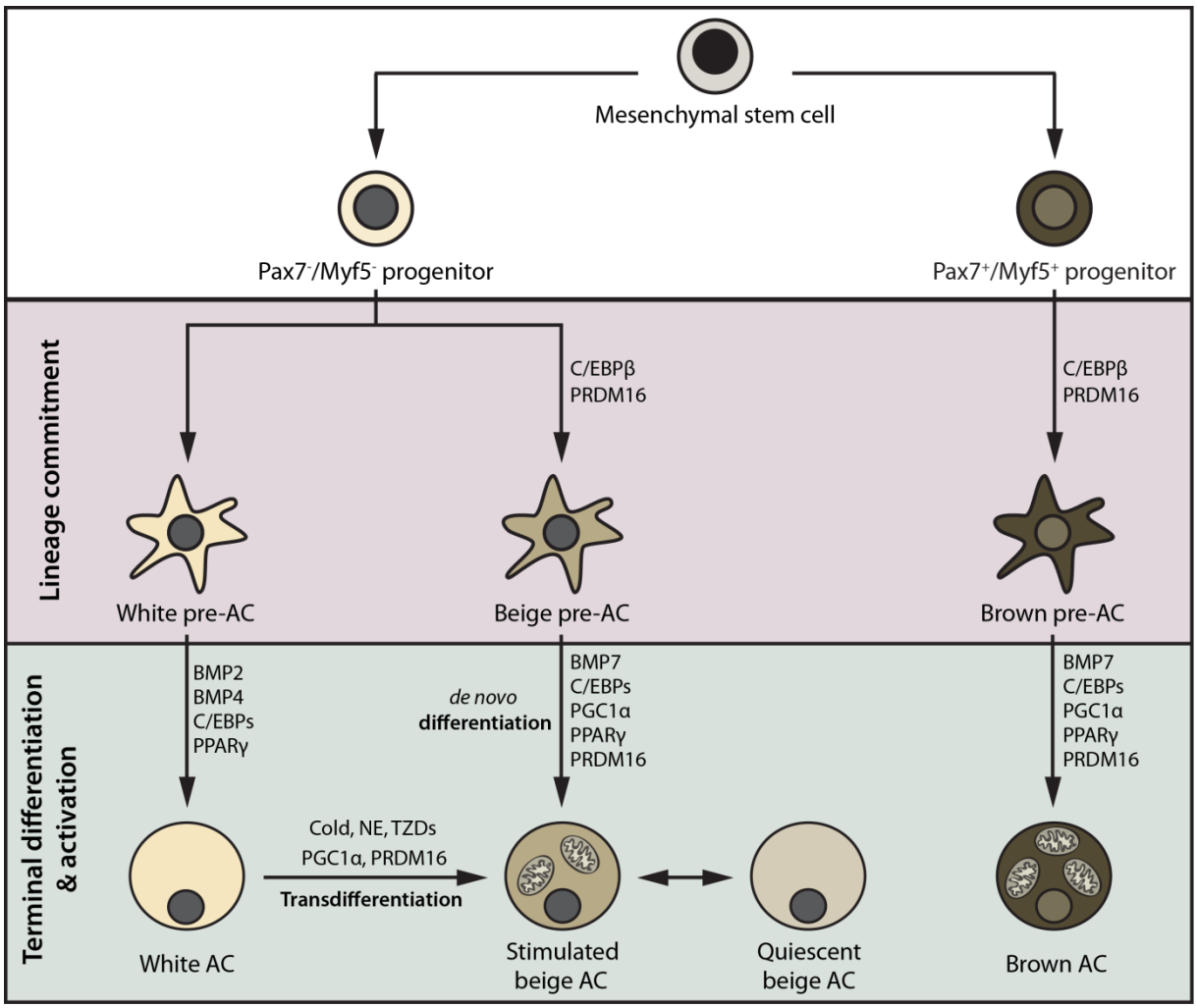

Figure 3: Differentiation of white, beige and brown adipocytes. ACs are derived from mesenchymal stem cells. White and beige ACs originate from a Pax7$^-$/Myf5$^-$ progenitor while classic brown ACs share a common Pax7$^+$/Myf5$^+$ progenitor with skeletal muscle cells. Under appropriate conditions, these early progenitors become commited white, beige or brown AC precursor cells. During terminal differentiation, expression of PPARγ and C/EBPs determines both white and brown fat development. High expression levels of PRDM16 and subsequent expression of PGC1α and C/EBPβ drive the precursor cells towards beige or brown AC fate. Apart from *de novo* differentiation of beige ACs from a commited precursor, transdifferentiation of white to beige ACs has been described under various stimuli such as cold exposure. Beige ACs may respond in an adaptive manner to the energy status of an individual: in the absence of appropriate stimuli these cells remain quiescent and deposit energy. Under negative energy balance or upon other adrenergic stimuli, activated beige ACs dissipate stored energy.

Adipogenesis of both white and brown fat cells is driven by the transcription factors peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs). There is compelling evidence that both cell types share mesodermal origin. Despite these similarities, it has recently been confirmed, that white and brown ACs are derived from distinct precursor cells. At an early stage of differentiation, mesenchymal stem cells give rise to a population of myogenic factor 5 (Myf5)-expressing cells and a subset of Myf5-negative cells[130–132]. Myf5$^+$ cells are capable of differentiating into brown ACs whereas white ACs develop from the Myf5$^-$ population[130,131]. In the presence of appropriate stimuli, these early adipose precursor cells (adipoblasts) become committed white and brown preadipocytes. At this stage, precursor cells retain their proliferative capacity and cell cycle arrest is required before committed preadipocytes differentiate into mature fat cells.

Differentiation occurs in a tightly controlled sequence of events: initial growth arrest, clonal expansion, early and late differentiation[131,133,134]. Growth arrest is mediated by the transcription factors PPARγ and C/EBPα which transactivate the expression of AC-specific genes. Morphologically, cells lose their fibroblast-like structure and adopt a spherical appearance; on a molecular level, expression of the preadipocyte marker Pref-1 is markedly reduced[131]. At later stages of terminal differentiation, lipolysis-associated genes, as well as genes involved in glucose metabolism and *de novo*-lipogenesis are expressed and cells accumulate lipids to become mature ACs[60,128,131,133–136]. While PPARγ and C/EBPs play important roles in driving both white and brown fat development, there are a number of transcriptional components directing mesenchymal stem cells towards a brown AC fate[120,134,136–138]. Brown AC precursor cells transiently express *Myf5* and *Pax7* thereby sharing a developmental origin with skeletal muscle cells. A master regulator in the determination and differentiation of brown fat cells is the transcriptional coregulator PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16) which inhibits the myogenic pathway in favor of brown fat differentiation[137–139]. PRDM16 coactivates C/EBPβ thereby inducing expression of *Pparγ* and *PPARγ coactivator 1α* (*Pgc1α*). Additionally, PRDM16 directly interacts with PPARγ and PGC1α to further drive brown AC differentiation and activation[137,139]. PGC1α is a transcriptional coactivator and plays a central role in mitochondrial biogenesis in various tissues, in particular in highly metabolically active organs such as heart, kidney, brain, liver, skeletal muscle and BAT[140–142]. It is critically involved in AC cell fate decision by specifying the genes targeted by transcription factors such as PPARγ thus inducing brown AC differentiation. Unexpectedly, ablation of PGC1α does not abolish BAT development as its functional loss is most likely largely compensated by the actions of PGC1β[143,144]. However, cold exposure or adrenergic stimulation require and strongly induce *PGC1α* expression in BAT. Simultaneously, phosphorylation by p38 directly activates and stabilizes PGC1α. Together, this results in mitochondrial biogenesis as well as induction of mitochondrial and thermogenesis-related genes such as *Ucp1* leading to enhanced uncoupling of respiration. Thereby, this transcriptional coactivator provides a direct link between external stimuli and adaptive thermogenesis[59,101,141,145–147]. Importantly, is has been shown that adenoviral delivery of PGC1α to human differentiated, white ACs *in vitro* as well as to murine WAT *in vivo* could induce expression of *Ucp1* underlining the role of PGC1α in the acquisition of a brown AC-like phenotype[140,148].

1.1.6 WAT browning and beige/BRITE adipose tissue

Besides classical white and brown fat cells, a third type of ACs has recently been identified[149]. While these cells resemble white ACs as they have very low basal expression of *Ucp1*, they share common biochemical features and thermogenic capacity with brown fat cells. Under appropriate stimulation, these cells bear the hallmarks of brown ACs[69,149–151]. As opposed to BAT which is present as a distinct depot in rodents in the interscapular, cervical, axillary and perirenal region, BRITE (brown-in-white) or beige ACs are interspersed within WAT, particularly in the inguinal depot[146,149]. Interestingly, external stimuli such as cold

exposure or adrenergic stimulation with the Adrb3-agonist CL316,243 (CL) results in increased *Ucp1* expression of beige ACs and a browning phenotype of WAT[101,129,145,152–156].

Recent studies using transgenic mice have shown that PRDM16 plays a critical role in the induction of a thermogenic program during scWAT browning. PRDM16 is both necessary and sufficient for brown-like adipogenesis in WAT and has thereby been identified as a mediator of adaptive thermogenesis in this tissue[126,138,154,157,158]. Browning was not reported in visceral WAT in these mice, where considerably lower endogenous levels of PRDM16 were detected[157,159]. Interestingly, whole body energy expenditure was greatly elevated in transgenic mice expressing PRDM16 in scWAT that moreover exhibited resistance to DIO and improved glucose tolerance. On a transcriptional level, PRDM16 interacts with PPARγ and PGC1α, thereby coordinating repression of WAT-related genes while inducing BAT genes including thermogenic and mitochondrial genes (Fig. 2)[129,131,138,146,154,157,158]. PGC1α is a master regulator of the thermogenic program of brown and beige ACs in particular in response to adrenergic stimulation induced by cold exposure or Adrb3-agonists[145,146]. Loss of this transcriptional coactivator dramatically reduces cold-induced thermogenic capacity *in vivo* while ectopic expression in WAT induces expression of *Ucp1* and other BAT-specific genes[129,146,154].

Despite certain similarities with classical brown ACs, beige fat cells are derived from different precursor cells and are characterized by a distinct gene expression pattern[149–151]. Whereas BAT ACs are derived from a $Myf5^+$ lineage, beige ACs originate from a $Myf5^-$ negative precursor[146,151]. To date, the origin of beige ACs has not been unraveled conclusively and there are two distinct hypotheses regarding the emergence of these cells in WAT depots[129,131,146,150,151,154,159]: (i) transdifferentiation and (ii) *de novo* differentiation. The transdifferentiation hypothesis suggests that most or all white ACs possess an inherent ability to adopt a brown-like phenotype under appropriate stimuli[69,131,154,160]. According to this theory, interconversion between white and brown-like fat cells may be bidirectional and could provide an adaptive mechanism to respond to the nutritional and energy status of the organism. In times of energy surplus, these cells might possess a white AC-like phenotype with energy-storing properties while they might undergo browning in case of a negative energy balance or adrenergic stimulation and dissipate stored energy[156,161]. However, the *de novo* differentiation hypothesis is supported by accumulating evidence that brown-like cells emerging in WAT originate from a distinct progenitor cell. This subpopulation of precursor cells differs from classical white preadipocytes by expressing the selective cell surface markers CD137 and TMEM26[151,153]. At a basal state, such as under thermoneutral conditions, these beige ACs may appear unilocular and feature low expression of thermogenic genes. Adrenergic stimulation will induce morphological alterations towards a multilocular lipid distribution. Simultaneously, the thermogenic gene program is induced, including expression of high levels of *Ucp1*, allowing beige ACs to switch from an energy-storing to an energy-dissipating state[151]. At this writing, there is evidence supporting both hypotheses and it remains elusive whether transdifferentation, *de novo* differentiation of beige precursor cells, or a combination of both accounts for WAT browning under adrenergic stimulation.

