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THE ROLE OF GROWTH FACTORS IN WHITE FAT BROWNING AND METABOLIC DISEASE

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THE ROLE OF GROWTH FACTORS IN WHITE FAT BROWNING AND METABOLIC DISEASE

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Science is the great antidote to the poison of enthusiasm and superstition.

Adam Smith

ABSTRACT

The adipose tissue is composed of a variety of cell types that constantly cross-communicate with each other to allow the tissue to operate and to adapt to various external stimuli. The diversity of these cell populations and their production of secretory factors define not only adipose tissue morphology, but also its function. This regulatory network is particularly important in times of adipose tissue remodeling and failure to adapt may result in severe malfunction of adipose tissues. Data presented in this thesis provide evidence that stromal vascular cells of the adipose tissue have important functions in adipose tissue remodeling and metabolic activation. We specifically focus on the role of various tyrosine kinase growth factor families such as VEGF, PDGF and FGF and their capacity to alter the adipose tissue microenvironment by their ability to remodel blood vessels or promote differentiation of vessel-associated cells into adipocytes.

In **paper I**, we identified a miRNA-327-FGF10-FGFR2 autocrine regulatory loop that is fundamental for white adipocyte browning. We provide the first evidence that a miRNA-dependent mechanism can control the differentiation of PDGFR-α^+ cells into thermogenic beige cells. We further demonstrated that FGF10 is not only important for white adipocyte differentiation, but additionally possesses the ability to recruit and activate beige adipocytes. Finally, systemic inhibition of miRNA-327 induced white adipocyte browning, thereby improving whole-body metabolic rates and norepinephrine-induced thermogenesis in an FGF10 dependent manner. Our data suggest a therapeutic potential of FGF10 modulating miRNAs for treatment of obesity and related metabolic diseases.

Paper II represents a detailed characterization of the function of VEGFR1 in the adipose tissue during browning and brown adipose tissue activation. Since VEGFR1 acts as a decoy receptor for VEGF, loss of VEGFR1 results in an increase of VEGF-VEGFR2 complex formation and thereby robust angiogenesis. The generation of two distinct endothelial cell specific knockout mouse strains allowed us to demonstrate that loss of VEGFR1 in endothelial cells is sufficient to induce adipose tissue angiogenesis and thereby white adipocyte browning and brown adipose tissue activation. Loss of VEGFR1 in adipocytes or myeloid cells, however, had no detectable effect on adipose tissues. Endothelial specific VEGFR1 KO mice were resistant to diet-induced obesity, resulting in improved insulin sensitivity and a reduction of ectopic lipid accumulation in the liver. Therefore, VEGFR1 blockade in endothelial cells represents an attractive approach to treat obesity, type 2 diabetes and liver steatosis.

In **paper III** we demonstrate that angiogenic endothelial cells produce PDGF-CC, which results in white adipose tissue browning. We show that such white adipose tissue browning can be prevented by: 1) inhibition of angiogenesis by VEGFR2 blockade, 2) systemic knockout of *Pdgfc*, or 3) by treatment with a PDGFR-α neutralizing antibody; all resulted in the inability of WAT to undertake a beige phenotype. We could demonstrate that endothelial cells influence adipocyte function in a paracrine fashion by secreting PDGF-CC that drives

PDGFR-α^+ preadipocytes into beige adipocyte differentiation. Hence, we conclude that an increase in PDGF-CC levels or the activation of PDGFR-α downstream signaling in preadipocytes might be a novel treatment options for obese patients.

In **paper IV** we asked the question why age is such a strong indicator for obesity and insulin resistance, and if blood vessels play a role. We produced evidence that continuous age-related changes in the adipose vasculature modulate fat mass, adipocyte function, insulin sensitivity and blood lipid profiles. In middle-aged mice, blood vessel numbers were low and VEGFR1 expression levels high, resulting in reduced vascular plasticity. Surprisingly, middle-aged mice on high-fat diet, but not standard chow, are highly sensitive to anti-VEGF treatment, which resulted in reduced body weight, improved HOMA-IR and enhanced glucose clearance. These findings indicate that low vascular plasticity in middle-aged individuals increases their risk to suffer from obesity and type-2 diabetes.

Collectively, this thesis work uncovers important players in the cross talk between the adipose tissue vasculature and adipocytes, which lays the ground for the development of novel pharmaceutical approaches.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Fettvävnader är viktiga i både lagring och användning av energi, och dessa vävnader består av olika typer av celler som kommunicerar med varandra. Sammansättningen av vävnaden, samt cellernas kommunikation spelar en avgörande roll för fettvävnadens korrekta funktion. Samspelet definierar bland annat om fettceller hellre lagrar eller bryter ner fett, och är speciellt viktig t.ex. under fastande, överdrivet kaloriintag, fysisk träning eller om kroppen är utsatt för extremt varma eller kalla temperaturer. Komplikationer som typ 2-diabetes eller kardiovaskulära sjukdomar kan förekomma under sådana förhållanden om fettvävnaden inte kan anpassa sig. Blodkärl levererar syre och näring till cellerna och är därför viktiga för kommunikation mellan celler i fettvävnaden och resten av kroppen. I vår forskning försöker vi förstå hur blodkärl kommunicerar med omgivningen i fettvävnader under speciella förhållanden, med avsikten att förändra blodkärlen för att förbättra eller förhindra fetma och diabetes.

I den första publikationen identifierade vi en signal – så kallad FGF10 – som är särskilt viktig för kommunikation i fettvävnaden när kroppen utsätts för kalla temperaturer. Genom att öka den signalen lyckades vi öka förmågan av fettvävnader att producera värme när möss utsattes för kalla temperaturer (4 °C). Intressant nog kunde vi visa att den signalen kan leda till ett ökat antal värmeproducerande fettceller även vid rumstemperatur. Dessa resultat är viktiga eftersom värmeproduktion i fettvävnaden resulterar i ökat energibehov i hela kroppen och därför förbrukas fler kalorier hos möss med ökad mängd av den signalen.

I 2:a publikationen analyserade vi en signalmottagare som finns på utsidan av cellerna och därigenom kan ta emot signaler från andra celler. Vi upptäckte att blockering eller borttagning av denna mottagare resulterade i fler antal blodkärl i fettvävnaden. Genom olika experiment såg vi hur ett ökat antal blodkärl ledde till: 1) mer värmeproducerande celler i fettvävnader vid exponering för kyla, och 2) ett motstånd mot viktökning i möss som matades med kaloririk mat. Dessa möss hade därmed minskad risk att utveckla diabetes eller fettlever. Vi drar slutsatsen att blockering av denna mottagare kan vara till nytta för patienter som lider av fetma, typ 2-diabetes eller fettleversjukdom.

I 3:e publikationen visar vi att celler som utgör själva blodkärlen kan producera och skicka ut en signal som kan förändra fettcellernas funktion. Om antalet blodkärl minskas drastiskt eller denna signal avlägsnas kan fettceller inte längre producera värme när möss utsätts för kyla. Om istället signalen är högre än normalt kan vissa celler som tätt omger blodkärlen genomgå förändring till att bli fettceller och därigenom producera värme.

I 4:e publikationen studerade vi blodkärlens roll i fettvävnaden i olika åldersgrupper och kunde hitta skillnader i antalet av blodkärl i fettvävnader och förmåga att anpassa sig till förändringar beroende på ålder. Möss i medelåldern hade färre blodkärl och en minskad förmåga att ändra antal av blodkärl. Foder med högt kaloriinnehåll gjorde däremot fettvävnaden av möss i medelåldern mera anpassningsbara. Intressant nog, så ökades möjligheten av blodkärl att anpassa sig i möss i medelåldern med fetma drastisk, vilket resulterade i lägre kroppsvikt och

minskad risk för att utveckla diabetes efter blodkärlsförändrande medicinering. Dessa resultat tyder på att minskad förmåga av blodkärl att anpassa sig till förändringar hos individer i medelåldern ökar risken för fetma och diabetes.

Sammanfattningsvis avslöjar denna avhandling viktiga delar i kommunikationen mellan blodkärl och fettceller, som i framtiden kan ligga till grund för utvecklingen av nya läkemedel för att behandla fetma och relaterade sjukdomar.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

Fettgewebe besteht aus einem Gemisch verschiedener Zellarten, die miteinander kommunizieren. Die Zusammensetzung dieser Zellen, sowie die Art und Weise, wie sie miteinander kommunizieren, ist wichtig für die korrekte Funktion des Fettgewebes. Diese Interaktion bestimmt unter anderem, ob Fettzellen Fett speichern oder abbauen und ist besonders wichtig während des Fastens, übermäßiger Kalorienzufuhr, intensiver körperlicher Aktivität und wenn der Körper extrem heißen oder kalten Temperaturen ausgesetzt ist. Wenn während dieser Situationen Probleme in der Kommunikation zwischen Zellen auftreten, kann das Fettgewebe Schwierigkeiten haben, sich anzupassen und Komplikationen wie Typ-2-Diabetes oder Herz-Kreislauf-Erkrankungen können auftreten. Blutgefäße versorgen die Zellen mit Sauerstoff und Nährstoffen und sind daher besonders wichtig für die Kommunikation zwischen Zellen im Fettgewebe untereinander und mit dem Rest des Körpers. In unserer Forschung versuchen wir zu verstehen, wie Blutgefäße mit Fettzellen kommunizieren. Wir versuchen außerdem diese Kommunikation so zu beeinflussen, dass bereits bestehende Krankheiten im Fettgewebe behoben werden können.

In der ersten Veröffentlichung identifizierten wir einen Kommunikationsfaktor, FGF10, der besonders wichtig für die Anpassung des Fettgewebes an kalte Temperaturen ist. Weiters fanden wir einen Weg, die Menge dieses Faktors zu erhöhen, was zu einer Steigerung der Wärmeproduktion im Fettgewebe führte. Nicht alle Fettzellen können Wärme produzieren und wir konnten zeigen, dass der identifizierte Kommunikationsfaktor sogar bei Raumtemperatur dazu führt, dass eine erhöhte Anzahl an wärmeproduzierenden Fettzellen im Körper gebildet wird. Dieses Ergebnis ist wichtig, da die Wärmeproduktion im Fettgewebe zu einem erhöhten Energiebedarf im gesamten Körper führt. Daher können Medikamente, die diesen Kommunikationsfaktor aktivieren, zu potentiellem Gewichtsverlust führen.

In der zweiten Veröffentlichung analysierten wir einen Faktor an der Zelloberfläche, der die Möglichkeit hat, Signale von anderen Zellen zu empfangen. Wir entdeckten, dass das Blockieren oder Entfernen dieses Faktors zu einer erhöhten Anzahl an Blutgefäßen im Fettgewebe führte. Wir setzten Mäuse, denen dieser Faktor in den Blutgefäßen fehlt, kalten Temperaturen aus oder fütterten diese mit kalorienreichem Futter. Die Neubildung von Blutgefäßen führte bei Kälteeinwirkung zu erhöhter Wärmeproduktion im Fettgewebe und zu einem Gewichtsverlust bei Mäusen, die mit kalorienreichem Futter gefüttert wurden. Diese Mäuse hatten auch ein verringertes Risiko, an Diabetes oder Fettleber zu erkranken. Wir schließen daraus, dass das Blockieren dieses Faktors bei Patienten mit Adipositas, Typ-2-Diabetes oder Fettlebererkrankungen nützlich sein kann.

In der dritten Veröffentlichung zeigen wir, dass Blutgefäßzellen einen Kommunikationsfaktor erzeugen und absondern können, der die Funktion von Fettzellen verändern kann. Wenn die Anzahl der Blutgefäße drastisch abnimmt oder dieser Faktor entfernt wird, können Fettzellen keine Wärme mehr erzeugen und deren Körpertemperatur nimmt ab. Wenn erhöhte Mengen dieses Faktors vorhanden sind, können sich Blutgefäß-assoziierte Zellen von diesen lösen und

sich zu Fettzellen umwandeln. Dadurch wird mehr Energie in Form von Wärme vom Körper abgegeben, was zur Gewichtsreduktion führt.