There is accumulating evidence that – in contrast to BAT identified in human infants – the molecular signature of the BAT recently detected in adult humans resembles more closely murine beige than classical brown fat[151,162]. Indeed, it has been reported in multiple studies that PET-scans reveal a rather low quantity of thermogenically active BAT in adult humans. Stimulation by acute cold exposure could activate the tissue and substantially increase uptake of the glucose analog FDG[117,121–123,126,162,163]. Considerable variations in BAT activity were detected even within the same subject due to seasonal variations in ambient temperature[118].

1.1.7 Current treatment strategies for obesity and metabolic disorders

As metabolic disorders such as T2D are in the majority of cases caused by overweight or obesity, therapeutic approaches aim primarily at lowering the patient's body weight. Lifestyle interventions such as low-calorie diet or exercise regimes have proven very efficient in weight loss therapy. However, these approaches are only successful given compliance of the patient and long-term adherence to these interventions[164,165]. In order to be considered clinically meaningful and significantly improve metabolic health, body weight has to be reduced by at least 5 % within one year[164,165]. Importantly, to achieve and sustain significant body weight loss, patients usually have to rely on long-term drug treatment. Several anti-obesity drugs have been withdrawn from the market due to serious long-term adverse effects that were not observed during trial periods prior to their approval[164–166]. For instance, sibutramine, an oral appetite suppressant, has been used in obesity treatment and showed efficacy for clinically significant weight loss. However, sibutramine has been withdrawn from the market in 2010 since the compound was associated with severe cardiovascular toxicity[166–171]. Similarly, the appetite suppressant rimonabant has been discontinued due to adverse effects on the central nervous system[166,170,172]. At this writing, orlistat (Xenical®) is the only anti-obesity drug approved by the Food and Drug Administration. The compound functions as a lipase inhibitor. Thus, uptake of dietary lipids is inhibited and they are secreted via the feces. However, the drug is associated with gastrointestinal side effects that can be improved when consuming a low-fat diet[164,166,169,173–175]. In terms of morbid obesity and related health risks, bariatric surgery is the most efficient weight loss approach resulting in a nearly 25 % reduction of overall mortality[165,176]. It is also the most effective way of treating T2D in morbidly obese subjects. However, this procedure is highly invasive and linked to risks associated with any major surgery[165]. This clearly demonstrates that there is an urgent need for the development of novel therapeutic approaches for obesity and associated disorders.

1.1.8 Therapeutic implications of human BAT

The finding, that adult humans possess a low but significant amount of BAT which responds to activation, has provided new hope for the development of novel anti-obesity drugs. Recent studies have identified a number of molecules, pharmacological and nutritional agents as well as secreted proteins that contribute to WAT browning[155]. Interestingly, human studies have detected 63 g of BAT in the supraclavicular-paracervical depot of one particular subject. In relation to the total body weight of an adult human, this amount of tissue seems almost

negligible. However, according to estimations, this small amount of human BAT may be sufficient to substantially increase the individual's energy expenditure. There is evidence that cold-induced NST results in a 40% increase over basal energy expenditure[177,178]. Notably, the primary substrate for BAT-thermogenesis is FFAs and only a fraction of its metabolism can be attributed to glucose functioning as a substrate for BAT thermogenic activity. According to calculations, merely 2% of thermogenesis in fully activated BAT is fueled by glucose[179]. During acute cold exposure, the main energy source is the combustion of lipids stored within brown ACs themselves. Prolonged BAT activation however, results in rapid depletion of the BAT lipid-pool and requires mobilization of lipids from the periphery. Circulating FFAs derived from WAT lipolysis as well as gut chylomicrons and hepatic lipoproteins provide fuel for BAT thermogenesis[177,178,180]. Assuming full activation of the tissue, this may result in the consumption of energy equivalent to 4.1 kg of WAT per year[122,162]. Consequently, BAT activation or potential WAT browning in adult humans may have substantial benefits for metabolic health.

1.1.7.1 Environmental influences and physiological stimuli

Cold exposure

Cold exposure is a classical, physiological activator of BAT and also functions potently in BAT recruitment[118,146,155,181]. Acute effects of cold exposure activate BAT while elevated *Ucp1* expression, mitochondrial biogenesis and BAT hyperplasia are observed after chronic exposure. In addition to activation of classical BAT depots, chronic cold exposure also results in the emergence of beige ACs in WAT depots[103,118,145,182,183]. Cold-induced alterations in BAT activity have been shown to correlate negatively with body fat content in human subjects. A study exposing male subjects to controlled cold, has shown that both blood flow within BAT as well as metabolic activity of the tissue were greatly elevated[177]. The cold stimulus inducing BAT activation is received via thermo transient receptor potential (TRP)-channels which are members of the TRP ion channel family. In particular TRPM8 and TRPA1 seem to be sensitive to low ambient temperatures. Activation of these receptors occurs at temperatures of 20 °C and below. Indeed, human studies have confirmed BAT activation when subjects are exposed to temperatures of this magnitude[118,184,185].

Diet-induced thermogenesis (DIT)

Ingestion of a meal is followed by a thermogenic response consisting of two components. This thermic effect of food however, is generally rather small accounting for approximately 10 % of total energy intake. The obligatory component is attributed to the energy required for absorption and digestion of food, as well as synthesis and storage of absorbed nutrients as fat, carbohydrates and proteins[186,187]. Additionally, the facultative component further increases postprandial energy expenditure. There is evidence from both rodent and human studies, that this DIT component is mediated by sympathetic activation and BAT activity[178,188,189]. However, further studies are required to confirm a potential link between BAT and DIT[178,190]. Excessive caloric intake as well as diet composition is thought to affect the magnitude of DIT. Both caffeine and capsaicin, the active substance of hot peppers, increase DIT

presumably involving sympathetic activation[188,191–193]. Capsaicin elicits its function via binding to TRPV1, a heat-sensing member of the TRP ion channel family[118,184,185]. Furthermore, macronutrient composition of the diet also influences the magnitude of DIT. High protein/high carbohydrate proportions appeared to result in greater energy expenditure 24 hrs postprandial compared to meals with a high fat content. This increase in metabolic rate appeared to be mainly due to differences in DIT[194].

Exercise

Several studies indicate that in addition to cold- and diet-induced thermogenesis, prolonged exercise can also activate BAT thermogenesis[155,195]. This observation seems counterintuitive in light of muscle-induced heat generation occurring during exercise. Nevertheless, sympathetic BAT activation as well as recruitment of UCP1-expressing ACs to WAT depots has been described upon physical activity[195]. Recently, Irisin, a skeletal muscle-derived secreted hormone, has been identified. Secretion of this myokine is elevated upon exercise stimulus in rodents and humans via *Pgc1α* expression. Irisin is a hormone derived from proteolytical processing of the *Fndc5* gene product. In adipose tissue, Irisin stimulates *Ucp1* expression and induces scWAT browning via *Pparγ* upregulation. Even moderately increased Irisin levels mediate a marked increase in whole body energy expenditure, ameliorate glucose homeostasis, and protect from to obesity-induced insulin resistance[152]. Exogenous delivery of this myokine may have great therapeutic potential in obesity and diabetes treatment.

1.1.7.2 Endogenous secreted factors

The family of natriuretic peptides (NPs) consists of atrial NP (ANP), brain NP (BNP) and C-type NP. ANP and BNP are primarily released from the atria and ventricles and therefore termed cardiac NPs. Cardiac NPs regulate ion homeostasis, body water and excretion of sodium in the urine (natriuresis) in the body. In adipose tissue, NPs are involved in the regulation of lipolysis. Recent studies indicate a role of these peptides in BAT activation, WAT browning and regulation of thermogenic programs, thereby increasing whole body energy expenditure[156,196]. Another example of secreted factors that induce browning is Fibroblast Growth Factor 21. Under certain conditions such as cold exposure, this endocrine hormone is secreted by the adipose tissue and regulates scWAT browning in a PGC1α-dependent manner[154].

1.1.7.3 Thiazolidinediones/Glitzaones

Compounds of the class of Thiazolidinediones (TZDs) are used in the treatment of T2D. They are potent insulin sensitizers and exert their anti-diabetic effect via activation of PPARs, particularly PPARγ. However, the mechanism through which TZDs such as rosiglitazone mediate WAT browning remains to be elucidated[155,197]. It has been suggested that the underlying mechanism may involve stabilization of the PRDM16 protein thereby increasing its half-life[198].

1.1.7.4 Genetic models

Besides a number of secreted factors, several genetic models with an altered phenotype of BAT or beige fat have been described. AC-specific expression of the transcription factor forkhead box C2 under the ap2 promoter leads to scWAT browning, increase in *Ucp1* expression, as well as protection from obesity and diet-induced insulin resistance[156,199]. Interestingly, due to a hierarchical order of sympathetic activation, most agents that have a browning effect on WAT also recruit classical BAT. To date, only four compounds out of the rather large amount of browning agents have been identified to selectively induce browning of WAT without parallel BAT activation[156].

1.2 VASCULAR FUNCTIONS IN THE ADIPOSE TISSUE

1.2.1 Angiogenesis and Vascular endothelial growth factor (VEGF)

In the body of vertebrates, blood and lymphatic vessels play a crucial role in modulating a variety of physiological and pathological processes. Both systems are interconnected via the thoracic duct draining lymph into the blood circulation[200]. Angiogenic growth of lymphatic and blood vessels involves several tightly regulated steps including proliferation and migration of progenitor cells as well as tube formation and vessel maturation[200].

Hemangiogenesis, which will be referred to as angiogenesis in the following, is the growth of blood vessels by sprouting from pre-existing vessels. Sprouting angiogenesis stands in contrast to vasculogenesis, which describes the *de novo* formation of ECs by differentiation from committed mesodermal precursors[201–203]. Both vasculogenesis and angiogenesis are essential and rate-limiting during development and they are coordinated by a delicate interplay of various signaling molecules[201,204]. In growing tissues and organs, major blood vessels such as arteries and veins expand through remodeling and circumferential growth, while capillaries, microvessels that mediate gas and nutrient exchange, form networks via angiogenesis[200]. This occurs not only during embryogenesis and early development but also in the adult organism where angiogenesis plays a role in the female menstrual cycle, wound healing, pathogenesis of many diseases as well as adipose tissue expansion[200,202,205]. Disruptions and dysregulation of vessel growth can have detrimental consequences and both excessive as well as insufficient angiogenesis contribute to the pathogenesis of a number of disorders such as cancer, ischemia, atherosclerosis, neurodegeneration, obesity and T2D[63,202,206]. On a mechanistic level, angiogenic blood vessel growth is a multistep process initiated by a shift in the balance of pro- and anti-angiogenic factor expression. The signaling tissue secretes pro-angiogenic factors such as VEGFA which binds to VEGF receptor 2 (VEGFR2) expressed on ECs[207]. Upon ligand binding to this tyrosine kinase receptor, ECs are activated. VEGF-stimulated ECs release matrix metalloproteases leading to degradation of the basement membrane and extracellular matrix (ECM) consisting of laminins, heparan sulfate proteoglycans (HSPGs), collagen type IV and other components[208]. Thereby, stromal angiogenesis stimulators such as basic fibroblast growth

factor and platelet-derived growth factor (PDGF) that had been trapped by proteoglycans, are mobilized[200,207–209]. In response to VEGFR2 activation and ECM degradation, VEGFA functions as a chemoattractant and its spatial concentration gradient – along with other pro-angiogenic factors – controls EC proliferation and migration towards the signaling tissue[200,204,208,210,211]. To create functional blood vessels, endothelial tube formation is required resulting in the development of a vascular lumen. Finally, increasing oxygen delivery decreases local *Vegfa* expression thereby promoting attenuation of angiogenesis[200]. Mural cells including pericytes (PCs) and vascular smooth muscle cells are recruited to the newly formed blood vessel providing stability. Deposition of ECM components in the basement membrane further promotes vessel maturation[200,206,207,212,213]. In pathological angiogenesis, vessel maturation is often abnormal resulting in unstable, disorganized and dysfunctional vascular networks[213].