In der vierten Veröffentlichung definieren wir die Rolle von Blutgefäßen im Fettgewebe in verschiedenen Altersgruppen. Es konnten altersabhängige Unterschiede in der Anzahl und Anpassungsfähigkeit von Blutgefäßen festgestellt werden. Mäuse mittleren Alters hatten weniger Blutgefäße und eine verringerte Fähigkeit, die Anzahl der Blutgefäße zu verändern. Überraschenderweise machte hochkalorische Nahrung die Blutgefäße von Mäusen mittleren Alters anpassungsfähiger. Dadurch konnte durch medizinische Behandlung die Anzahl von Blutgefäßen in Mäusen mit Adipositas drastisch verringert werden, was in dieser speziellen Gruppe zu verringertem Körpergewicht und geringerer Wahrscheinlichkeit der Entwicklung von Diabetes führte. Diese Ergebnisse zeigen, dass eine verminderte Fähigkeit von Blutgefäßen, sich an Veränderungen anzupassen, das Risiko erhöht an Fettleibigkeit und Typ-2-Diabetes zu erkranken.

Zusammenfassend beschreibt diese Arbeit wichtige Akteure in der Kommunikation zwischen Blutgefäßen und Fettzellen, die die Basis für die Entwicklung neuer Medikamente zur Behandlung von Fettleibigkeit und damit verbundenen Stoffwechselerkrankungen bilden könnten.

LIST OF SCIENTIFIC PAPERS

I. **Carina Fischer**, Takahiro Seki, Sharon Lim, Masaki Nakamura, Patrik Andersson, Yunlong Yang, Jennifer Honek, Yangang Wang, Yanyan Gao, Fang Chen, Nilesh J. Samani, Jun Zhang, Masato Miyake, Seiichi Oyadomari, Akihiro Yasue, Xuri Li, Yun Zhang, Yizhi Liu and Yihai Cao. A miR-327-FGF10-FGFR2-mediated autocrine signaling mechanism controls white fat browning. Nature Communications. 2017 Vol. 8: 2079.

II. Takahiro Seki, Kayoko Hosaka, **Carina Fischer**, Sharon Lim, Patrik Andersson, Mitsuhiko Abe, Hideki Iwamoto, Yanyan Gao, Xinsheng Wang, Guo-Hue Fong and Yihai Cao. Ablation of endothelial VEGFR1 improves metabolic dysfunction by inducing adipose tissue browning. The Journal of Experimental Medicine. 2018 Vol. 215: 611.

III. Takahiro Seki, Kayoko Hosaka, Sharon Lim, **Carina Fischer**, Jennifer Honek, Yunlong Yang, Patrik Andersson, Masaki Nakamura, Erik Näslund, Seppo Ylä-Herttuala, Meili Sun, Hideki Iwamoto, Xuri Li, Yizhi Liu, Nilesh J. Samani and Yihai Cao. Endothelial PDGF-CC regulates angiogenesis-dependent thermogenesis in beige fat. Nature Communications. 2016 Vol. 7: 12152.

IV. Jennifer Honek, Takahiro Seki, Hideki Iwamoto, **Carina Fischer**, Jingrong Li, Sharon Lim, Nilesh J. 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 Sciences of the United States of America. 2014 Vol. 111: 14906.

LIST OF RELATED PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. J. Honek*, S. Lim*, **C. Fischer***, H. Iwamoto, T. Seki & Y. Cao. Brown adipose tissue, thermogenesis, angiogenesis: pathophysiological aspects. Horm Mol Biol Clin Investig 2014 Vol. 19: 5.
*Equal contribution

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III. K. Hosaka, Y. Yang, T. Seki, **C. Fischer**, O. Dubey, E. Fredlund, J. Hartman, P. Religa, H. Morikawa, Y. Ishii, M. Sasahara, O. Larsson, G. Cossu, R. Cao, S. Lim & Y. Cao. Pericyte-fibroblast transition promotes tumor growth and metastasis. Proc Natl Acad Sci U S A 2016 Vol. 113: E5618.

IV. Y. Yang, Y. Zhang, H. Iwamoto, K. Hosaka, T. Seki, P. Andersson, S. Lim, **C. Fischer**, M. Nakamura, M. Abe, R. Cao, P. V. Skov, F. Chen, X. Chen, Y. Lu, G. Nie & Y. Cao. Discontinuation of anti-VEGF cancer therapy promotes metastasis through a liver revascularization mechanism. Nat Commun 2016 Vol. 7: 12680.

V. P. Andersson, Y. Yang, K. Hosaka, Y. Zhang, **C. Fischer**, H. Braun, S. Liu, G. Yu, S. Liu, R. Beyaert, M. Chang, Q. Li & Y. Cao. Molecular mechanisms of IL-33-mediated stromal interactions in cancer metastasis. JCI Insight 2018 Vol. 3: e122375

CONTENTS

LIST OF ABBREVIATIONS

AC	Adipocyte
AdipoR	Adiponectin receptor
A-FABP	Adipocyte-Fatty acid binding protein
APC	Adipocyte progenitor cells
AR	Adrenergic receptor
AT	Adipose tissue
ATP III	Adult Treatment Panel III
BA	Brown adipocyte
BAT	Brown adipose tissue
BMI	Body mass index
BMP	Bone morphogenetic protein
C/EBP	Ccaat-enhancer-binding protein
CD	Cluster of differentiation
CRP	C-reactive protein
CT	Computer tomography
CVD	Cardiovascular disease
DGCR8	DiGeorge syndrome critical region gene 8
DIO	Diet induced obesity
FDA	Food and drug administration
FDG-PET	Fluorodeoxyglucose-positron emission tomography
FGF	Fibroblast growth factor
FOXO	Forkhead box protein O
GDP	Gross domestic product
HDL	High-density lipoprotein
HFD	High fat diet
Hh	Hedgehog
MEF	Mouse embryonic fibroblast
miR	MicroRNA
MSC	Mesenchymal stem cell
Myf	Myogenic factor

NCEP	National Cholesterol Education Program
NE	Norepinephrine
NG	Neuro-glial antigen
NRP	Neuropilin
OV	Parapoxvirus Orf virus
PDGF	Platelet+derived growth factor
PGC	Peroxisome proliferator-activated receptor gamma coactivator
PlGF	Placental growth factor
PPAR	Peroxisome proliferator-activated receptor
PRDM16	PR domain containing 16
RISC	RNA-induced silencing complex
RTK	Receptor tyrosine-kinase
Sca	Stem cell antigen
SMA	Smooth muscle actin
T2DM	Type 2 diabetes mellitus
TG	Triglyceride
TGF	Transforming growth factor
UCP1	Uncoupling protein 1
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WA	White adipocyte
WHO	World Health organisation
Wnt	Wingless/integrated

1 INTRODUCTION

1.1 OVERWEIGHT AND OBESITY WORLDWIDE

Overweight and obesity is considered a 21st century epidemic with more than 2.1 billion people suffering worldwide[1]. According to the World Health Organization (WHO), overweight is defined as having a body mass index (BMI) greater than or equal to 25; whereas obesity is reached when the BMI is greater than or equal to 30[2]. The trends of increasing body weights are interestingly also reflected in the population suffering from undernourishment - defined as insufficient energy intake resulting in a BMI less than 18.5. There has been a steady decline in undernourishment and a steep increase in obesity between 1990 and 2015, as shown in Fig. 1[3-6].

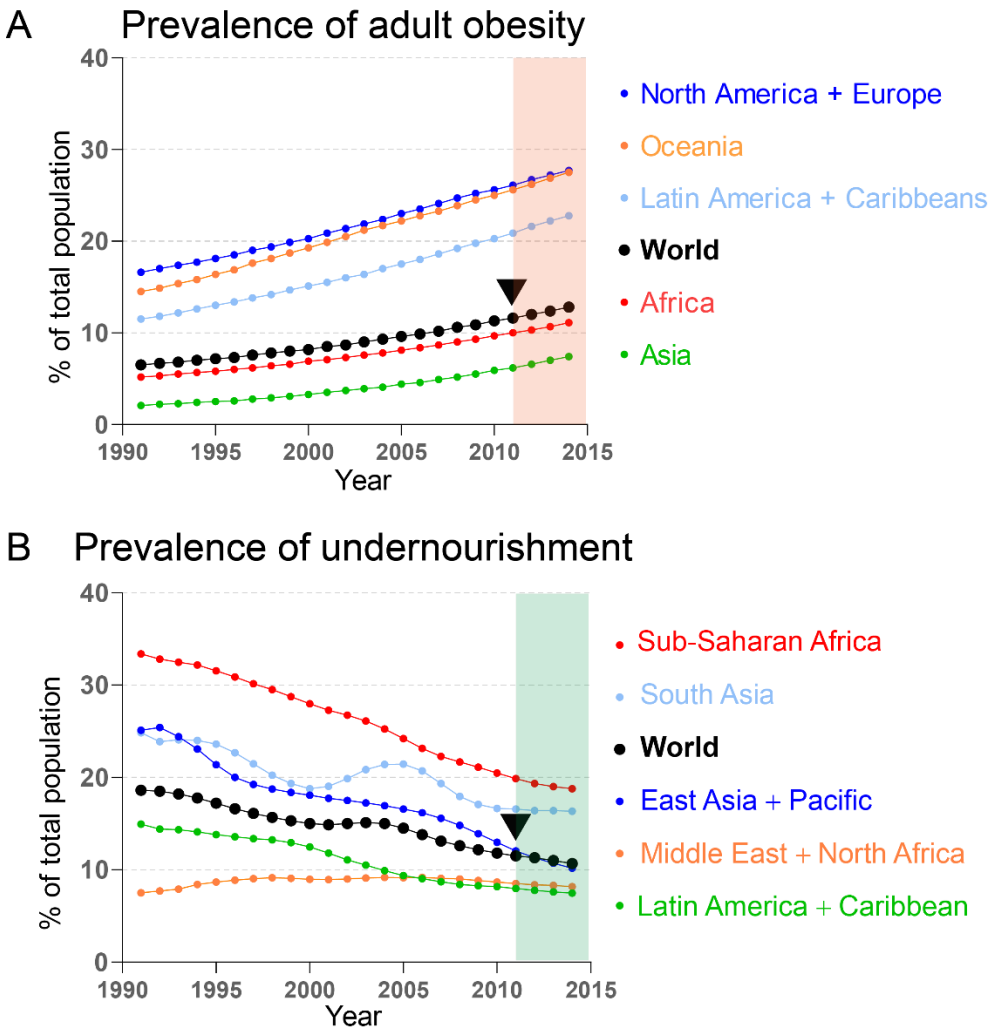

*Figure 1: **Obesity and undernourishment worldwide**. A) Prevalence of obesity by region, measured as percentage of adults aged 18 years and old with BMI greater than 30 kg/m². B) Share of population with caloric intake insufficient to meet the minimum energy requirements defined as necessary for a given population. The black triangle marks the turning point were more obese compared to undernourished people were observed for the first time. Data acquired from Our World in Data. Source: UN Food and Agricultural Organization/WHO. License: CC BY-SA3-6.*

Obesity is associated with a great impact on the society. In fact, McKinsey company, using the WHO disease database, ranked obesity as one of the top three global social burdens with a global economic impact of 1.72 trillion € in 2012. This corresponds to 2.8% of the global gross domestic product (GDP) and does not take into account the large costs of treating associated diseases such as diabetes, cardiovascular disease and depression[2]. Additionally, recent predictions project that around 20% of the world's population will be obese by 2025, which would double the economic impact[7]. Combined efforts are clearly needed to globally tackle malnutrition and reduce the burden for the individual as well as the society.