Equivalent to hemangiogenesis, the term lymphangiogenesis describes the formation of new lymph vessels from pre-existing lymphatic vessels[200]. The lymphatic system plays an important role in interstitial fluid homeostasis, immunity as well as metabolism and has recently gained attention regarding metastatic spreading of tumor cells[214–216]. Although involving a different set of growth factors, receptors and signaling pathways, lymphangiogenesis follows a similar mechanism as hemangiogenesis including lymphatic EC (LEC) proliferation, sprouting, migration and tube formation[216]. While hemangiogenesis is potently stimulated by VEGFA, lymphangiogenesis is mainly induced via the VEGFC/VEGFR3 signaling axis[200,217].

1.2.2 VEGFs and their receptors

Both blood vessel angiogenesis and lymphangiogenesis are regulated by members of the VEGF family[210]. This group of growth factors comprises the following members (Fig. 4): VEGFA, VEGFB, VEGFC, VEGFD and placenta-derived growth factor (PlGF) 1 and 2 sharing a common homology domain[218]. The existence of VEGFA splice variants further increases the functional diversity of this growth factor family[204,219,220]. The isoforms $VEGFA_{145}$, $VEGFA_{189}$ and $VEGFA_{206}$ contain two heparin-binding domains and are consequently tightly bound to HSPGs. Isoforms that are sequestered in the ECM by HSPG binding can be mobilized via proteolysis. $VEGFA_{165}$ and $VEGFA_{121}$ are lacking one or both heparin-binding domains, respectively, and are therefore diffusible isoforms[218]. The major functions of VEGFA include proliferative effects on ECs and induction of vascular permeability. It also plays a role in EC survival, sprouting, migration and tube formation[210,219]. This factor is critically involved in both vessel development and maintenance[210]. VEGFA binds to VEGFR1, VEGFR2, neuropilin (NRP) 1 as well as NRP2. VEGFR2 has lower affinity for VEGFA compared to VEGFR1. However, VEGFR2 seems to be of greater importance regarding VEGFA-mediated mitogenic effects and vascular permeability[219]. *Vegfa* expression is particularly critical during early development and even heterozygous deletion is embryonically lethal. Isoform studies indicate that $VEGFA_{165}$ is the major effector of VEGF action[219,221]. The growth factor features highest

expression levels during developmental stages and expression decreases in most tissues during the postnatal period.

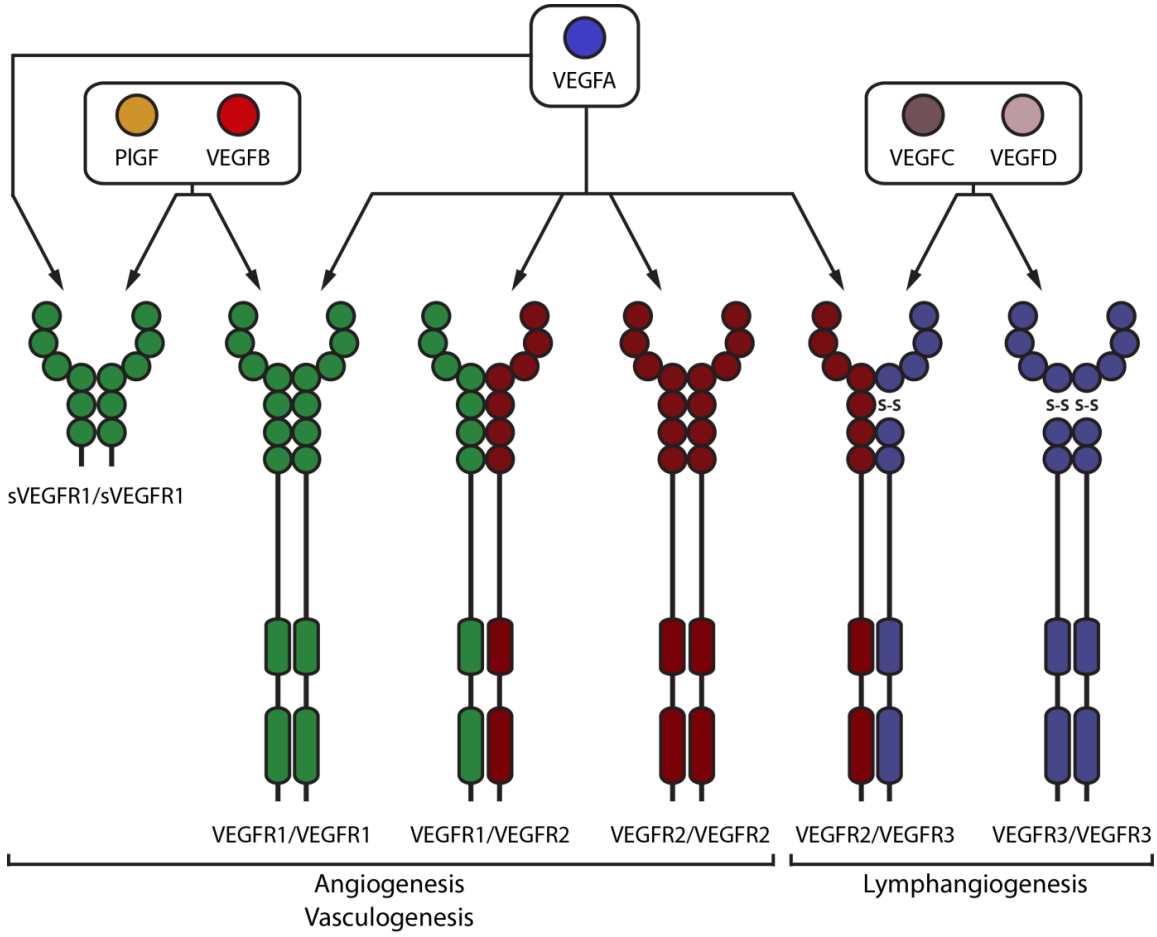

Figure 4: VEGF family and their receptors. The members of the VEGF family and their receptors regulate angiogenesis, vasculogenesis and lymphangiogenesis. Ligand binding triggers the formation of receptors homo- or heterodimers resulting in their activation and intracellular signal transduction. In addition to membrane-bound receptors, this family comprises a soluble form of VEGFR1 (sVEGFR1), lacking the membrane-binding domain.

Certain physiological processes and disease states requiring neovascularization may involve upregulation of *Vegfa* in the adult organism[222]. Low oxygen tension results in hypoxia which is an important trigger of *Vegfa* expression in several tissues as well as in tumors and other pathological settings. Oxygenation can be achieved through diffusion until the tissue reaches a particular size. However, oxygen diffusion limits are typically around 100 – 200 µm and tissues exceeding this size require a functional vascular network to maintain physiologically appropriate oxygen levels[222,223]. Neovascularization is an adaptive response to tissue hypoxia and is observed in several pathological conditions such as coronary heart disease or tumor angiogenesis. The molecular mechanisms underlying hypoxia-induced angiogenesis have been studied extensively and are well understood. On a transcriptional level, responses to tissue hypoxia are mainly mediated by hypoxia inducible factors (HIFs) and in particular HIF-1, a heterodimer consisting of HIF-1α and the aryl hydrocarbon receptor nuclear translocator (Arnt)[222,224,225]. The HIF family of transcription factors regulates the expression of a number of genes involved in apoptosis, cell cycle, energy

metabolism and angiogenesis. The *Vegfa* gene is one of the HIF target genes that contains hypoxia response elements (HREs) in its 5' and 3' untranslated regions[222,224,226]. Under normoxia, the HIF-1α subunit is highly unstable. The protein is hydroxylated by prolyl hydroxylase domain-containing (PHD) enzymes using oxygen as a substrate, thereby initiating ubiquitination. Consequently, HIF-1α readily undergoes proteasomal degradation. Reductions in oxygen tension, however, result in attenuated hydroxylation of HIF-1α due to depletion of the PHD substrate and in this manner, hypoxia allows for intracellular HIF-1α accumulation[222,224,227]. Upon translocation of HIF-1α into the nucleus, where it forms a complex with Arnt and the coactivator protein CBP/p300, the transcriptional complex binds to HREs in the promoter and enhancer regions of target genes including *Vegfa*[222,224]. In addition to increasing *Vegfa* expression levels, hypoxic conditions have a stabilizing effect on *Vegfa* mRNA. Furthermore, there are a number of growth factors and cytokines such as PDGF-BB or transforming growth factor-β that upregulate *Vegfa* gene expression.

While VEGFA is extensively studied, the role of VEGFB, which binds to VEGFR1 and NRP-1, is poorly understood. Studies indicate that the growth factor is dispensable for angiogenesis but critical for vascular maintenance and survival of vascular ECs, PCs, smooth muscle cells (SMCs), as well as vascular stem and progenitor cells[228]. VEGFB is produced by the heart, skeletal muscle and BAT, all of which are metabolically highly active tissues. Recently, it has been shown that VEGFB induces transendothelial transport of lipids via upregulation of fatty acid transport proteins and thereby coordinates the transport of lipids to peripheral energy-expending organs in a paracrine manner[229].

PlGF shows merely weak angiogenic activity. However, after heterodimer formation with VEGFA, this growth factor acts as a mitogenic factor on ECs and stimulates angiogenesis via signaling through VEGFR2[222].

VEGFC and VEGFD are ligands for VEGFR3 and are involved in lympangiogenesis. Similar to the fundamental role of VEGFA in hemangiogenesis, VEGFC governs the key events in the formation of new lymphatic vessels including sprouting, survival and proliferation of LECs. The lymphangiogenic activity of VEGFD however, is not crucial for lymphatic development. Although these ligands exert their actions mainly via binding to VEGFR3, they can also signal through VEGFR2[200,222].

1.2.3 Vascular endothelial growth factor receptors (VEGFRs)

The three members of the VEGFRs are receptor tyrosine kinases (RTKs) consisting of seven extracellular immunoglobulin-like domains, a transmembrane regions and an intracellular tyrosine kinase domain[218]. In general, downstream signaling of RTKs is initiated by binding of a covalently linked ligand homo- or heterodimer[225,230]. Ligand binding results in dimerization of two receptor monomers, transautophosphorylation of intracellular tyrosine residues, and consequently RTK activation. Upon receptor activation, downstream signaling molecules are recruited and further transmission of the signal is

propagated through Src homology-2 (SH2) and phosphotyrosine binding (PTB) domain-containing proteins[225,230]. Alternatively, the activated receptor can recruit adaptor proteins such as growth factor receptor-bound protein (Grb) 2 that mediate protein-protein interactions and thereby assembly of signaling complexes. Via this elaborate signaling cascade, extracellular ligand binding is translated into appropriate intracellular responses.

VEGFR1 is expressed by hematopoietic stem cells, monocytes, macrophages as well as vascular ECs and binds VEGFA, VEGFB and PlGF (Fig. 5). This receptor exists both as a membrane-bound form and as a truncated, soluble splice variant (sVEGFR1).

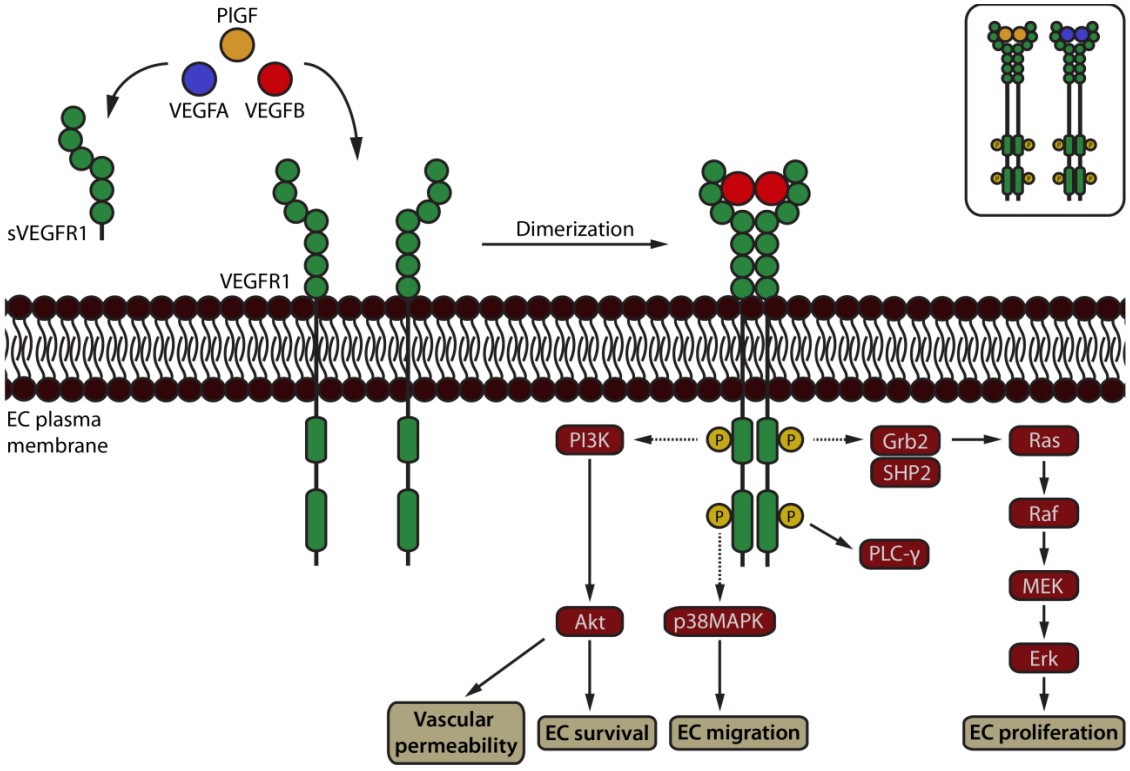

Figure 5: Downstream effects of VEGFR1-mediated signaling. VEGFR1 exists as a membrane-bound receptor and as a soluble splice variant (sVEGFR1) that can bind the ligands VEGFA, VEGFB and PlGF. Upon ligand binding, VEGFR1 dimerizes resulting in transautophosphorylation of tyrosine residues. An intracellular signaling cascade is triggered regulating EC survival, proliferation, migration and vascular permeability. Importantly, tyrosine kinase activity of VEGFR1 is rather weak and this receptor mainly functions as a decoy receptor preventing binding of VEGFA to VEGFR2.

The precise function of this receptor has not been fully elucidated yet. Depending on developmental stage as well as cell type, VEGFR1 can exert different or even opposing functions. Deletion of the *Vegfr1* gene is embryonically lethal and the receptor appears to act as a negative regulator of VEGFA during early development[225,230–232]. It has been proposed that VEGFR1 may function as decoy receptor by binding VEGFA with high affinity thereby preventing the interaction of this ligand with VEGFR2. There is compelling evidence that the soluble VEGFR1 isoform exerts an inhibitory effect on EC mitogenesis thus negatively regulating VEGFR2 and suppressing angiogenesis[218,225,231,233]. Membrane-bound VEGFR1 interacts with a number of downstream signaling pathway components

such as PI3K, phospholipase C (PLC)-γ, Src-homology domain 2-containig phosphatase (SHP2) and Grb2[234].

In contrast to VEGFR1, functions of VEGFR2 are much better understood. This receptor binds VEGFA, VEGFC and VEGFD. During adulthood, *Vegfr2* is almost exclusively expressed in vascular ECs[232]. VEGFR2 is critically involved in developmental angiogenesis, physiological and pathological neovascularization. The affinity of VEGFA for this receptor is ten times lower compared to VEGFR1; nevertheless, VEGFR2 is the key regulator of mitogenesis, angiogenesis and vascular permeability[225,230,232]. Downstream signaling of VEGFR2 involves several major signaling cascades (Fig. 6): the PI3K-Akt pathway mediates EC survival and vessel permeability, the p38 MAP kinase pathway governs EC migration and the Ras-Raf-Mek-Erk pathway stimulates EC proliferation[225,235].

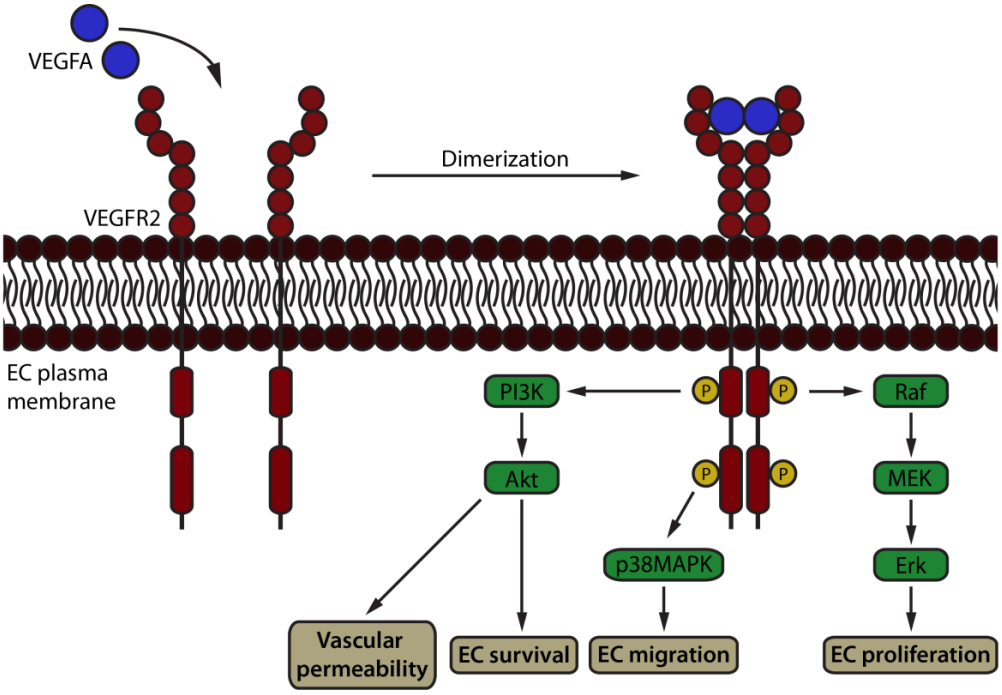

Figure 6: Downstream effects of VEGFR2-mediated signaling. VEGFR2 is almost exclusively expressed on vascular ECs and binds VEGFA with lower affinity than VEGFR1. However, VEGFR2 is the key regulator of EC mitogenesis, vascular permeability and angiogenesis. Upon ligand binding, VEGFR2 dimerizes and transautophosphorylation of intracellular tyrosine residues propagates growth factor signaling of this receptor tyrosine kinase.

Expression of *Vegfr3* is generally restricted to LECs and this receptor binds exclusively VEGFC and VEGFD. Although the downstream signaling pathways induced upon VEGFR3 activation remain to be unraveled, it has been suggested that signals are transduced via the PI3K-Akt and the Ras-Raf-Mek-Erk pathway. However, other signal transducers such as PLC-γ, SHP2, c-Jun N-terminal kinases (JNK) 1/2 and the transcription factors STAT3 and STAT5 might be involved in VEGFR3-mediated signaling (Fig. 7)[231,236–240].

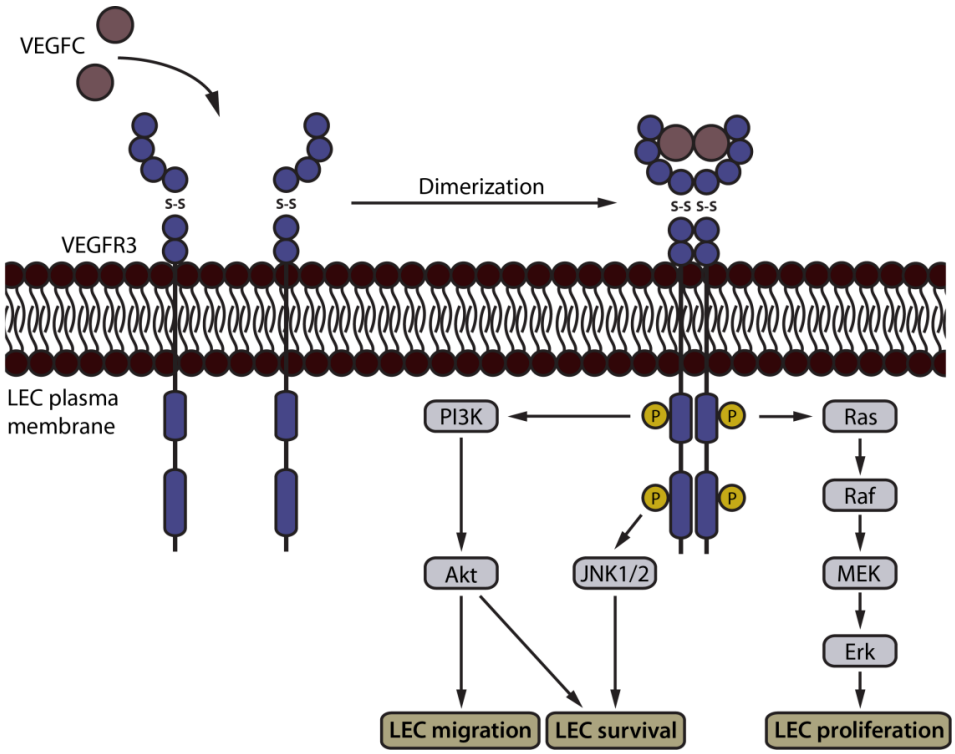

Figure 7: Downstream effects of VEGFR3-mediated signaling. VEGFR3 is expressed on LECs and mediates lymphangiogenesis. The receptor dimerizes upon binding of its ligands VEGFC and VEGFD leading to receptor activation. Intracellular signaling involves the PI3K/Akt and Erk pathways resulting in LEC survival, proliferation and migration.