This thesis focuses on the treatment of overweight, obesity and related diseases. Our goal is to generate knowledge that will help to halt, or even reverse weight gain globally and thereby increase quality of life for a big fraction of the world's population.

1.1.1 Obesity prevention

Although many efforts have been made to slow down or even halt the obesity epidemics, the proportion of adults defined as obese has reached a devastating level worldwide, affecting people in both wealthy and poor countries (Fig. 2).

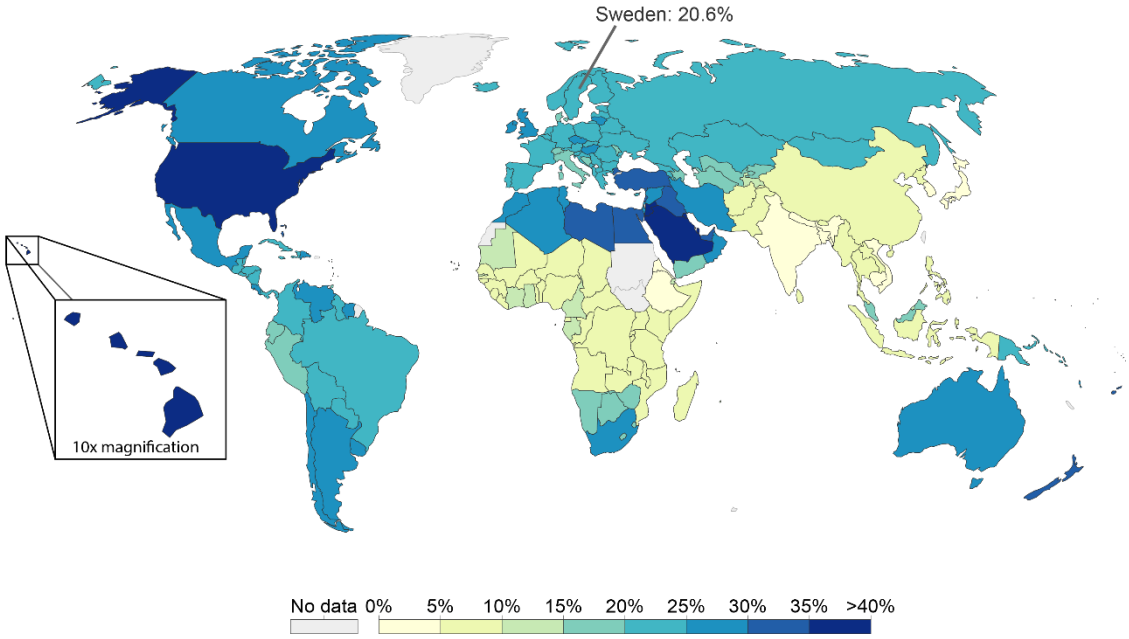

Figure 2: ***Share of adults defined as obese, 2016.*** *Percentage of defined population with BMI of 30 kg/m^2 or higher. Exact percentage in Sweden highlighted in black. Source: WHO, Global Health Observatory. License: CC BY-SA*[3-6]*.*

The fundamental cause of overweight and obesity is the imbalance between caloric intake and caloric expenditure. This effect can be attributed to an increased intake of energy-rich foods that contain high fat and high sugar, along with a decrease in physical activity, both at the workplace and due to improved infrastructures.

Being overweight or obese can have a serious impact on one's health. Common health consequences of an increased BMI are an enhanced risk of chronic diseases such as cardiovascular diseases, diabetes, sleeping disorders, musculoskeletal disorders and some cancers. Many obese patients suffer from mental health problems, such as low self-esteem and depression, in addition to the severe physical burden (reviewed in[8]).

Several daily factors are influencing how we consume and what we eat. Advertisement and preferential food placement of energy-rich food in stores have been shown to be linked to childhood obesity and food preferences in young people[9]. To counterbalance this, strategies to reduce the incidence of obesity have been developed, including: 1) regular monitoring of caloric intake, body weight and activity levels, 2) dietary education and control such as healthy diets in schools, 3) physical activity programs, and 4) higher taxes on unhealthy foods. However, despite these efforts, obesity incidence is still on the rise.

Why do overweight and obesity levels still rise? One major problem is that many countries do not classify obesity as a disease. Therefore, people suffering from obesity do not get the necessary support from society and most importantly, through the health care system. It is true that based on the first law of thermodynamics, surplus energy uptake and insufficient energy expenditure over time leads to excessive accumulation of energy in the form of fat in the body and thereby to obesity. However, this view is too simplistic. We now know that many factors play determining roles in the development of obesity, such as genetic predisposition, environmental factors, fat distribution (waste-to-hip ratio), overall diet quality, as well as neuronal and endocrine communication through a variety of neuropeptides, hormones and growth factors that collectively are essential to control food intake and energy expenditure[1].

1.1.2 Metabolic syndrome

The "metabolic syndrome" is defined as a cluster of conditions that result from excess adiposity and overnutrition. According to the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III), patients are classified to have metabolic syndrome if at least three of the following conditions are present: hyperglycemia/insulin resistance, hypertension, atherogenic dyslipidemia, visceral obesity, reduced levels of low-density lipoprotein (HDL) or increased levels of triglycerides (TGs) in the blood[10,11]. The concept to define a metabolic syndrome is used to easily identify patients at higher risk of type 2 diabetes mellitus (T2DM) or cardiovascular disease (CVD), and thereby motivate patients and their physicians to take appropriate steps to reverse obesity (reviewed in[10]).

1.1.3 The role of aging in obesity and metabolic disease

Aging refers to the deterioration of homeostatic processes in the body over time, leading to functional decline and increased risk of disease and death. Aging is a universal contributor to

metabolic decline and related diseases such as T2DM, CVD and stroke[12]. Both, ectopic fat deposition and insulin resistance are closely linked to the aging process, and visceral fat depletion or caloric restriction have been demonstrated to improve insulin sensitivity and increase life span[13]. There are several biochemical pathways activated by caloric restriction, many of which alter the metabolic state of cells and activate signaling pathways important for glucose sensing and aging. Longevity genes are another group of genes linked to aging and metabolic alterations. Disruption of longevity genes such as sirtuins, p66Shc and mTOR, has been linked to the development of metabolic syndrome, suggesting that the loss of homeostasis in these signaling pathways is sufficient to cause metabolic alterations even in younger individuals (reviewed in[14]).

One of the most important determinants of cardiovascular health is a person's age. Beside pathological chances of the heart itself, impaired endothelial function can be observed in the aged population. Age-related pathological changes in the vasculature include atherosclerotic plaques, ischemic tissue and arterial stiffening caused by fibrosis, hypoxia and senescence of endothelial cells. Both, increased and decreased vascular density, might be detected in aging individuals. In patients with insufficient vascular growth or perfusion, hypertension and ischemic tissues are mature complications. Age-related excessive growth and remodeling, on the other hand, is commonly observed in the vasculature of the eye, resulting in macular degeneration (reviewed in[15]). The important angiogenic factor, vascular endothelial growth factor A (VEGF), together with Ang1, is particularly important in blood vessel survival and plasticity in adults[16] and anti-VEGF is commonly used for the treatment of age-related macular degeneration[17]. Obesity and cardiovascular health are strongly linked, the effect of VEGF and VEGF-related signaling on age-related complications of the adipose tissue (AT), however, has not been studied yet in detail. It would be of great interest to determine how VEGF levels change during aging and how VEGF signaling can influence vascular function, metabolic activity and insulin sensitivity in aging ATs.

1.1.4 Clinical treatment options for obesity and related diseases

Lifestyle interventions, dietary changes and exercise are the first-line treatment choice for obesity[18]. These measures often lead to a reduction of body weight. For lifestyle interventions, e.g., a meaningful weight loss can be achieved, but maintaining weight loss over time has proven itself difficult[19]. Medical or surgical interventions are clinically approved in addition to the previous measures and are reserved for patients with moderate- to high-risk obesity that show comorbidities or have failed to reduce their weight by first-line interventions[20]. Drugs that are food and drug administration (FDA) approved for the treatment of obesity either inhibit lipid uptake in the intestine (e.g. Orlistat) or reduce appetite and food intake (e.g. Lorcaserin and Liraglutide). These drugs result in an average weight loss of less than 10%[1]. Bariatric surgery, on the other hand, a surgical procedure with the purpose to restrict the amount of food the stomach can hold, is much more effective, with an average weight loss of 23% after a 2 year period[21]. Surgery also positively affects a number of comorbidities such as T2DM and

sleep apnea and patients report a consistent improvement in quality of life[1]. Most of the surgical procedures are however permanent and possible long-term consequences such as fat malabsorption, protein-energy malnutrition and micronutrient deficiencies can occur, resulting in potential life-long requirement of oral supplementations[22]. While surgery is still the best option for patients with obesity, there is a strong need to develop novel, non-invasive, and more efficient drugs. Understanding the AT and pathological changes during obesity are essential to reach this goal. One promising novel approach is the conversion and activation of white adipocytes (WAs) into metabolically more active beige adipocytes (ACs) together with the activation of preexisting brown adipocytes (BAs). The following chapter will explain the different types of ACs and their function in energy storage, heat production and cell-cell communication.

1.2 ADIPOSE TISSUE

AT is a type of loose connective tissue that contains ACs; cells specialized in storing energy in form of triglycerides. In mammals, AT is mainly located subcutaneously, adjacent to organs and in the bone marrow[23]. For many years, it was believed that AT was solely important for insulation, mechanical support, and energy storage in times of surplus and energy source in times of starvation. However, since the discovery in 1994 that leptin is exclusively produced and secreted by ACs[24], it is recognized and classified as an endocrine organ. A great number of AT-derived secretory factors have since been identified, including cytokines, metabolites, hormones and growth factors[25]. With recent knowledge, it has become clear that ATs are critically involved in the regulation of energy homeostasis, the immune system, wound healing, reproduction and blood vessel biology[26].

1.2.1 Energy storage and heat production

There are two types of ACs, white and brown ACs. White ACs have leptin as their characteristic protein. Their main function is energy storage, which is accomplished by a centrally contained single lipid droplet. Histologically, their nucleus and cytoplasmic content is pushed towards the cell wall; therefore, their appearance is white under the light microscope. Brown ACs, on the contrary, have multiple smaller lipid droplets, abundant mitochondria and uncoupling protein 1 (UCP1) as their characteristic protein[27,28]. Their high mitochondrial content let them appear brown under the light microscope and their main function is to use energy to produce heat by a process called thermogenesis[29]. Brown AT (BAT) exists at many sites in most new-born mammals, but can even be found at later age, especially in hibernating animals[30] and rodents[31]. With the help of fluorodeoxyglucose-positron emission tomography / computer tomography (FDG-PET/CT), active and functional BAT was recently detected and characterized in adult humans[32]. First clinical studies demonstrated that activation of thermogenesis in humans increases resting metabolic rates by around 200 kcal/day and significantly improves insulin sensitivity[33]. Since then, BAT is recognized as powerful novel

therapeutic target for the treatment of a variety of metabolic diseases[34]. In addition to BAT, a subset of WA has the ability to increase their mitochondrial content and act as thermogenic cells. These cells are commonly known as beige ACs and evolve within white adipose depots[35]. Due to the limited amount of BA in adult humans, additional WA activation might be necessary to achieve desired clinical outcomes[36]. A more detailed characterization of beige and brown AT development and activation is however essential to overcome current therapeutic challenges such as the identification of the most beneficial method of BAT activation for the treatment of obesity and T2DM as well as the reduction of cardiovascular side effects[33].

1.2.2 White fat browning

Browning is the process in which beige ACs are generated in WAT following stimuli such as cold temperature, adrenergic activation or exercise[37]. These beige ACs can be generated by both *de novo* differentiation of preadipocytes[38] and transdifferentiation of preexisting WAs and are characterized by a distinct morphology with a more centralized nucleus, multilocular lipid droplets as well as an increase in mitochondrial number and activity (reviewed in[37]). An essential function of beige ACs is the ability to, upon adrenergic stimulation, activate UCP1 expression. UCP1 is located in the inner membrane of mitochondria and is essential for the generation of heat by a process called non-shivering thermogenesis. This process leads to a reduction of ATP production in the mitochondria and thereby to increased energy requirements in order to maintain the cell's energy needs[39].

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) - probably the most important regulatory protein in thermogenesis - can activate the expression of UCP1 and key mitochondrial enzymes when ectopically introduced into WAs[40]. Physiologically, PGC-1α is activated upon adrenergic stimulation when norepinephrine (NE) binds to the β3-adrenergic receptor (β3-AR) on ACs. A large number of browning factors, which are factors that enhance beige AC number, have been identified. Some examples are: 1) myokines that are activated downstream of PGC-1α upon exercise (e.g. irisin and β-aminoisobutyric acid); 2) factors that regulate UCP1 synthesis (e.g. thyroid hormones); 3) proteins involved in adipogenesis (e.g. bone morphogenic proteins (BMPs) and diverse growth factors); 4) molecules that act in the central nervous system (e.g. leptin and melatonin) or; 5) other factors involved in one of the many steps required for beige AC differentiation and activation (reviewed in[41]).

If any of these factors will be powerful and specific enough to treat metabolic disorders in humans, however, remains unclear.

1.2.3 Adipogenesis and adipose tissue maintenance

ACs are derived from mesenchymal stem cells (MSCs) that first commit to precursor cells, then preadipocytes and lastly to terminally differentiated mature ACs in a process called adipogenesis[42].

MSCs are multipotent stromal cells that have the ability to become osteoblast, chondrocytes, myocytes and ACs[43,44]. All white precursor cells are myogenic factor 5 negative ($Myf5^-$) MSC. Dr. Cannon's research group could however demonstrate that brown AC precursors harbour myogenic gene expression patterns like Myf5 and can commit to both the fate of myoblasts and brown preadipocytes[45], suggesting distinct origins for white compared to brown ACs. It was further demonstrated that the expression level of ccaat-enhancer-binding protein (C/EBP-β) and PR domain containing 16 (PRDM16) determines if $Myf5^+$ cells commit to brown preadipocytes or myoblasts[27].

For both, white and brown ACs, further commitment to preadipocytes seems to be regulated by transforming growth factor-β (TGF-β) family proteins, such as BMP7 in brown progenitors[46] or BMP2[47] and BMP4[48] in white progenitors. A complex interplay of transcription factors and signalling pathways are typically in place to allow preadipocytes to further differentiate into mature ACs[42]. Studies in preadipocyte cell lines demonstrated that hormonal induction of AC differentiation was followed by expression of the early differentiation factors C/EBPβ and C/EBPδ which induce the transcription of C/EBPα and peroxisome proliferator-activated receptor gamma (PPARγ)[49]. The latter two factors transform the cells into mature ACs and stay active for the life of the AC[50]. Further studies showed that PPARγ alone can initiate the adipogenic program and retroviral overexpression results in cells that possess many features of mature ACs[51,52]. Additionally, PPARγ could induce adipogenesis in C/EBPα deficient mouse embryonic fibroblasts (MEFs). Therefore PPARγ can be seen as the proximal effector in AC differentiation[50]. In addition to these transcription factors, a variety of signalling pathways such as insulin-like growth factor, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), wingless/integrated (Wnt) - β-catenin and hedgehog (Hh) have been identified to be crucial to control preadipocytes commitment to mature ACs[42,53].

Whether or not ACs of different depots need distinct signals to differentiate, and how many steps MSCs have to undergo to differentiate into mature ACs, is not yet clear. The reason being a limited number of cell-specific markers and a lack of unique morphological characteristics in progenitor cells and preadipocytes[53].

1.2.4 Plasticity in the adipose tissue

In obese individuals, body fat mass can reach up to 40% of whole-body composition[54]. Adipocyte size, however, doesn't increase linearly with obesity level but rather shows large inter-individual variations[55]. The cellular and molecular mechanisms controlling AC size and

AT growth in different individuals are largely unknown, it is however well understood that AC hypertrophy is associated with metabolic complications, independent of body composition and fat distribution[56]. An elegant approach developed by Peter Arne's research group revealed that the turnover rate of ACs in adult humans is about 10% per year[55]. In mice, this number is estimated to be around 10-15% per month, which reflects a slightly higher turnover rate in mice compared to humans considering the shorter lifespan of mice[57]. In addition, turnover rates are sensitive to pharmacological, physiological and dietary stimuli[58]. Inducible genetic depletion of all mature AC in mice demonstrated further, how plastic adult AT is. Depleted ACs could completely regenerate within weeks[59]. This enormous plasticity generates a promising niche for clinical interventions and gives hope to people suffering from metabolic diseases.

1.3 ADIPOSE TISSUE VASCULATURE

Vascularization defines the growth and function of all tissues and organs. The delivery of oxygen and nutrients as well as the removal of waste products are strongly dependent on blood vessel number and organisation. Hormones and growth factors delivered through the blood stream effect AT function and AC activation[60]. The interplay between AT vasculature and ACs is specifically interesting because AT is very plastic throughout adult life and this plasticity is strongly dependent on its vasculature[61-64]. The increase in volume of pre-existing AC (hypertrophy) and the formation of new small ACs (hyperplasia), for example, are tightly linked to blood vessel structure and angiogenesis[65]. Vascularisation develops in concert, or even guide adipogenesis[66]. The importance of blood vessels in AT can be further reflected by the fact that every AC is in direct contact with a blood vessel at all times. Consequently, AT represents one of the most vascularized organs of the human body[67]. Furthermore, there is a strong correlation between vessel density and metabolic activity in ACs. Increasing blood vessel number in AT alone is sufficient to increase AC metabolism and leads to a brown-like phenotype in WAT. Blocking angiogenesis, on the other hand, will block AC browning[64].

It has been postulated for decades that adipocyte progenitor cells (APCs) are located in the proximity of blood vessels[68], however only recent lineage tracing approaches could verify that. Perivascular smooth muscle cells[69], fibroblasts[38] and pericytes[70] are able to differentiate into ACs *in vivo.* Especially cells derived from the stromal vascular fraction (SVF) with high expression levels of cluster of differentiation 34 (CD34), PDGFR-α and stem cell antigen 1 (Sca1) display a high potency to differentiate into ACs. Additional markers, such as CD24, neuron-glial antigen 2 (NG2), smooth muscle actin (SMA) and PDGFR-β can be expressed on APCs and might help to understand tissue specific differences in preadipocyte populations *(reviewed in*[71]*).*

Through such experiments, it is clear that blood vessel number and organisation play a crucial role in adipogenesis and AT remodelling. Understanding the crosstalk between ACs and AT vasculature is crucial as large AC size, defective blood vessels and low metabolic activity in

the AT correlate with dysfunction and metabolic disease. Therefore, a healthy vascular network in AT is crucial for metabolic health[54].

1.3.1 Adipocyte-vascular crosstalk

The AT SVF cell population includes endothelial cells, fibroblasts, B- and T-lymphocytes, macrophages, myeloid cells, pericytes, smooth muscle cells, and adipose stromal/stem cells [72]. Different cell types in the AT communicate with each other via cell-cell contact or by secreting factors such as cytokines[73], hormones and growth factors[74]. Several proteomic profiling approaches have recently been performed to define the complete secretome of the AT and around 600 secretory factors have been identified so far[75]. Adipose-derived secretory factors modulate adipogenesis[76], immune cell recruitment and activation[77], as well as AC metabolism and function[78]. Crucial adipokines secreted by ACs, such as leptin and adiponectin, are widely studied and their functions and expression patterns have been characterized in detail[79]. Interestingly, however, ACs only make up less than 50% of cells in the AT[80]. Moreover, WAT expansion and remodelling leads to changes in progenitor cell population and cell composition in the AT[81]. Only a few studies have addressed the question how SVF cells respond to alterations in AC metabolism and tried to explain the crosstalk between different cell types in the AT.

The following chapters describes the interplay between ACs and vascular cells in the AT. A special focus will be directed to vessel modulating adipokines and important growth factor families such as VEGF, FGF and PDGF families.

1.3.2 The impact of adipokines on the vasculature

Adipokines are defined as biologically active molecules that are secreted by the AT and involved in various processes within the AT as well as systemically[82].

One of the most studied adipokines is adiponectin - a protein hormone critically involved in glucose and lipid homeostasis – with important additional effects being exerted directly on the vasculature. It acts through binding to its cell surface receptors; Adiponectin receptor 1 (AdipoR1) (skeletal muscle), AdipoR2 (liver), and T-cadherin (cardiovascular system)[83]. Circulating in the bloodstream, adiponectin protects the endothelium and has strong anti-inflammatory properties[84]. It can further promote endothelial progenitor cell migration upon vascular damage, reduce the production of reactive oxygen species, and thereby diminish oxidative stress[85]. In contrast to the majority of adipokines, adiponectin levels are inversely correlated to body weight[86]. Furthermore, high adiponectin levels are linked to lower risk of hypertension[87]. Therefore, adiponectin can be considered as one of the beneficial adipokines.

Adding to the list of adipokines with beneficial effects are; omentin - an adipokine that induces insulin-mediated signalling, and whose expression is negatively correlated to BMI and

coronary atherosclerosis[88], and apelin - which has a positive effect on the aortic wall, causing its relaxation[89].

In contrast, there are also several adipokines that are harmful for the vasculature. Leptin, for example, represents a strong risk factor for atherosclerosis[90] and thrombosis[91] and has pro-inflammatory properties by increasing C-reactive protein (CRP) transcription[92]. The primary function of leptin is to regulate food intake and energy expenditure. In obese patients, a resistance to leptin is gradually acquired, resulting in an inability to feel sated. Adipocyte fatty acid binding protein (A-FABP) is a pro-inflammatory cytokine. It is upregulated in obese individuals and strongly linked to vascular dysfunction. High A-FABP levels lead to endothelial dysfunction, atherosclerosis, hypertension, and coronary heart disease[93]. Other adipokines related to cardiovascular disease include resistin, a pro-thrombotic adipokine[94] and chimerin, which has been linked to early atherosclerotic plaque formation[95].

1.4 ADIPOSE TISSUE GROWTH FACTORS

1.4.1 VEGF signaling and angiogenesis in adipose tissues

The VEGF growth factor family consists of VEGF, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) and the corresponding tyrosine-kinase receptors VEGFR1, VEGFR2 and VEGFR3. In some cases, neuropilins (NRPs) can act as co-receptors by interacting with heparin binding VEGF isoforms and PlGF. VEGF, VEGF-B and PlGF are further alternatively spliced and exist in soluble and heparin binding forms. They bind the receptors as homodimeric polypeptides, although naturally occurring heterodimers of VEGF and PlGF have been described[96].

Signals are transmitted when VEGF ligands bind to their corresponding receptors, resulting in receptor dimerization and conformational changes in the receptor monomers[97]. The ATP binding site in the intracellular kinase domain gets exposed, followed by binding of ATP and auto- or transphosphorylation of the tyrosine residues on the receptor itself or downstream signal transducers[98]. The signal transduction is tightly controlled by dephosphorylation or internalization and degradation of the receptors[99].

Activation of different VEGFR by specific ligands leads to distinct outcomes. When VEGFR1 interacts with VEGF-B or PlGF, it has an important role in tissue homeostasis and regeneration[100]. VEGF has around 10-times higher binding affinity for VEGFR1 compared to VEGFR2. Due to the weak tyrosine-kinase activity of VEGFR1, angiogenesis is inhibited when VEGF binds to VEGFR1 instead of VEGFR2. Therefore VEGFR1 is sometimes referred to as "decoy" receptor for VEGF[101]. The interaction of VEGF with VEGFR2 however, results in robust vasculogenesis during embryonic development and angiogenesis in adults[102]. VEGFR3 is the main growth factor receptor controlling lymphangiogenesis by interaction with VEGF-C and VEGF-D[103,104].