1.2.4 Platelet-derived growth factors (PDGFs) and their receptors

The PDGF family consists of the ligands PDGFA, PDGFB, PDGFC and PDGFD as well as their tyrosine kinase receptors PDGFRα and PDGFRβ. These RTKs are structurally related to VEGFRs and consist of five extracellular immunoglobulin loops and an intracellular kinase domain[241,242]. PDGFs are mitogens for a number of mesenchymal cell types and bind as homo- or heterodimers to their respective receptors. Ligand binding induces receptor dimerization, autophosphorylation and activation[241,242]. The growth-factor mediated signals are further transduced through protein-protein interactions involving SH2 and PTB domains that provide docking sites for recruited effector proteins[241,243]. Several *in vitro* and *in vivo* studies have identified PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC as ligands for PDGFRα while PDGF-BB and PDGF-DD signal through PDGFRβ[242]. Downstream signaling of both receptors involves activation of the PI3K/Akt and MAPK signaling pathways as well as STAT signaling resulting in cell proliferation and survival thereby mediating the mitogenic effect of PDGFs[243,244]. It has been shown that PDGF-BB-mediated signaling induces growth of angiogenic vessels through stimulation of EC proliferation and survival via signaling through both PDGFRα and PDGFRβ[245–247]. Furthermore, PDGF-BB/PDGFRβ regulates maturation of newly formed blood vessels via recruitment of PCs and SMCs[213,247,248].

Notably, a subpopulation of PDGFRα-expressing bipotential AC progenitors that can differentiate into both white and brown ACs under appropriate stimuli has recently been

identified[249]. Detailed molecular studies including identification and origin of the ligand are required to further elucidate the mechanism underlying PDGFRα-mediated determination of preadipocyte fate.

1.2.5 Reciprocal interaction between adipose tissue and the vasculature

The adipose tissue, both WAT and BAT, is highly vascularized. Each AC is surrounded by a blood vessel, reflecting the close functional relationship between adipose tissue and the vasculature[41,63,202,205,212,250,251]. Maintaining appropriate blood flow and supply with endothelial-derived factors is crucial for adipose tissue expansion, remodeling and metabolic functions[202,212,250]. Similarly, the adipose tissue produces and releases a myriad of vasoactive adipokines that affect angiogenesis and vascular functions in an endocrine and paracrine manner. Consequently, disruptions in the secretion of these molecules is critically involved in linking obesity to endothelial dysfunction[63,202,212,250]. Endothelial dysfunction is defined as increased vasoconstriction and impaired vasodilatation and is associated with a number of pathological conditions such as hypertension, atherosclerosis, adipose tissue inflammation and insulin resistance[250,252].

The critical role of microvessel growth in the development of adipose tissue is reflected by the spatio-temporal link between adipogenesis and angiogenesis during embryonic development. In early adipose tissue organogenesis, fat cell clusters, so-called "primitive organs" develop through formation of vascular structures and subsequent migration of preadipocytes into fat cell clusters. During the fetal stage, size and number of these primitive fat organs increase[253–256]. Studies suggest that plasminogen activator inhibitor-1 coordinates adipogenesis and angiogenesis in the formation of primitive fat organs by regulating migration of preadipocytes and ECs[257]. There are indications that ACs and ECs may share a common progenitor with potential to differentiate into either lineage depending on external stimuli thus strengthening the concept of a close interrelationship of these two cell types[258]. Furthermore, a recent study revealed a perivascular origin of preadipocytes. Both stromal vascular cells as well as a subset of capillary ECs was found to express *Zfp423*, a gene regulating preadipocyte determination[212,255,258–260]. However, a recent study challenges the hypothesis of a potential pericytal or endothelial origin of AC precursor cells supporting the view that a mesenchymal Pref-1 positive progenitor gives rise to ACs[261].

Adipose tissue growth and remodeling does not only occur during early development but also plays a role during the postnatal period and adulthood. Adipose tissue is highly plastic, experiencing continuous expansion and shrinkage throughout the lifetime depending on nutritional status of an individual[202,205,212]. The adipose vasculature is critically involved in adipose tissue homeostasis by exerting a number of functions. Blood vessels transport albumin-bound FFAs as well as lipoproteins containing TGs and cholesterol[262]. Moreover, the vasculature supplies oxygen and nutrients, but also removes waste products. These functions are especially important in metabolically highly active BAT[63]. Moreover, the vasculature delivers plasma containing growth factors, hormones, immune cells and

cytokines to the adipose tissue and thereby contributes to maintaining AC survival and function. Additionally, activated ECs also produce a number of factors which modulate adipose tissue growth and homeostasis in a paracrine manner[63,212]. In terms of adipose tissue expansion, blood vessels play a dual role. Firstly, the vasculature supplies circulating stem cells with adipogenic potential, such as bone-marrow derived stem cells and mesenchymal stem cells originating from other tissues[63,212]. Secondly, recent studies demonstrated that murine and human adipose progenitor cells reside in the mural compartment of the adipose vasculature[259,260,263]. Owing to microvessel fenestration, the vasculature is also involved in mediating systemic functions of adipose tissue via secreted adipokines such as adiponectin or leptin which act on the hypothalamus[63,264,265]. It is noteworthy that AC-derived factors have been well studied regarding their role in promoting angiogenesis. However, to date, the molecular mechanisms underlying EC-mediated modulation of adipocyte growth and function remain poorly understood.

1.2.6 Targeting the adipose vasculature modulates adipose tissue function and whole body metabolism

Due to the fact that adipose tissue growth is critically dependent on angiogenesis, anti-angiogenic therapy appears to be a promising approach to prevent or reverse adipose tissue overgrowth, obesity and related metabolic disorders. However, both inhibition and stimulation of angiogenesis has been reported to contribute to a leaner phenotype and improved metabolic profile in several pre-clinical mouse models. These findings are based on both pharmacological interventions as well as genetic VEGF gain-of-function and loss-of function approaches[145,205,251,266–269]. The metabolic status of adipose tissue appears to be a critical determinant whether positive or negative modulation of angiogenesis should be achieved to treat obesity. In metabolically quiescent WAT, angiogenesis may promote adipogenesis and energy deposition whereas microvessel growth in metabolically active BAT facilitates energy expenditure[63]. It has been established that dysfunction of adipose tissue rather than its absolute mass determines metabolic health[3,4]. Chronic deprivation of oxygen through anti-angiogenic therapy may have detrimental system effects on metabolic health as the dysfunctional adipose tissue may cause ectopic lipid deposition. Paradoxically, hypoxia-induced adipocyte death could counteract adipose tissue expansion and thereby have beneficial effects. Interestingly, the effects of vascularization on obesity and metabolic health seem to be stage-dependent. Inhibiting angiogenesis at early stages of obesity development aggravates adipose tissue inflammation and insulin resistance, potentially by shifting adipokine production towards a diabetogenic profile. Thereby, a reduction of adipose vascularization at early stages of obesity appears to have detrimental local and systemic effects[251,266,267]. However, suppression of angiogenesis when obesity is already established appears to reverse metabolic defects such as glucose intolerance or insulin resistance potentially by triggering apoptosis of malfunctioning adipocytes thereby inducing adipose tissue regression[251,266,267]. The underlying molecular mechanisms remain elusive and further detailed studies are required in order to define a promising therapeutic approach for the treatment of obesity by modulating the adipose vasculature.

2 AIMS OF THIS THESIS

The aims of this thesis were to establish appropriate models to study adipose tissue angiogenesis and lymphangiogenesis and to use these models to investigate WAT browning as well as to study the effects of targeting the adipose vasculature on obesity and metabolism.

- In **paper I** we aimed at establishing a non-invasive, physiologically relevant model of BAT activation and WAT browning. We intended to optimize available methodology to study adipose tissue and adipose vasculature morphologically. Furthermore, we provide approaches to study the physiological outcome of cold-induced alterations in WAT and BAT.

- The aim of **paper II** was to evaluate the effect of ageing on the adipose vasculature and AC function. We investigated the relationship between blood vessels in various adipose depots and insulin sensitivity. We further studied the systemic effect of targeting the adipose vasculature with anti-angiogenic agents in both lean and obese mice.

- In **paper III** we investigated the reciprocal crosstalk between ACs and vascular ECs. A variety of factors secreted by the adipose tissue that modulate vessel growth and function via paracrine signaling has been described in the literature. However, our study aimed at identifying EC-derived factors that might modulate adipogenesis and AC function.

- The aim of **paper IV** was to develop an *in vivo* model that would allow the investigators to study the lymphangiogenic potential of various growth factors and molecules. This model could be used in future studies to define a potential role of lymphatic vessels in modulating adipose tissue and metabolism.

3 METHODS

In the following, the most important methods used in the constituent papers are described in detail.

3.1 MOUSE COLD ADAPTATION ASSAY

In this assay, the effect of cold exposure on UCP1-dependent NST as well as transformation of WAT to beige adipose tissue can be studied. The control group of mice is maintained at their thermoneutral temperature around 30 °C. At this temperature, mice do not require any thermoregulatory activity in order to sustain their body temperature. Another group of animals is first adapted at moderate cold (18 °C) for a minimum of one week. For wild type mice, one week of adaptation is sufficient to activate their NST capacity so that they will tolerate subsequent cold exposure. Certain genetically modified strains, such as UCP1-/- mice however, require longer adaptation for up to three weeks. After adaptation at moderate temperature, mice can be transferred to the cold (4 °C). For this assay as for all experiments involving animals, it is critical to adhere to ethical regulations and guidelines and humane considerations are required in order to prevent unnecessary suffering of the mice.

3.2 INDIRECT CALORIMETRY

3.2.1 Measurement of basal metabolic rate

Mice are placed into a metabolic chamber with free access to food and water and a regular 12h-light:12h-dark cycle. The temperature of the metabolic cage is adjusted to the desired value. During the following 24 hrs, oxygen (O_2) consumption and carbon dioxide (CO_2) production are measured. The obtained values can be used to determine whole body energy expenditure and the Respiratory Exchange Ratio (RER). The RER is the ratio between CO_2 production and O_2 consumption, which provides information about the primary source of energy for the mice. A RER of 0.7 implies that fat is primarily used as an energy source while RER $\geq$ 1 indicates that carbohydrates serve as main fuel. A mix of fat and carbohydrates is reflected by a RER of 0.85.

3.2.2 Measurement of NE-induced NST

Mice are anesthetized with pentobarbital to avoid muscle movement during this assay and placed in a metabolic chamber with an internal temperature of 33 °C to maintain mouse body temperature. For approximately 30 min, O_2 consumption and CO_2 production are measured continuously. After a stable baseline is obtained, 1 mg/kg NE is injected subcutaneously at the dorsal midline of the mouse and the respiratory parameters are measured for about 90 min. This assay measures NST and thereby provides information about BAT activation and UCP1 activity.

3.3 INTRAPERITONEAL INSULIN TOLERANCE TEST (IPITT)

Prior to this physiological assessment of insulin sensitivity, mice are fasted for 4 – 5 hrs with free access to drinking water. At the end of the fasting period, the baseline blood glucose level (t=0) is assessed with a glucometer. For this purpose, the mouse tail vein is punctured with a sterile needle and a drop of blood is applied to a glucose test strip. For the IPITT, 0.5 Units of Insulin are injected intraperitoneally into the mouse. The dose of insulin depends on the mouse strain and potential treatment that the animal has undergone previously which might render the mouse more or less sensitive to insulin. It is therefore critical to establish the proper insulin dose in a pilot experiment in order to prevent the mice from becoming severely hypoglycemic during the IPITT. A drop of blood is collected 15, 30, 60 and 120 min post insulin injection and the respective blood glucose levels are determined using a glucometer. After completing this experiment, mice are returned to their home cage with free access to food and water and monitored closely until they have recovered.