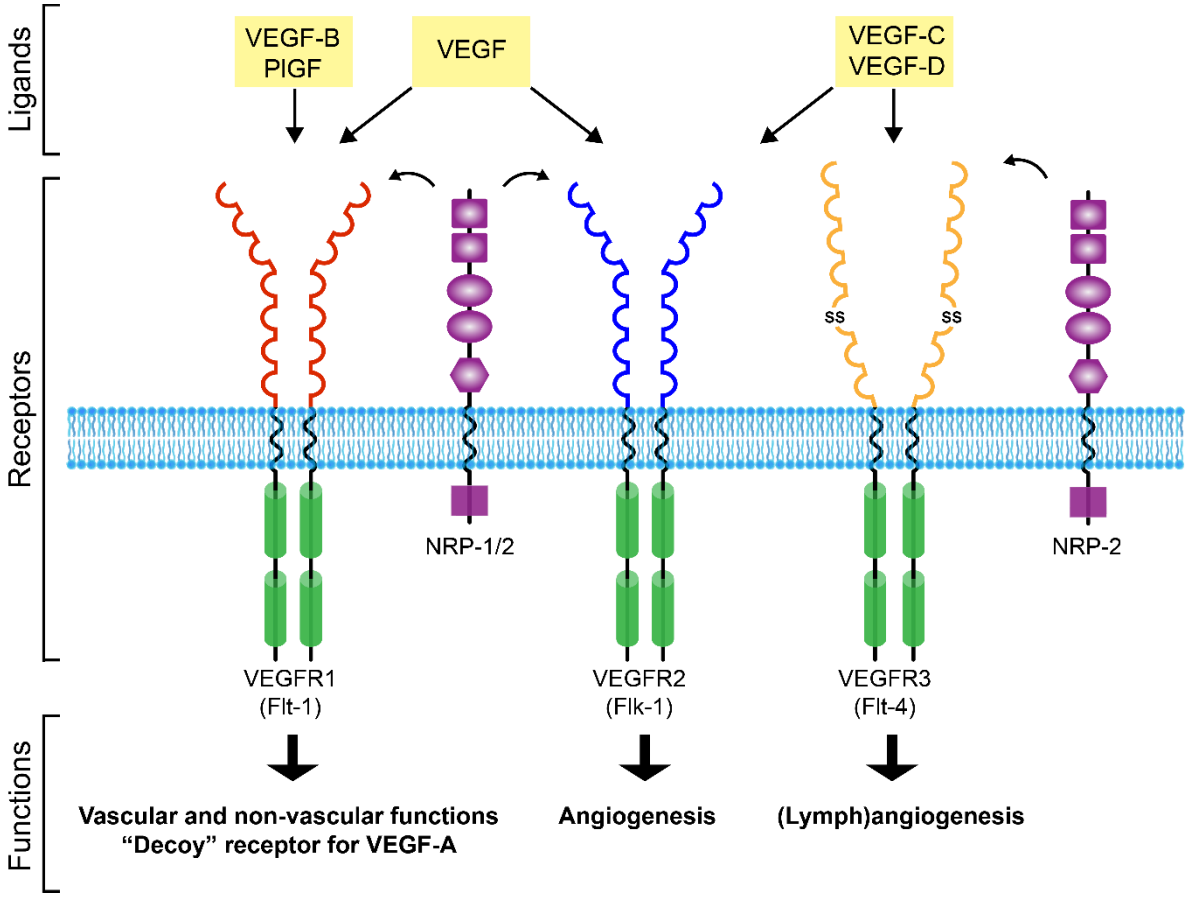

*Figure 3: **VEGF signaling.** Representation of the interaction between VEGF ligands and their receptors and possible functional outcomes.*

In the AT, VEGF-A mainly originates from ACs and adipose specific depletion of VEGF results in reduced vascular density, hypoxia, apoptosis, inflammation and metabolic defects in mice fed with high fat diet (HFD)[105]. Overexpression, on the other hand, results in increased adipose vasculature, reduced hypoxia and reversed metabolic defects in mice fed on HFD[105]. A doxycycline inducible mouse model further demonstrated that adipose specific overexpression of VEGF results in rapid browning and increases fat graft survival and activation[106].

As mentioned previously, VEGFR1 acts as a counter player to VEGFR2. Overexpression of VEGF-B resulted in increased VEGF /VEGFR2 signalling and angiogenesis in ATs and depletion of VEGFR1 further promoted this effect, resulting in increased AT function in obese mice[107]. This suggests VEGF-B/VEGFR1 as a therapeutic target to increase vascular density and AC metabolism in order to counteract obesity and diabetes.

Lymphatic vessels have an essential role in lipid transport[108] and immune cell trafficking[109]. How these vessels influence adipose metabolism, however, is not fully understood[110]. VEGF-C and VEGF-D levels have been correlated to obesity and unfavourable lipid parameters.

Therefore, Michael Detmar's research group utilized a soluble VEGFR3 (acting as a ligand scavenger) overexpressing mouse model to demonstrate that decreased VEGFR3 signalling and resulting decreased lymphatic vessel numbers protect against obesity-induced insulin resistance and hepatic lipid accumulation[111]. To further evaluate their findings, VEGF-C overexpressing mice were generated. Increased levels of VEGF-C resulted in weight gain, insulin resistance and ectopic lipid accumulation[112]. In both cases the effect could be linked to macrophage polarization and number[111,112]. Therefore, it can be concluded that VEGFR3 mediated lymphangiogenic signalling is unfavourable in obese patients and attenuation of it could be a promising strategy to improve diabetes and metabolic syndrome. VEGFR3 signalling was described to provide a holistic view on the VEGF signalling family, lymphatic vessel biology is, however, outside the scope of this thesis and will not be discussed further.

1.4.2 PDGF signaling and adipocyte progenitor cell fate

The PDGF family consists of four ligands (PDGF-A, PDGF-B, PDGF-C and PDGF-D) that act via two receptor tyrosine-kinases (RTKs), PDGFR-α and PDGFR-β[113]. The possible PDGF-PDGFR interactions include homo- and heterodimer formation of both ligands and the receptors and are multiple and complex. How many of these combinations are physiologically relevant, however, is still unclear. Knockout of PDGF receptors in mice demonstrated that PDGFR-β and PDGF-B are essential for the development of perivascular cells, whereas PDGFR-α and PDGF-A are more broadly required for successful embryogenesis[114].

PDGFR-β could be identified as marker for proliferating and renewing adipogenic progenitors. PDGFR-β^+ cells are located in the perivascular niche and contribute to WA development *in vivo*[115]. Additionally, in 2012, PDGFR-α^+ cells were defined as bipotential AC progenitor cells that can differentiate into both BA and WA, suggesting a role for both PDGFR-α and PDGFR-β in adipogenesis[116]. In adult mice, PDGFR-β^+ cells seem to differentiate into ACs when challenged with HFD or adrenergic stimulation[70]. If PDGF signalling is over-stimulated, however, these cells are forced to differentiate into fibroblasts instead of pericytes or ACs[117]. This has been observed in obese patients. Onogi et al. described that despite the physiological role of PDGF-BB-PDGFR-β^+ in AT neovascularization and pericyte recruitment, the increased recruitment of PDGF-B expressing pro-inflammatory M1-macrophages in obese patients can lead to pericyte detachment and fibrosis[117]. This observation could be repeated by increasing PDGFR-α kinase activity in mice, which resulted in severe perivascular fibrosis and failure to develop WAT[118]. This demonstrates that both increased PDGFR-α and PDGFR-β signalling forces progenitor cells to differentiate into fibroblasts.

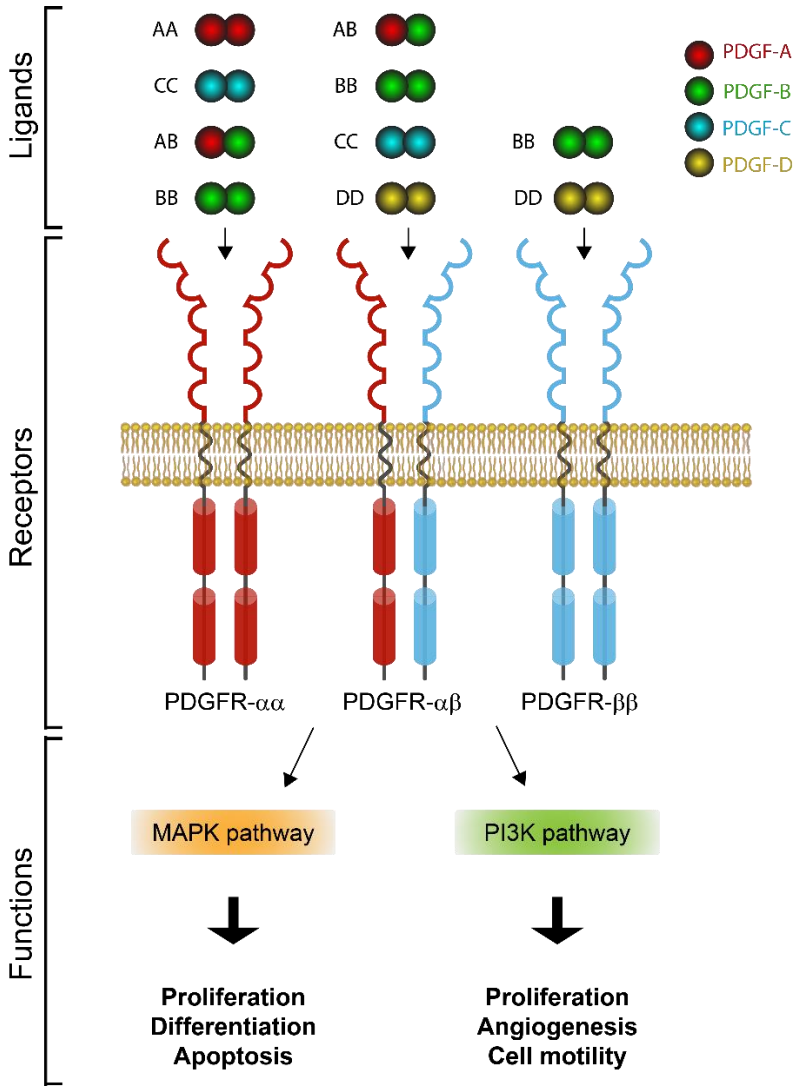

Figure 4: ***PDGF signaling.*** *Representation of the interaction between PDGF ligands and their receptors and possible functional outcomes.*

Traditionally, PDGFR-β is defined as mural cell marker[119], and PDGFR-α as fibroblast marker[116], but their similar mesenchymal phenotype and plastic receptor expression patterns (sometimes both PDGFR-α and PDGFR-β can be found on the same cell and might even heterodimerize[120]), do not allow cell type classification based on PDGFR expression only. A recent study demonstrated that PDGFR-α expression precedes PDGFR-β expression in subcutaneous adipose depots, but only in a small subset of visceral adipose stromal cells, suggesting additional tissue specific differences. A recent study could confirm that HFD feeding or thermoneutrality increased the recruitment and differentiation of PDGFR-β^+ cells into ACs and resulted in bigger ACs and obesity. This study could further demonstrate that depletion of PDGFR-β^+ cells, on the other hand, protected against diet induced obesity (DIO)[121]. Future research will help to further define the physiological functions of PDGFs and PDGFRs in adipose derived MSCs and adipose progenitor cells and will hopefully clarify the fate of PDGFR-α^+ and PDGFR-β^+ cells in distinct ATs *in vivo*.

1.4.3 FGFs that act as adipokines

The FGF family comprises 22 members with high sequence similarity, however, diverse mechanisms of action. Most FGFs are canonical FGFs that act as extracellular proteins binding one of the four FGFRs (FGFR1-4) and function in either paracrine or endocrine manner. FGF11-14, however, are intracellular FGFs that act independently of receptor binding[122]. All four receptors contain three extracellular immunoglobulin-like domains IgI, IgII, and IgIII that are important in receptor dimerization. FGFR1–3 can alternatively use part of the IgIII domain to generate FGFR1-3a, b and c isoforms that are expressed in different tissues and have distinct binding specificities[123].

Knockout (KO) studies in mice have shown that FGFs are important for early embryonic development and organogenesis. In adult tissues, they mediate metabolic functions, tissue repair and regeneration[124]. Three FGFs have been identified as adipokines; FGF1, FGF10 and FGF21. FGF1 binds to all FGFRs and has been demonstrated to be highly induced in WAT upon HFD feeding. Furthermore, the PPARγ-FGF1 axis has been shown to be critical for maintaining metabolic homeostasis and insulin sensitization. FGF1 KO mice are viable, but display severe insulin resistance, inflammation, vascular defects and aberrant AC size distribution upon HFD feeding[125].

FGF10 is another adipokine that binds with high affinity to FGFR-2b in the target tissue. FGF10 KO mice die shortly after birth due to multi-organ failure[126]. FGF10 is abundantly expressed in WAT, especially by preadipocytes, and loss of this factor *in vivo* results in reduced proliferation of preadipocytes and great impairment of WAT development[127,128].

FGF21, which binds FGFR-1c, mainly acts as a hepatokine and is involved in glucose and lipid metabolism. Fasting or ketogenic diet increases FGF21 expression, resulting in augmented glucose and lipid metabolism in hepatocyte and WAs. FGF21 transgenic mice are resistant to DIO. Furthermore, administration of recombinant FGF21 to *ob/ob* (leptin deficient) and *db/db* (leptin receptor deficient) mice reduces their glucose levels to near normal levels[129,130] by inducing PPARγ and thereby promoting insulin sensitivity[131]. In addition, FGF21 is synthesized in BAT in response to cold exposure where it activates mitochondrial uncoupling[132].

Collectively, these findings suggest FGFs and downstream signalling as potential novel targets for therapeutic interventions for the treatment of obesity, diabetes and related metabolic diseases.

1.5 MICRORNAS IN ADIPOSE CONTEXT

1.5.1 What are microRNAs?

The cell has developed intricate strategies to regulate and fine-tune its transcriptional output. One of these strategies are noncoding or non-messenger RNAs, a group of diverse molecules with structural, enzymatic and regulatory functions. MicroRNAs (miRNAs) belong to the group of small non-coding RNA molecules, are about 22 nucleotides in length and have been shown to regulate RNA silencing and control post-transcriptional regulation of gene expression (reviewed in[133]).

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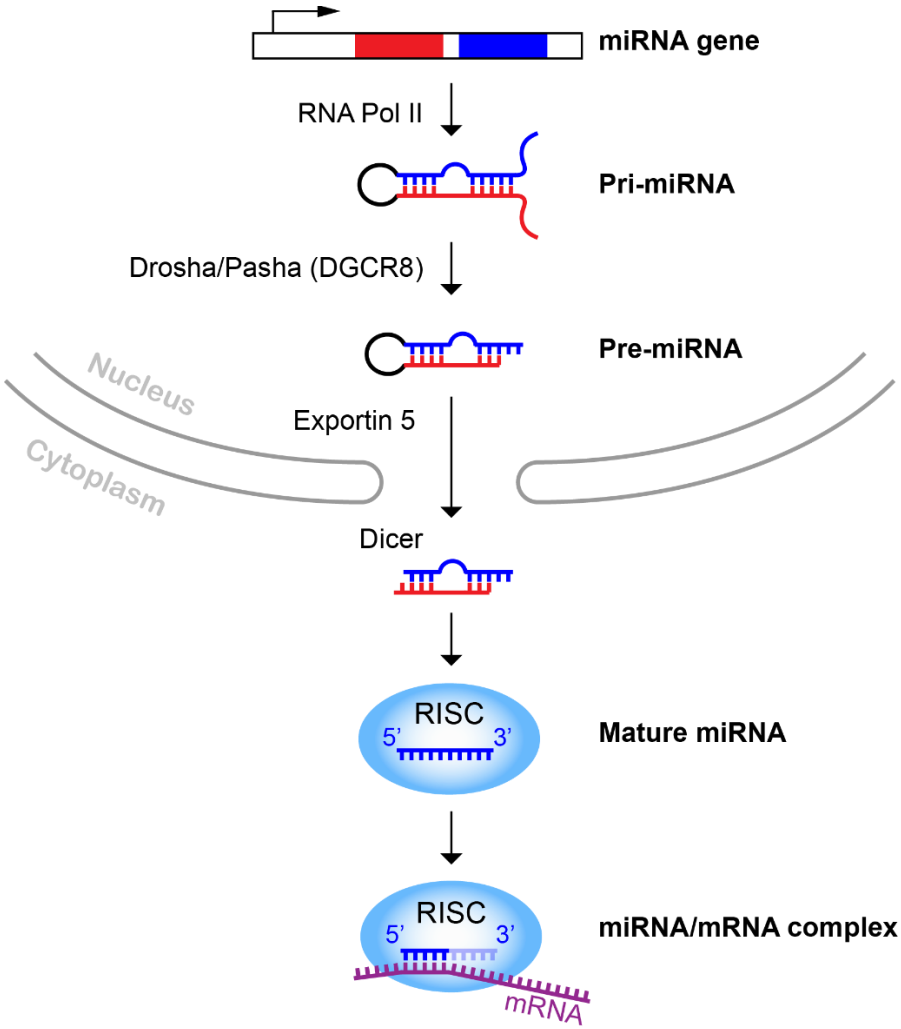

*Figure 5: **MiRNA synthesis and target interaction.** Representation of the miRNA synthesis process and relocation to the cytoplasm that allows target silencing.*

MicroRNAs themselves are transcribed by polymerase II or polymerase III to primary transcripts (pri-miRNAs) in the nucleus. Drosha and its co-factor DiGeorge syndrome critical region gene 8 (DGCR8) cleave pri-miRNAs at the bottom of their stem loops to 70-nucleotide precursors (pre-miRNAs). Exportin5 is responsible for their export to the cytoplasm where

Dicer generates a 20-25 nucleotide long miRNA duplex. The active strand of the miRNA duplex is retained in the RNA-induced silencing complex (RISC) to base pair with target mRNAs leading to Argonaut-dependent mRNA cleavage or translational repression[134]. Figure 5 shows a simplified schematic of the process by which miRNAs are generated. Since the discovery of the first miRNA in 1993[135], hundreds of miRNAs have been identified that directly regulate the expression of over 30% of human and mouse genes and 60% of human protein-coding genes have been under selective pressure to maintain a conserved sequence in the 3' untranslated region (UTR) to base pair with miRNAs[136].

1.5.2 MiRNAs in adipogenesis, browning, and metabolic disease

To define the function of miRNAs in AT development, adipose specific Dicer KO mice were generated. These mice had defects in WAT development and reduced thermogenic activity in BAT and died within three weeks after birth[137]. In contrast, KO of Dgcr8 in all adiponectin positive cells resulted in a viable and fertile mouse strain that demonstrated defects in brown fat development and browning, suggesting that miRNAs are especially important to metabolically highly active brown and beige ACs[138]. MiR-133 and miR-155 have since been identified to negatively regulate BA differentiation and browning *in vivo*[139,140]. On the contrary, the miR-193b-365 cluster and miR-196a induce BA differentiation and WA browning[141,142]. This highlights that different miRNAs, depending on the specific targets, will have a unique impact on AT differentiation and browning. Thus, every single miRNA must be evaluated separately to draw conclusions.

In DIO, a subset of miRNAs is deregulated in both human and mice. These miRNAs vary drastically between different depots; however, they mainly seem to be involved in immune response, lipolysis and AC differentiation[143-145]. Furthermore, significant positive correlation between the upregulation of a subset of miRNAs and insulin resistance could be revealed in obese patients[146]. Therefore, it is reasonable to speculate that miRNAs have pathophysiological functions, and might be associated with impaired adipogenesis, insulin resistance and obesity-related inflammation. This suggest miRNAs as viable targets to combat T2DM and other metabolic complications associated with obesity.

2 AIMS

The overall aim of this thesis was to define the role of growth factor signaling and angiogenesis in AT activation, metabolic disease and aging.

The specific aims were:

I: To define the function of growth factor targeting miRNAs in AT browning

II: To characterize the role of VEGFR1 in AT browning and metabolic disease

III: To identify SVF-derived secretory factors that induce AT browning

IV: To evaluate age-related changes in the adipose vasculature and their consequences on AC function.

3 RESULTS AND DISCUSSION

Blood vessels are not solely important for coping with metabolic changes in the AT, they also play a prominent role in modulating adipocyte function[67]. By secreting paracrine factors, ECs communicate directly with ACs to e.g. activate them during adrenergic stimulation[63]. A collection of studies further demonstrated that perivascular cells serve as the progenitor pool for ACs and control AT plasticity[115,121,147]. The publications included in this thesis focus on the role of blood vessels in the regulation of AC differentiation and function.

3.1 PAPER I: A MIR-327-FGF10-FGFR2-MEDIATED AUTOCRINE SIGNALING MECHANISM CONTROLS WHITE FAT BROWNING

Many stimuli such as cold exposure, adrenergic activation, exercise, and diet have been identified that have the ability to activate WAs to take on a brown-like phenotype[148]. The molecular mechanisms why some cells are recruited and commit to a beige adipose lineage, however, are not fully understood. One group of possible modulators of AC differentiation are miRNAs – small non-coding RNAs that have a function in RNA silencing and post-transcriptional gene regulation. Overexpression of miRNAs that target important regulators of adipocyte differentiation such as C/EBPβ resulted in a substantial reduction of fat mass *in vivo*[139], proving evidence for their ability to alter differentiation potential in ACs.

In this publication, the aim was to identify secretory factors that have the ability to drive ACs into differentiation under browning conditions and find molecular regulatory mechanisms that lead to their activation. Therefore, we performed microarray analysis in parallel to a miRNA array on SVF-derived cells from browning WAT. We could identify a group of growth factors that were upregulated upon browning and corresponding downregulated miRNAs that target these growth factors. Several members of the FGF family were induced in the SVF of browning WAT and we could successfully identify miR-327 as a miRNA that targets FGF signaling and is suppressed during adrenergic stimulation. FGF10-FGFR2 signaling drives preadipocytes into differentiation and we could demonstrate that miR-327 controls this process by modulating FGF10 levels, both *in vitro* and *in vivo*. *In vivo*, blockade of miR-327 increased FGF10 levels in WATs and resulted in a reduction of adipocyte size and induction of UCP1 expression. Genetic diminishment of FGF10 by utilizing Fgf10+/- mice blocks the miR-327 inhibitor-induced activation of WAT, suggesting FGF10 as the mature effector in this process. Our data suggest an autocrine regulatory pathway in preadipocytes of WAT where miR-327 expression levels control FGF10 protein levels and thereby downstream FGFR2 signaling, adipocyte differentiation and browning.