3.4 QUANTITATIVE DETECTION OF BLOOD LIPID COMPONENTS, GLUCOSE AND INSULIN

Immediately after sacrificing the mice with an overdose of CO_2, blood is collected by cardiac puncture and serum is prepared. Serum levels of cholesterol, glucose, glycerol, non-esterified free fatty acids (NEFA) and TGs were quantitatively assessed using colorimetric assays. Circulating insulin was detected in mouse serum and quantified using a sensitive Mouse Insulin ELISA Kit. To determine fasting glucose and insulin levels, mice were fasted for 4 – 5 hrs prior to euthanasia.

3.5 HISTOLOGY, WHOLE MOUNT ANTIBODY STAINING AND IMMUNOHISTOCHEMISTRY

At the experimental endpoint, mice were sacrificed and adipose tissues as well as other organs were dissected and collected immediately. Tissues were fixed in 4 % paraform-aldehyde (PFA) overnight and then transferred to phosphate-buffered saline (PBS). A portion of the tissue was embedded in paraffin for microtome sectioning and immunohistochemistry staining while another portion was used for whole mount antibody staining.

For whole mount staining, sections of about 1 mm thickness were prepared by carefully cutting the tissue with a sharp scalpel blade. The tissue was digested with Proteinase K (20 mg/ml) and permeabilized with methanol. After thoroughly rinsing, unspecific binding sites were blocked with 3% milk in PBST prior to incubation with a primary antibody overnight. The following day, the tissue was washed and incubated with an appropriate fluorescence-linked secondary antibody. It is possible to use a combination of different antibodies generated in different species to obtain double or triple staining of several cellular components. Positive signals were detected using confocal microscopy.

For immunohistochemistry, 5 µm thick sections of paraffin-embedded tissue were prepared using a microtome. Tissue sections were rehydrated in an ethanol/water series. After antigen unmasking, unspecific binding sites were blocked with 5% normal goat serum and sections

were incubated with primary antibodies at appropriate dilutions in a humid chamber overnight. The following day, sections were washed and incubated with suitable secondary antibodies. The tissue was then mounted in mounting medium containing DAPI to visualize cell nuclei and the sections were analyzed using a fluorescence microscope.

3.6 MICROSCOPY

Imaging was done using fluorescence or confocal microscopy. Fluorescence microscopy allows the analysis of thin tissue sections e.g. to detect co-localization of certain intracellular markers. Confocal microscopy permits the analysis of whole mount tissue by subsequently scanning several layers of defined thickness and at defined step size. The stack of images is then merged into a sharp three dimensional image. Thereby, structure and organization of the vasculature can be studied in great detail. The images obtained by fluorescence or confocal microscopy were analyzed in a qualitative and quantitative manner using the software Adobe Photoshop Creative Suite 5.

3.7 RNA EXTRACTION AND QUANTITATIVE REAL TIME PCR (QRT-PCR)

Immediately after euthanasia, adipose tissues were dissected and snap frozen in liquid nitrogen. For RNA isolation, WAT and BAT were homogenized and total RNA was isolated using an RNA isolation kit. Approximately 1.5 µg total RNA were reversely transcribed and the cDNA was used for subsequent qRT-PCR. Each 20 µl reaction contained 1 µl cDNA, 10 µl SYBR Green reagent, and 150 nM forward and reverse primers, respectively. The protocol contained 40 cycles with each cycle comprising denaturation for 15 s at 95 °C, annealing for 1 min at 60 °C, and extension for 1 min at 72 °C. Threshold cycle (Ct) values were determined for all samples. The housekeeping gene actin was used as an internal control and all Ct values of the target genes were normalized to the Ct value of actin. The 2-ΔΔCt method was used to calculate the relative abundance of transcripts.

4 RESULTS AND DISCUSSION

4.1 PAPER I: COLD-INDUCED ACTIVATION OF BROWN ADIPOSE TISSUE AND ADIPOSE ANGIOGENESIS IN MICE

Several research groups have shown that cold exposure activates rodent BAT and induces a transformation of scWAT to a beige/BRITE phenotype via sympathetic activation[101,103,145,270]. Adipose tissue plasticity is associated with vascular alterations due to a switch in the balance between pro- and anti-angiogenic factor expression[202,212]. Along with morphological changes, functional alterations of the adipose tissue can be observed during cold adaptation and may result in modulation of whole-body energy homeostasis. In this paper, we describe a mouse model to systematically study cold-induced adipose tissue angiogenesis and BAT activation in a non-invasive manner. This protocol evaluates step by step the investigation of WAT, BAT and the adipose vasculature on a morphological level and provides tools to study the functional consequences of temperature-induced modulation of the adipose tissue phenotype. Commonly encountered problems are addressed in a separate troubleshooting section.

In the model described in this protocol, mice are divided into two groups. One group of mice is maintained at their thermoneutral temperature of 30 °C while the second group of mice is adapted to a low ambient temperature. For this purpose, mice are initially acclimatized at moderate cold (18 °C) for one week before transition to 4 °C. Both groups should be exposed to the respective temperature for the same amount of time to allow for comparable results of the experiment. At the experimental endpoint, several parameters can be studied including metabolic rate, non-shivering thermogenic capacity, macroscopic and microscopic adipose tissue morphology as well as gene expression. Depending on the parameter of interest, the duration of this experiment should be adjusted. The cold-induced angiogenic response as well as BAT activation and scWAT browning are already evident after a few days of exposure. Prolonged exposure may further enhance the observed phenotype. When studying gene expression, it is recommended to study differential gene regulation both at an early and a late time point. Some genes may only be transiently upregulated; they may be induced after acute cold exposure but return to a basal level after prolonged, chronic exposure. Nevertheless, this model provides a tool to unravel the molecular mechanisms involved in microvessel growth.

To study the effects of cold exposure on whole body energy homeostasis, we describe measurement of basal metabolic rate as well as UCP1-dependent NST. Basal metabolic rate is typically measured in non-sedated mice over a time period of 24-72 hrs. During the measurement, mice are maintained in their home cage which is placed inside a metabolic chamber with a fixed temperature, a 12h-light:12h-dark cycle and free access to food and water. To determine adaptive thermogenesis, mice are anesthetized to prevent muscle movement which may alter the results of this measurement and lead to high variability between individual mice. After obtaining a stable baseline, NST is stimulated by injection of NE and a rapid increase in O_2 consumption and CO_2 production can be observed. The magnitude of the response varies depending on BAT amount and activation status and allows conclusions regarding the animal's NST capacity. Typically, in cold-exposed wild type mice,

the observed response to NE injection is significantly increased compared to mice maintained under thermoneutral conditions. As mice are anesthetized during this procedure, the ambient temperature in the metabolic chamber is to be set to 33 °C to prevent hypothermia. Furthermore, if performing this experiment at temperatures below the thermoneutral zone, BAT and NST may already be activated prior to NE-injection leading to inaccurate results[100]. In this protocol, we further describe different staining methods to histologically analyze adipose tissues. To obtain information regarding the three-dimensional structure and integrity of the adipose vasculature, whole mount staining and subsequent confocal imaging provide powerful tools. In order to investigate intracellular components such as mitochondria or to study the vascular density in relation to the number of ACs, immunohistochemistry on paraffin or cryostat sections is preferable.

The protocol described here can be employed using mouse strains of different genetic backgrounds. It is highly recommended to modify the protocol according to the mouse strain that is used. When using animals with genetic deletions of thermogenesis-related genes such as UCP1 knockout mice, acclimation time has to be prolonged to three weeks to prevent death of the animals. Additionally, this model can be used to investigate the effect of existing or potential anti-obesity or anti-diabetic drugs in combination with cold exposure. A combination-based approach may have beneficial additive or synergistic effects on body weight reduction and improvement of metabolic health. Using this model, we studied the mechanisms underlying VEGF-related angiogenesis during browning of WAT (Paper III).

4.2 PAPER II: MODULATION OF AGE-RELATED INSULIN SENSITIVITY BY VEGF-DEPENDENT VASCULAR PLASTICITY IN ADIPOSE TISSUES

Obesity-related metabolic disorders account for the most common causes of death globally and pose a large burden on health care systems in various countries. Interestingly, the risk to develop T2D increases dramatically when individuals reach an age of 40 years. According to the Centre of Disease Control and Prevention, nearly two thirds of individuals diagnosed with T2D in 2011 were between 40 – 64 years old[271]. Although it is known that both ageing and obesity constitute major risk factors for the development of this disease, it remains enigmatic why the incidence of T2D dramatically increases in middle-aged subjects. There is evidence that ROS-induced mitochondrial damage is associated with age-related muscle insulin resistance[272]. Although little is known regarding the effect of ageing on the adipose vasculature *in vivo*, it has been reported that vascularization and angiogenesis are impaired in the adipose tissue of older mice[42,273].

In this paper, we provide evidence that alterations in the adipose vasculature and *Vegf* expression during ageing, correlate with the risk of developing T2D. In the first part of our study, we investigated age-related alterations of the adipose vasculature as well as AC morphology and functions. One mouse day roughly corresponds to 50 human days, assuming a lifespan of 2 years for mice and 100 years for humans, respectively. For this ageing-study, we included C57Bl/6 mice of ages 1, 4, 10, 12, and 16 months which corresponds to 4-, 17-, 42-, 50-, and 70-year old humans. In our study, average AC diameters in WAT gradually

increased from 1 mo to 10 mo of age where they reached a maximum of 106 (± 2.7) μm in scWAT and 116 (± 0.6) μm in epiWAT. The observed AC hypertrophy correlated with an increase in body weight and BMI with increasing age. When mice exceeded the age of 10 mo, average AC size decreased while body weight and BMI stabilized. We also found that blood vessel density in several WAT depots of healthy, lean mice varied markedly over the mouse life span. Microvessel density was highest in the youngest mice (1 mo-old) and steadily decreased reaching a minimum in middle-aged animals. In accordance with these findings, VEGF protein levels in WAT were lowest at the age of 10 mo. After 10 mo of age, WAT microvessel density gradually increased. Similar alterations in vessel density and AC diameter were observed in interscapular BAT (intBAT); however, these effects were more subtle compared to WAT. Interestingly, total intBAT mass increased with age and the tissue mass in 16-mo old mice was approximately two times higher compared to the youngest age group in our study. However, despite this increase in total mass, UCP1 mRNA and protein levels were markedly reduced at 12 and 16 mo compared to younger mice at 4 mo of age. This indicates that BAT activation was lower in older mice. To gain further molecular insight into the mechanisms underlying age-related alterations of the adipose vasculature, we studied the expression of *Vegfr1* and *Vegfr2* in WAT and BAT. We found that *Vegfr1* expression was highest at 10 mo of age. This finding is in accordance with the hypothesis that this receptor mainly functions as a decoy receptor for VEGFA and acts as a negative regulator of angiogenesis. On the contrary, *Vegfr2* expression was highest in the youngest animals and decreased with age reconciling with its role as a transducer of VEGF-induced angiogenesis.

Next, we studied age-related changes in AC functions such as lipolysis and insulin sensitivity by analyzing serum levels of several blood lipids as well as fasting glucose and insulin levels. While no overwhelming age-related changes in serum TGs, NEFA, glycerol, cholesterol or fasting glucose could be detected, circulating insulin was markedly increased with age, resulting in substantially reduced insulin sensitivity in older animals. At the age of 10 mo, mice exhibited a strong elevation in circulating insulin levels and greatly increased HOMA-IR values compared to younger animals indicating insulin resistance. This onset of insulin resistance was negatively correlated with adipose vascularization as vascular density in both epiWAT and scWAT was lower at this age compared to all other age groups included in this study. We concluded that VEGF modulates angiogenesis in the adipose tissue and that lower vascular density is associated with an increased risk of developing insulin resistance and T2D. To validate this hypothesis and to further study the role of VEGF signaling in age-related development of insulin resistance and T2D, we treated lean, healthy mice of 1-, 7- and 15-mo of age with specific anti-VEGF and anti-VEGFR2 neutralizing antibodies. Paradoxically, treatment with anti-VEGF antibody decreased adipose vascular density while improving insulin sensitivity via significantly lowering serum glucose levels in all studied age groups. The magnitude of this effect however varied greatly with age and middle-aged animals exhibited a rather moderate response as compared to younger and older mice. To get further insight into the mechanism of anti-VEGF-induced decrease of blood glucose, we performed an IPITT on vehicle- and anti-VEGF-treated animals of different age. In young

and old mice, glucose clearance following administration of 0.5 Units insulin per kilogram body weight was significantly accelerated in mice treated with anti-VEGF. In middle-aged mice, however, glucose clearance was not significantly altered after anti-VEGF treatment. These findings validate that middle-aged mice were less sensitive to anti-VEGF therapy compared to other age populations. The observation that anti-VEGF-mediated vascular reduction improved insulin sensitivity was unexpected and further studies are required to fully unravel the underlying complex mechanism.

To investigate whether anti-VEGF therapy might have beneficial effects on insulin sensitivity in a clinically relevant model of DIO, mice were fed a high fat diet to induce adipose hypertrophy and insulin resistance. Treatment of these animals with an anti-VEGF antibody expectedly reduced adipose vascular density. As seen in lean, healthy animals, VEGF-blockade could significantly improve DIO-associated insulin resistance as well as glucose clearance in an IPITT. In this model, anti-VEGF therapy markedly reduced body weight, BMI, as well as total mass of scWAT, epiWAT and intBAT. The average AC diameter in all studied adipose depots decreased significantly after anti-VEGF treatment. In BAT, anti-VEGF therapy reduced the amount of lipid deposition and increased mitochondrial content. These findings suggest that anti-VEGF agents might offer a novel opportunity to improve insulin sensitivity in obese patients. Further studies using a mouse model with fully developed T2D will be necessary to determine whether anti-VEGF therapy is effective under established disease conditions.

Taken together, our findings further demonstrate that adipose vasculatures show differential responses to anti-VEGF treatment in various age populations. This phenomenon might have therapeutic implications for treatment of obesity and diabetes with anti-VEGF-based anti-angiogenic drugs. Currently, several anti-VEGF based drugs are used in clinical settings in the treatment of cancer patients as well as in ophthalmological disease therapy[274,275].

4.3 PAPER III: ENDOTHELIAL PDGF-CC MEDIATES VEGF-ANGIOGENESIS-DEPENDENT THERMOGENESIS IN BROWNING FAT

The adipose microenvironment contains various cell types such as AC, vascular ECs, stromal cells and inflammatory macrophages[63,202]. A crosstalk between these distinct cell populations coordinates adipose tissue growth, expansion and remodeling. ACs might communicate with ECs in a paracrine manner by producing a myriad of cytokines and factors[63,212]. It is likely that ECs modulate AC functions via soluble or membrane-bound factors; however, little is known about this potential crosstalk. In this paper, we studied the role of angiogenesis in modulating AC functions. We show that ECs are critically involved in WAT browning following adrenergic activation. Our findings indicate that EC-derived PDGFC induces differentiation of progenitors into ACs. Pharmaceutical blockade of PDGFRα as well as deletion of the *Pdgfc* gene could greatly abolish the paracrine regulatory effect and markedly reduce WAT browning.

It has previously been shown that PDGFRα$^+$ cells constitute bi-potential AC progenitors that can differentiate into both WAT and BAT ACs *in vitro*[2,249]. However, the identity and source of the ligand have not been described yet.

WAT browning mediated by adrenergic activation is well established. In our study, administration of the specific β3-adrenergic agonist CL induced a strong angiogenic response in gonadal WAT (gWAT) of mice. We detected increased vessel density in this adipose depot already after 2 days of treatment and a 3-fold increase was seen at day 10 of CL treatment. We found that *Vegf* expression was markedly increased in the adipocyte fraction but not in the stromal vascular fraction (SVF) of gWAT upon CL treatment. Angiogenic effects were also observed in other WAT depots as well as in intBAT. A similar angiogenic response was observed in scWAT and BAT of cold-exposed mice while the increase in vascular density in gWAT was only moderate. Local adenoviral delivery of VEGF (AdVEGF) to mice induced *Vegf* expression, angiogenesis and browning in gWAT. However, thermogenic capacity of these mice remained unaltered leading to the conclusion that whole body energy homeostasis is not significantly affected by gWAT browning. This finding implies WAT depot-specific differences regarding the contribution to whole body energy expenditure.

Additionally, CL treatment induced browning of gWAT and scWAT and markedly increases *Ucp1* expression levels of these depots. In cold-exposed animals, only scWAT exhibited a browning phenotype. Interestingly, UCP1-deficient mice showed a VEGF-dependent angiogenic response as well as WAT browning while NST was abolished in these animals. These findings suggest that CL-mediated adipose angiogenesis is UCP1-independent and suggest a hypoxia-independent mechanism of *Vegf* upregulation since this growth factor is highly expressed upon adrenergic stimulation even in the absence of BAT or beige thermogenesis. There is strong evidence from various studies that PGC1α is involved in hypoxia-independent upregulation of *Vegf* expression[145,276]. To further delineate the mechanism underlying CL-induced adipose angiogenesis, we analyzed expression levels of *Vegfr1* and *Vegfr2* in the CD31$^+$ EC fraction. We found that both receptors showed marked upregulation after CL-treatment. Blockade of VEGFR2- but not VEGFR1-mediated signaling with specific antibodies completely abolished gWAT angiogenesis after adrenergic activation. Additionally, browning of WAT, UCP1 expression and NST were strongly suppressed when blocking this receptor indicating a critical role of VEGFR2-VEGF-signaling in this process. This observation was strengthened using a genetic model with endothelial deletion of VEGFR2. These findings pointed at a paracrine regulation of AC functions by VEGFR2-activated ECs. As VEGFR2 expression is largely restricted to ECs, our findings highlight the role of the vasculature in WAT browning.

To define potential VEGF-induced EC-derived factors involved in the paracrine modulation of AC function, we performed genome-wide gene expression analysis. We found that PDGFC was among the most upregulated growth factors in the SVF of CL-treated gWAT. Additionally, *Pdgfc* was strongly induced in the SVF of gWAT upon AdVEGF transduction suggesting that this factor indeed plays a role in gWAT browning. Expression of *Pdgfc* was

restricted to $CD31^+$ ECs and ablated in VEGFR2-deficient mice indicating that VEGFR2 signaling is required for CL-induced *Pdgfc* expression in ECs. In mice deficient for PDGFC, CL treatment induced an angiogenic response in the adipose tissue; however, browning and *Ucp1* expression were strongly suppressed. This effect could be rescued by local delivery of AdPDGFC to gWAT and scWAT of these mice. A pharmacological approach using specific antibodies against both PDGFRα and PDGFRβ suppressed CL-induced browning of WAT but not angiogenesis. The suppressive effect was more pronounced when blocking PDGFRα. This observation was verified in a physiologically relevant model by treating cold-exposed mice with these specific antibodies. Interestingly, only PDGFRα blockade led to a significant reduction of NST capacity. To further delineate the mechanism underlying PDGFC-regulated WAT browning, we studied the identity of $PDGFR\alpha^+$ cells in the adipose tissue. Interestingly, PDGFRα was expressed exclusively on adipose stromal cells and expression could be increased by CL-treatment. We therefore hypothesized that this population of $CD34^+$, $Sca1^+$ and $PDGFR\alpha^+$ cells constitutes a pool of adipose progenitor cells that can differentiate into UCP1-expressing beige ACs under appropriate stimuli such as treatment with PDGFC. Taken together, we concluded that endothelial PDGFC contributes to regulating the paracrine activation of $PDGFR\alpha^+$ beige AC precursor cells via VEGF-VEGFR2 signaling.

4.4 PAPER IV: MOUSE CORNEAL LYMPHANGIOGENESIS MODEL

While hemangiogenesis in the adipose tissue is being extensively studied, knowledge about adipose lymphangiogenesis specifically in respect to WAT browning is scarce. Several *in vitro* assays are established to study LEC proliferation or migration; however, lymphatic vessel maintenance requires stabilization and remodeling of the vessels which can only be investigated *in vivo* in the context of a functional lymphatic network. Others have described a mouse cornea suture model in which lymphangiogenesis is induced by inflammation. In this model, a suture is placed in the epithelial layer of the mouse cornea resulting in an inflammatory response. However, in this setting, a number of cytokines and factors concertedly induce lymphangiogenesis and it is not possible to delineate the effect of a single, specific factor. While this model is suitable for testing the effect of potential novel drugs on inhibiting lymphangiogenesis independent of targeting a particular factor, it cannot be used to study the role of a specific cytokine in inducing lymphatic vessel growth.

In this paper, we present an *in vivo* model to study the lymphangiogenic potential of specific cytokines and other factors in the mouse cornea. This model also provides an opportunity to address the mechanisms underlying remodeling of lymph vessels as well as functional aspects of the lymphatic network. As opposed to blood vessels, lymphatic vessels are not perfused with blood and consequently remain invisible to the naked eye. To visualize lymphatics, specific markers including lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), VEGFR3 or podoplanin are commonly used. The major drawback of immunohistochemical staining of lymphatic vessels is that the markers do not distinguish between pre-existing and newly formed lymph vessels. Moreover, some markers are not suitable to use for detection of lymphatics in all tissues. The marker LYVE-1 for instance does not provide reliable results

when used in the adipose tissue. In addition to lymphatics, LYVE-1 also stains other components such as adipose macrophages. In our cornea model, we take advantage of the avascular nature of the cornea under physiological conditions. Consequently, all lymphatic vessels that are detected in this assay constitute newly formed vessels and therefore this model allows us to study lymphangiogenesis in a quantitative manner.

The procedure consists of four steps: (1) micropellet preparation, (2) corneal implantation, (3) immunohistochemistry and (4) data analysis. For this purpose, uniform slow-release micropellets are prepared containing equal amounts of factors to ensure reproducibility and robustness of this assay. The pellet is then surgically implanted in the mouse cornea by creating a micropocket on one eye of the animal. After a defined period of time, animals are euthanized and the cornea is carefully dissected. Whole mount immunohistological staining can be performed using lymphatic markers such as LYVE-1. Depending on the experimental question, double or triple staining with other specific antibodies (e.g. anti-CD31) can be performed. Confocal images can be acquired and the average lymphatic vessel area can be quantified.

In addition to studying the potential lymphangiogenic effect of a single factor or several factors in combination, this model can also be used to test potential novel drugs that might inhibit lymphangiogenesis or modulate remodeling and function of lymphatic vessels. Dysfunctional lymph vessels lead to the formation of lymphedema due to inadequate drainage. In this condition, excessive amounts of lymph accumulate in the interstitial space. In severe cases, persistent lymphedema will result in proliferation of fibrous tissue and may eventually lead to a condition termed Massive Localized Lymphedema which is often observed in morbidly obese patients[277–279]. Lymphedema also constitutes a problem in cancer treatment, particularly in breast cancer patients following partial or total mastectomy[280]. Delivery of lymphangiogenic factors might restore lymph vessel structure and function and thereby offer an attractive potential novel treatment option for patients with lymphatic dysfunction. On the other hand, inhibiting lymphangiogenesis may provide a powerful approach to prevent metastatic spreading of disseminated tumor cells from the primary tumor in cancer patients.