In more details, our first approach was to set a time-point during the treatment with the β3-adrenergic receptor agonist CL 316,243 (from now on referred to as CL) and the exposure to cold, where both angiogenesis and AC browning are already detectable in WAT but remodeling still occurs. Only 5 days of CL treatment and 2 weeks of 4°C exposure were sufficient to substantially activate both angiogenesis and white AC browning in scWAT and

visWAT, with augmented amounts of mitochondria and UCP1 in AC and increased numbers of ECs and perivascular stromal fibroblasts present in the browning tissues. Therefore, we chose these two time points for all following *in vivo* functional analysis. We know from previous studies that the expression levels of a number of secretory factors peak at around 1-5 days of CL treatment and thereafter return to their physiological levels[62,63]. Thus, we performed miRNA array analysis on 3-day CL treated SVFs of visWAT compared to vehicle treated controls. From a number of up-, and downregulated miRNAs, we extracted growth factor targeting, downregulated miRNAs and confirmed their expression levels by qPCR analysis. Targets were identified with a number of online tools such as miRWalk, TargetScan and microRNA.org. One of the most downregulated miRNAs, miR-327, was predicted to bind to FGF10, which is highly expressed in AT and has previously been described to modulate preadipocyte differentiation[128]. Consequently, we cloned the two predicted miR-327 binding sites in the 3'UTR of *Fgf10* into a dual luciferase reporter vector and measured luciferase activity in the presence of miR-327. A significant reduction of luciferase signals could be measured for both binding sites, and specific point mutations of the binding site hindered miR-327 from binding, thereby preventing miR-327 mediated repression of luciferase signals. These data confirm that miR-327 has two binding sites in the 3'UTR of *Fgf10* mRNA. Extraction of FGF family members from a microarray conducted with 3-day CL treated visWAT SVFs compared to vehicle control confirmed that *Fgf10,* as well as its receptor *Fgfr2,* were upregulated upon early browning of WAT. Considering the mode of action of miRNAs, the analysis of mRNA expression levels does not fully reflect their repressive potential. Therefore, we performed western blot analysis to define FGF10 protein levels in scWAT, visWAT and iBAT. Interestingly, protein levels of FGF10 in scWAT and visWAT were extremely low at thermoneutral temperatures and increased considerably after CL treatment or cold exposure. FGF10 could be detected in all samples from iBAT but did not change markedly upon adrenergic stimulation. These findings indicate an important function of FGF10 during WAT browning.

To pinpoint which cells in the SVF express *Fgf10*, $CD45^+$ immune cells, $CD31^+$ vascular ECs, PDGFR-α^+ stromal fibroblasts, and PDGFR-α^- cells were isolated by magnetic cell sorting and expression levels of *Fgf10* was measured. *Fgf10* was almost exclusively produced by PDGFR-α^+ stromal fibroblasts, a cell type with the capacity to differentiate into ACs. *Fgfr2* and miR-327 showed similar, however less specific, expression patterns with the highest expression measured in PDGFR-α^+ cells. These data suggest an autocrine regulatory loop where levels of miR-327 control protein levels of FGF10 and thereby FGFR2 mediated downstream tyrosine kinase signaling in PDGFR-α^+ cells. To confirm this initial hypothesis, we treated 3T3-L1 preadipocytes with miR-327 mimics and inhibitors. FGF10 levels were markedly reduced after miR-327 mimic treatment and increased after treatment with a miR-327 inhibitor. Furthermore, miR-327 mimic treatment resulted in decreased phosphorylation of the downstream FGFR2 mediators FGFR substrate 2-alpha (FRS2-α) and Akt, but not Erk. These findings confirm that miR-327 targets FGF10 and blocks FGF10-induced FGFR2-Akt signaling. Following the general notion that Akt phosphorylation is important for

preadipocyte differentiation[149], we performed a differentiation assay with 3T3-L1 preadipocytes and primary preadipocytes isolated from scWATs. In both cases, miR-327 mimic administration resulted in a great inhibition of preadipocyte differentiation potential where only a very low number of lipid-loaded ACs could be detected in the mimic treated, compared to control treated samples. Inhibition of miR-327, on the other hand, resulted in a significant increase in preadipocyte differentiation and lipid deposition. We further measured differentiation markers in primary preadipocytes and differentiated WA treated with miR-327 mimics. All differentiation markers were drastically reduced in mimic treated samples. Furthermore, analysis of browning markers was performed after differentiation of miR-327 mimic treated cells with or without 4h isoproterenol stimulation to activate uncoupling in these cells. Consistent with previous results, browning markers were significantly less expressed in mimic treated cells compared to controls.

To further define the role of miR-327, FGF10, FGFR2 and downstream Akt signaling in the regulation of preadipocyte differentiation, we performed preadipocyte differentiation assays with 3T3-L1 cells under a variety of conditions. First, we conducted a rescue experiment, where we applied recombinant FGF10 to miR-327 mimic treated cells. Exogenously applied FGF10 allowed these cells to differentiate into ACs in greater numbers. However, if FGFR2 was blocked or Akt was inhibited simultaneously, cells failed to differentiate. This indicates that FGF10 acts through the FGFR2-Akt pathway to induce differentiation. Loss of function experiments revealed that knockdown (KD) of *Fgf10* completely blocked differentiation and simultaneous treatment with the miR-327 inhibitor failed to rescue this phenotype. The addition of conditioned medium from control or miR-327 inhibitor treated cells, however, enabled cells to differentiate similar to untreated cells. These data verify that FGF10 is crucial for adipocyte differentiation and that extracellular FGF10 is the key mediator. Next, we checked the influence of FGF10 and miR-327 levels on preadipocyte proliferation at different time-points during the differentiation process. 3T3-L1 preadipocytes were treated with FGF10 recombinant protein, *siFgf10*, miR-327 mimic or miR-327 inhibitor, but none of these treatments resulted in altered proliferation. SiFgf10 and miR-327 mimic treatment blocked differentiation, therefore some proliferative preadipocytes could be detected at day 6 of differentiation, whereas other groups were already terminally differentiated. These data confirm that FGF10 levels have no effect on preadipocyte proliferation.

To validate if increased levels of miR-327 can modulate WAT browning *in vivo*, miR-327 expressing adenovirus was directly injected into scWAT and visWAT of immunodeficient NSG mice, followed by CL treatment or cold exposure. In both cases, excessive amount of miR-327 lead to increased adipocyte size and a decrease in mitochondrial number and uncoupling. Additionally, FGF10 protein levels were drastically decreased in CL and 4 °C treated scWATs overexpressing miR-327. Furthermore, increased scWAT mass, reduced oxygen consumption, attenuated NE-induced thermogenesis and lower core body temperatures could be detected in mice with increased miR-327 levels upon cold exposure. To characterize systemic effects of miR-327 inhibition, we injected a miR-327 inhibitor to C57/Bl6 mice. Interestingly, FGF10 protein levels were highly elevated in 30 °C exposed

mice, which demonstrated reduced adipocyte size and increased numbers of mitochondria and augmented UCP1 levels. Exposure to cold temperatures in combination with miR-327 inhibition resulted in robust browning, increased mitochondrial staining and *Ucp1* expression. Furthermore, increased energy expenditure and NE-induced thermogenesis could be detected in 30 °C and 4 °C exposed mice treated with miR-327 inhibitor. To validate if this effect is dependent on FGF10, we additionally injected miR-327 inhibitor into $Fgf10^{+/-}$ mice. Compared to WT mice, miR-327 inhibitor effects were drastically reduced and only slight changes in adipocyte size could be detected. FGF10 and UCP1 levels were barely detectable and no changes in scWAT weight, body temperature or energy expenditure were observed. Since the miR-327 inhibitor was applied systemically, we additionally analyzed BAT activation and FGF10 levels in all inhibitor studies, however, could not detect any effects of miR-327 inhibition on iBAT.

Based on the collected evidence in this publication, we believe that miR-327 regulates FGF10 production in PDGFR-α^{+} preadipocytes under physiological conditions. Cold exposure or similar adrenergic stimulation reduces miR-327 levels, resulting in increased levels of FGF10 that can act in an autocrine fashion to activate FGFR2-Akt signaling, resulting in beige adipocyte differentiation.

3.2 PAPER II: ABLATION OF ENDOTHELIAL VEGFR1 IMPROVES METABOLIC DYSFUNCTION BY INDUCING ADIPOSE TISSUE BROWNING

Adipocytes produce important secretory factors such as VEGF that are crucial to switch on angiogenesis[105,150]. While the interaction of VEGF with VEGFR2 has been intensively characterized in the AT, how binding of VEGF to VEGFR1 effects angiogenesis and AT metabolism is less understood; controversial findings in diverse studies make it difficult to draw general conclusions[107,151].

We utilized various pharmacological and genetic approaches to block VEGFR1 or delete the VEGFR1 gene globally or in specific cell types such as EC, AC and myeloid cells to clarify some of these controversies. Systemic VEGFR1 blockade or deletion resulted in robust angiogenesis and a browning phenotype in the AT. EC specific KO of VEGFR1 was sufficient to generate a similar phenotype as seen in global VEGFR1 KO mice and protected the mice from DIO. On the contrary, conditional KO of VEGFR1 in AC and myeloid cells failed to induce angiogenesis and no changes in adipocyte size and activation could be detected. Our data strongly suggests that the deletion or inhibition of VEGFR1 in ECs modulates adipocyte metabolism and provides an appealing novel mechanism for the treatment of obesity and related diseases such as liver steatosis and T2DM.

Our study design is based on the initial finding that blockade of VEGFR1 induces angiogenesis. Indeed, 10-day anti-VEGFR1 treatment of C57/Bl6 mice resulted in a robust activation of

angiogenesis in scWAT, visWAT and iBAT[1]. Furthermore, vessel integrity could be confirmed by the quantification of vessel perfusion and leakiness. VEGFR1 has three ligands, VEGF, VEGF-B and PlGF. Expression analysis of these three factors after anti-VEGFR1 treatment demonstrated an increased level of *Vegfa*, suggesting that VEGF is the driver of angiogenesis upon VEGFR1 blockade. Consequently, we performed some combination studies where VEGF or VEGFR2 were blocked in combination with VEGFR1 blockage. Both VEGF and VEGFR2 neutralization fully reversed VEGFR1 induced angiogenesis. Furthermore, we could demonstrate that VEGFR1 blockade increases VEGFR2 phosphorylation, further suggesting VEGFR2 as the mediator of angiogenesis upon VEGFR1 inhibition. The effect of anti-VEGFR1 induced angiogenesis on ATs was a reduction in AC size and an increase in non-shivering thermogenesis. Both effects could be reversed by simultaneous blockade of VEGF or VEGFR2. A time-course experiment in 1-, 2-, 4-, 7- and 10-day anti-VEGFR1 treated animals demonstrated, that vessel numbers were significantly increased as early as treatment day 2, however, UCP1 expression levels and adipocyte size were not significantly altered until after the angiogenic period of 7 days. Therefore, we conclude that angiogenesis precedes AT alterations when VEGFR1 is blocked.

To strengthen our findings, we performed additional analyses of AT in tamoxifen-inducible VEGFR1 KO mice (*Flt*$^{f/f}$; Rosa26-Cre-ER^{T2}). Similar to the pharmacological data, 10 days after KO induction: 1) vessel numbers were drastically increased; 2) EC proliferation could be detected; 3) AC size was reduced; and 4) non-shivering thermogenesis was increased in both white and brown ATs. Due to the broad expression of VEGFR1 in different cell types[152], EC specific VEGFR1 KO mice were generated, *Flt*$^{f/f}$; *Tie2*-Cre-ER^{T2} and *Flt*$^{f/f}$; *Cdh5*-Cre-ER^{T2}. We used two different KO models because *Tie2* expression has been reported in glial cells in addition to ECs[153]. To exclude any effects generated by non-ECs, we extended our analysis with the more specific Cdh5-Cre strain. After tamoxifen induction, VEGFR1 expression was reduced to about 1/3 of the initial expression in isolated ECs. Both KO strains showed similar activation of angiogenesis and AC browning as the systemic KO mice. We extended our investigation on *Flt*$^{f/f}$; *Cdh5*-Cre-ER^{T2} mice by treating them with CL and could demonstrate that loss of VEGFR1 in EC augments angiogenesis and thermogenesis in scWAT, visWAT and iBAT. Additionally, *Flt*$^{f/f}$; *Cdh5*-Cre-ER^{T2} mice displayed resistance to DIO as evident in the metabolic analysis following HFD feeding. EC specific VEGFR1 KO mice had a similar food intake as WT mice, however less body weight and decreased scWAT, visWAT and iBAT fat weight. Moreover, blood parameters such as free fatty acids, glycerol, cholesterol, triglycerides, glucose and insulin were notably reduced, resulting in improved glucose tolerance and rapid glucose clearance in EC-VEGFR1 KO mice. Moreover, lipid staining of the liver revealed a reduction of ectopic lipid storage in hepatocytes. Essentially, loss of VEGFR1 in EC ameliorates dysfunctions in lipid and glucose metabolism and decreases lipid

[1] To simplify, scWAT, visWAT and iBAT are used in the thesis text to define the three analysed adipose tissues, although different nomenclatures might have been used in the original scientific publications.

deposition in the liver, revealing the potential of blocking VEGFR1 for the treatment of T2DM and fatty liver disease.