5 CONCLUSIONS AND FUTURE DIRECTIONS

This thesis describes the use of several mouse models to study the role of angiogenesis in the context of obesity and related metabolic disorders such as T2D. We also study the mechanism of WAT browning and the potential role of the vasculature in this process. This work describes two different approaches to induce WAT browning and BAT activation, cold exposure and pharmacological adrenergic activation using the specific Adrb3 agonist CL.

In Paper I, we describe a non-invasive, physiologically highly relevant model in which mice of different genetic backgrounds can be acclimated to cold ambient temperature. We describe various methods to study the effects of cold exposure on tissue morphology and composition as well as metabolic parameters such as whole body energy expenditure and NST-capacity. This model allows the investigator to combine cold exposure with drug treatment using small molecules, proteins or other compounds. It would be interesting to evaluate the effect of cold exposure in combination with currently used drugs for obesity or T2D therapy. As previous studies have shown that cold-induced WAT browning might be a beneficial approach for weight loss and improvement of metabolic health in healthy individuals, subpopulations with predisposition for arteriosclerosis appear to be at great risk for plaque instability due to cold-induced lipolysis following this regimen[281]. The mechanism underlying these differential effects remain to be elucidated and it would be interesting whether combining cold exposure with cholesterol-lowering drugs might abolish this effect. Furthermore, our model allows identifying and studying novel molecular players regulating WAT browning and metabolic status of an individual. These molecules might constitute potential targets for the treatment of obesity and related disorders.

Although this model provides an excellent opportunity to study adipose vasculature and metabolism in a non-invasive way, there are certain limitations and considerations that should be made to obtain reproducible, stable and scientifically meaningful results. A common disadvantage of general animal housing conditions with regard to metabolic studies is the fact that the ambient temperature of 22-23 ˚C is significantly lower than the animals' thermoneutral temperature. Thus, mice are facing a temperature challenge resulting in BAT activation and increase in NST capacity, which can lead to a misinterpretation of metabolic measurements. Additionally, ambient temperature-induced alteration of NST may mask potential true changes in metabolic rate due to genetic alterations or drug treatments[100]. Therefore, including appropriate controls maintained at thermoneutral temperature is critical when performing metabolic studies. Furthermore, when measuring NST-capacity, NE-injection itself induces a pharmacological, BAT-independent elevation of metabolism that may cause an overestimation of NST-capacity. To determine the true extent of adaptive NST, measurements obtained from the control group housed at thermoneutrality provide background values and can be used to normalize the data[100,282]. As prolonged housing at thermoneutrality markedly decreases UCP1 activity, measurements obtained from this control group cannot be attributed to adaptive NST and can be subtracted. Alternatively, UCP1-ablated animals may be used as a control[100]. Another possibility to avoid overestimation of

adaptive thermogenesis due to a pharmacological response to NE injection may be the use of specific Adrb3 agonists such as CL as the receptors targeted by this compound show significantly higher expression in adipose tissue compared to other organs. However, specific agonists only target Adrb3 and neglect the contribution of β1- and α-adrenergic receptors which may be required for a full thermogenic response[100,283,284].

There are different ways of representing results of metabolic measurements; most frequently they are given per animal or normalized per kilogram bodyweight. However, when comparing lean versus obese animals, the body weight difference is largely due to excess lipid accumulation in obese animals rather than alterations in lean body mass. Lipid droplets do not contribute to the thermogenic effect and thereby normalization based on body weight may result in incorrectly low metabolic rates of obese animals compared to their lean counterparts[100]. Consequently, it is favorable to present the results per animal. In specific cases – such as when substantial differences in muscle mass between experimental groups are observed – normalization based on lean body mass may provide more accurate estimations of the metabolic rate[100].

A challenge when measuring whole body energy expenditure is to determine whether potential alterations are due to increased BAT activation or attributable to beige adipose tissue. This question may be addressed using rodent models in which BAT depots have been surgically removed. Alternatively, beige AC-specific induction of *Ucp1* expression without affecting the expression level of this protein in classical BAT depots might help to further understand the contribution of beige adipose tissue to whole body energy expenditure[157]. To date, several studies indicate that beige ACs can compensate for the loss of brown fat cells[119,285]. Importantly, specific inhibition of scWAT browning and beige AC function leads to decreased thermogenic capacity and has detrimental consequences such as increased susceptibility to obesity, insulin resistance and further metabolic complications. These findings are rather unexpected as the total amount of UCP1 protein in beige cells is ten times lower compared to classical brown ACs[282]. Nevertheless, the results obtained from these studies highlight the importance of beige fat for whole body energy homeostasis [159].

In Paper III, we describe drug-induced WAT browning using the adrenergic agonist CL. Treatment with this compound largely mimics cold-induced effects on AC morphology, blood vessel density and metabolism. However, as opposed to cold exposure, drug-mediated adrenergic stimulation induces browning not only in scWAT but also in gWAT which represents visceral adipose tissue. Interestingly, subcutaneous and visceral adipose depots appear to have opposing effects on metabolic health. While scWAT seems to play a protective role, visceral adipose tissue mass is positively correlated with metabolic dysfunction[286]. Detailed mechanistic studies using both the cold model as well as CL-induced browning might unravel the inherent differences between these two types of WAT. Here, we identify endothelial-derived PDGFC as a paracrine signaling molecule mediating the transformation of WAT to a BAT-like phenotype. This work shows how the vasculature can directly regulate WAT browning. These findings may lead to the identification of novel

options for the treatment of obesity. The PDGFC-induced effect on WAT browning is mediated via signaling through mainly PDGFRα, but PDGFRβ is also involved. Further studies are required to define whether additional EC-derived factors play a role in this process. Moreover, it would be interesting to study a potential correlation between *Pdgfc* expression and BAT or beige adipose tissue mass in human individuals as well as in lean versus obese patients. Notably, although treatment with CL provides promising results in numerous animal models, attempts to use this drug in clinical settings have not been successful to date. This illustrates that there are considerable discrepancies between animal studies and human settings that require further investigation.

In Paper II we studied the role of ageing in relation to adipose tissue, in particular AC morphology and function as well as the effect on the adipose vasculature. Although it is well understood that both ageing and obesity constitute risk factors for the development of metabolic diseases such as T2D, the understanding of the role of the adipose vasculature is very limited. In this paper, we also describe a role of VEGF in modulating hyperglycemia and insulin sensitivity in mice. Decreased insulin sensitivity is a precondition of T2D and our findings show that it can be ameliorated by treatment with anti-VEGF agents. A number of anti-VEGF agents are currently used in clinical settings for the treatment of cancer patients[274,275]. Apart from the direct effect of VEGF inhibition on the tumor vasculature, additional mechanisms might contribute to the anti-tumor effect of this treatment. Interestingly, T2D and hyperglycemia constitute risk factors for several cancers such as colon, endometrial, kidney, pancreas, liver and breast cancer. Particularly in women, even moderately elevated blood glucose levels in non-diabetic individuals appear to increase the risk for developing cancer[287]. Cancer cells utilize glycolysis and subsequent lactate fermentation to obtain the energy required for their growth and proliferation (Warburg effect)[288,289]. It has been described that the glycolytic rate in cancer cells is up to 200 times higher compared to normal cells[288,290]. In most normal cells, pyruvate produced via cytoplasmic glycolysis is metabolized by mitochondrial oxidative phosphorylation generating 36 ATP molecules per glucose molecule. As opposed to this more energy-efficient pathway, cancer cells only carry out the first step – aerobic glycolysis – yielding merely 2 ATP molecules. However, it enables the cells to rapidly convert nutrients into biomass which is advantageous for high growth rates and has been described not only in tumors but in various highly proliferative tissues[291–293]. Given both epidemiological and metabolic data, it is reasonable to speculate that lowering blood glucose levels may offer an attractive approach to inhibit tumor growth by depletion of their primary energy source[293]. Our study demonstrates that VEGF-blockade significantly decreases blood glucose levels. These findings may offer an additional mechanism underlying the effect of VEGF-agents in cancer therapy. However, further detailed studies are required to address this question. Long-term anti-VEGF-induced hypoglycemia might have adverse systemic effects that could potentially counteract patient health and survival. As a consequence of drug-mediated hypoglycemia, breakdown of adipose tissue and muscles in an attempt to restore glycaemia may occur leading to or aggravating cancer cachexia to a potentially fatal extent[293]. Furthermore, selective starvation

of cancer cells appears challenging and inducing hypoglycemia severe enough to cause the death of cancer cells would most likely have detrimental effects on other tissues within the body. While most organs are capable of adapting to ketogenic conditions, brain cells are highly glucose-dependent and may suffer severe damage following induced hypoglycemia which can lead to seizures and coma[116,294,295]. A potential approach to resolve this problem might be the local administration of glucose to the brain[296]. Comprehensive studies are required to further investigate this possibility.

Whereas angiogenesis and the vasculature in adipose tissue are being extensively studied, only little is known regarding the role of lympatic vessels and lymphangiogenesis in this context. Particularly a potential involvement in WAT browning and modulation of whole body energy expenditure are of great interest. Lymphatic vessels as well as lymph nodes are in close spatial proximity of the adipose tissue, both subcutaneously as well as in the mesentery[297,298]. This close spatial proximity suggests that the lymphatics play a role in adipose metabolism. In fact, there is evidence for a reciprocal crosstalk between lymphatics and adipose tissue. Lymphatic vessels transport a variety of cargo such as immune cells, dietary lipids and proteins and may thereby – similar to blood vessels – play a critical role in maintaining functional adipose tissue[297,299,300]. *Vice versa*, when immune cell activation is required, increased lipolysis in the adipose tissue may provide energy to fuel the immune functions of the lymphatic system[297,298]. Animals with lymphatic deficiencies, such as mice with spontaneous heterogeneous mutations inactivating the gene encoding VEGFR3, exhibit adult-onset obesity and excessive depositions of subcutaneous adipose tissue. Additionally, disruption of lymphatic vessels induces AC differentiation-related complications resulting in hypertrophy and proliferation in the adipose tissue[301]. Although preliminary, there are indications that lymphangiogenesis is altered in the obese state[301,302]. Further evidence of the implication of lymphatic vessels has been obtained from mice with heterogeneous deletion of *Prox1*, a gene critically involved in LEC specification. Animals with only one allele of this gene exhibit disorganization and dysfunction of the lymphatic vasculature and develop adult onset obesity due to increased adipogenic differentiation[297,303,304]. Recent studies indicate that obesity predisposes individuals for lymphatic dysfunction and development of lymphedema[301,305]. Furthermore, it has been described that the function of the lymphatic system is compromised in a mouse model of high fat diet-induced obesity[301]. Further studies are warranted to elucidate this phenomenon and to unravel the mechanism underlying this observation. Our cornea lymphangiogenesis model described in Paper IV allows the investigator to study the angiogenic and lymphangiogenic potential of particular growth factors and molecules. This model could be used in the future to test the lymphangiogenic capacity of specific factors and adipokines that are differentially regulated upon cold exposure, drug-induced adrenergic activation and in obese or diabetic individuals. To study the lymphangiogenic capacity of adipocytes following adrenergic stimulation, cold-exposed or CL-treated adipose tissue could be implanted into the cornea using this model. For more detailed studies, that would allow to delineate which cells produce potentially lymphangiogenic factors, mature ACs and SVF could be separately implanted in the cornea.

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