To exclude the contribution of additional VEGFR1 expressing cell types to adipocyte browning, we further deleted VEGFR1 in ACs ($Flt^{f/f}$; Adipoq-Cre) and myeloid cells ($Flt^{f/f}$; LysM-Cre). Neither of these mouse strains exhibited an increase in angiogenesis, AC browning or thermogenesis. Furthermore, we did not see any increase in macrophage number in $Flt^{f/f}$; Cdh5-Cre-ER^{T2} mice treated with CL. Therefore, we conclude, that VEGFR1 KO in EC, not ACs or immune cells, influences angiogenesis and thereby modulates AT function.

In summary, the findings in this publication support the notion that VEGFR1 acts as a decoy receptor for VEGF. During homeostasis, only a fraction of VEGF binds to VEGFR1 with 10-fold higher affinity than VEGFR2. VEGF-VEGFR1 complexes result in relatively weak tyrosine kinase activation and thereby limit angiogenic potential in EC. Under such conditions, ATs are primed for energy deposition. Loss of VEGFR1 or blockade of the VEGFR1 binding site, on the contrary, allows a greater number of VEGF ligands to bind to VEGFR2, thereby transducing robust angiogenic signals. Proliferating EC secrete paracrine factors that trigger white ACs to take on a beige phenotype and induce mitochondrial uncoupling in BAs - resulting in increased energy expenditure.

3.3 PAPER III: ENDOTHELIAL PDGF-CC REGULATES ANGIOGENESIS-DEPENDENT THERMOGENESIS IN BEIGE FAT

The AT is an extremely plastic organ that can adapt quickly to extrinsic stimuli such as increased need to store lipids, elevated energy requirements or an increased need of the body to produce heat. The alterations in AC during these processes have been well characterized, however, how non-vascular cells are affected during these processes is poorly understood. A number of studies demonstrated that both AC expansion and adipocyte activation alter the cell composition in the AT – especially the number of vessels and vessel-associated cells (reviewed in[71] and[67]). Especially, induction of a beige phenotype in AC by β3-adrenergic stimulation or cold temperatures results in a drastic increase in blood vessels in the AT[62]. We could previously show that VEGF-VEGFR2 signaling is a key angiogenic stimulus during this process and is required for the induction of a brown-like phenotype in AC *in vivo*[154]. Despite this body of work, how these newly formed vessels interact with ACs to increase the number of beige ACs is not yet understood.

In this study we performed microarray analysis of the adipose SVF, which includes all cells of the AT except ACs. When looking closely at the expression pattern of growth factors present in the SVF during browning, we identified *Pdgfc* as one of the main factors upregulated during adrenergic stimulation. The PDGF family shares structural and functional similarities to the VEGF family and has previously been demonstrated to be important for preadipocyte differentiation. PDGFR-α is strongly expressed on preadipocytes that can give rise to white, beige and brown AC[116].

In this paper we aimed to identify the cellular source of PDGF-CC during browning and its effect on preadipocyte differentiation and activation. Performing *in vivo* gain- and loss-of-function experiments as well as cell specific mRNA expression profiling, we could demonstrate that ECs are the source for PDGF-CC during adrenergic stimulation. Both blockade and induction of VEGF signaling controlled endothelial PDGF-CC production. Loss of PDGF-CC did not alter vessel number or angiogenesis during browning, however, decreased beige AC numbers and non-shivering thermogenesis. Similarly, induction of PDGF-CC expression had no effect on vessel numbers, however induced a brown-like phenotype in WATs. Therefore, we conclude that EC derived PDGF-CC is an important mediated of vessel-AC communication that has an essential function during AC browning.

In our study, we first investigated angiogenic response in the visWAT of CL treated C57/Bl6 mice by performing a time-course experiment. Blood vessel numbers were significantly increased already 2 days after the first CL injection and tripled within 10 days of treatment. VEGF levels more than doubled within one day of treatment and remained high for around 5 days, further strengthening previous findings that angiogenesis is VEGF dependent during the browning process. To exclude any off-target effects of CL, we used β3-adrenoceptor KO mice and could confirm that CL does not cause any alterations in vessel number in these mice. In addition, cold exposure failed to activate browning and angiogenesis in visWAT, however, robust effects were observed in scWAT and iBAT. These data suggest that cold exposure was not sufficient to stimulate browning and angiogenesis in the visWAT in this particular experimental set-up. Accordingly, we used CL treatment for all further experiments.

To validate if angiogenesis is dependent on mitochondrial uncoupling, we used *Ucp1*$^{-/-}$ mice. While browning was significantly reduced in these animals, no differences in *Vegf* expression levels or vessel number were detected. By blocking VEGF signaling, we attempted to further specify its role in CL-induced angiogenesis and browning. We utilized specific neutralizing antibodies against VEGF and VEGFR2 and a VEGFR tyrosine kinase inhibitor (Sunitinib). All three agents significantly suppressed angiogenesis and thermogenesis in CL treated mice, further highlighting the importance of VEGF signaling during browning. In addition to the previously mentioned pharmacological approaches we generated inducible, EC-specific VEGFR2 KO mice and treated them with CL. Similar to previous data, both mouse strains exhibited a decline in angiogenesis and AC browning. Furthermore, we generated a gain-of-function model by transfecting either visWAT or scWAT with an adenovirus containing *Vegfa* and a GFP reporter construct. Local *Vegfa* overexpression alone could induce vessel number and browning in both visWAT and scWAT and even significantly increased oxygen consumption in mice when scWAT was transfected with the adenovirus. This demonstrates that both induction and reduction of angiogenesis has a substantial effect on WA activation and thermogenesis.

To get further insight into the mechanism by which angiogenesis modulates AC function we performed microarray analysis of visWAT SVFs of CL treated animals to look at upregulated secretory factors. PDGF-CC was found to be the most upregulated gene upon all growth factors

and cytokines, which could be confirmed with qPCR analysis. Subsequent *in vivo* loss-of-function and gain-of function experiments were conducted utilizing *Pdgfc*$^{-/-}$ mice and an adenovirus overexpressing *Pdgfc*. *Pdgfc*$^{-/-}$ mice showed no obvious phenotype and similar vessel numbers and non-shivering thermogenesis compared to WT mice. Upon CL treatment, however, an increase in AC size and a decrease in browning and mitochondrial marker expression could be detected, resulting in impaired non-shivering thermogenesis in browning WATs of *Pdgfc*$^{-/-}$ mice. Adenovirus-mediated, local delivery of PDGF-CC to visWAT and scWAT, could rescue this phenotype and resulted in an increase in the expression of beige AC markers and thermogenesis. These data validate that, upon adrenergic stimulation, EC derived PDGF-CC regulates thermogenesis in WATs downstream of VEGFR2-induced angiogenic signals.

In a DIO model, both *Vegf* and *Pdgfc* expression levels were markedly reduced. Therefore, we investigated if the positive effect of PDGF-CC on WA browning could be translated to obese settings. Indeed, adenovirus-induced local overexpression of *Pdgfc* in visWAT and scWAT of obese mice resulted in reduced fat mass and accelerated glucose clearance. PDGF-CC mainly binds to PDGFR-α to transduce downstream signals. We could show that PDGFR-α expression is increased in CL-treated visWAT and that PDGFR-α expression does not overlap with CD31 EC expression, indicating that non-EC in the AT increase the receptor expression during browning. We further isolated PDGFR-α^{+} cells from WATs from both, human and mice, and treated them with recombinant PDGF-CC. Upon differentiation, higher levels of UCP1 and other browning factors were detected. *In vivo* loss-of-function studies utilizing PDGFR-α and PDGFR-β neutralizing antibodies reflected less browning and inhibition of non-shivering thermogenesis in CL treated mice especially upon PDGFR-α inhibition.

In summary, we could demonstrate that VEGF-VEGFR2 interaction and induction of angiogenesis is necessary to activate white ACs to adapt a brown-like phenotype. These angiogenic ECs produce PDGF-CC, which binds to PDGFR-α on preadipocytes and drives their differentiation into beige AC.

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

Age is a major risk factor for obesity and secondary diseases such as T2DM, cardiovascular disease, hypertension and certain types of malignant diseases. Both incidence of obesity and diabetes is highest in middle aged individuals between around 40-60 years of age[155,156]. Although correlated to the BMI, the significant increase of T2DM prevalence in middle-aged individuals cannot be fully explained by increased body weight. One age-related risk factor for T2DM is the decline of pancreatic islet function and proliferative capacity[157]. Additionally, a large body of evidence suggest that metabolic abnormalities cause overproduction of reactive oxygen species that in turn lead to EC dysfunction, inflammation and diabetic vascular

disease[158]. There is, however, limited information on the role of the vascular network during the onset of diabetes.

In this paper, we provide evidence that vascular plasticity fluctuates with age and show that this is occurring in a VEGF-dependent manner. These fluctuations correlate with the risk of developing T2DM. We could further demonstrate that anti-VEGF treatment increases insulin sensitivity in age groups with high vascular plasticity and DIO mice. The first part of the study focuses on AC morphology and vessel number in different age groups. We chose C57Bl/6 mice of ages 1, 4, 10, 12 and 16 months, which corresponds to 4-, 17-, 42-, 50-, and 70-years in humans. We analyzed scWAT, visWAT and iBAT in these mice to evaluate differences in the vasculature in this broad set of adipose depots. In scWAT, AC size increased with age in accordance with BMI (from 1 month to 10 months), in contrast to vessel density, which was negatively correlated with BMI. In older mice, BMI stabilized, although we could demonstrate a decrease of AC size and increase of vessel number in the 12-, and 16-month old groups. VisWAT followed a similar trend. Vessel numbers were high in young and old age groups and had a low-peak in middle-aged mice. AC size was, similar to scWAT, negatively correlated to the vessel numbers. The decrease of adipocyte size and increase of vessel numbers in older age groups, however, was less pronounced. Similar but generally milder alterations of AC size and vessel density were measured in iBAT. Interestingly, iBAT weight almost doubled during a mouse's life span, *Ucp1* expression, however, did not correlate with the increase in tissue mass. Therefore, we investigated expression levels of *Ucp1* in iBAT and could confirm that less uncoupling protein was produced by iBAT in older age groups.

To address the role of VEGF signaling on age-related alterations of the adipose vasculature, VEGF, VEGFR1 and VEGFR2 protein levels were studied in WATs and iBAT. In accordance with the histological data obtained, VEGF levels reached a low-point in middle-aged animals and increased again thereafter. We could further show that VEGFR1 levels peaked in 10-month old mice. This finding correlates with the notion that VEGFR1 function as a decoy receptor for VEGF. VEGFR2 was highly expressed in young mice and showed declining expression with age, suggesting high demands for angiogenesis in adolescent mice. To elucidate the effect of VEGF signal inhibition on aging ATs, we injected VEGF- and VEGFR2 neutralizing antibodies to 1-month, 7-month, and 15-month-old healthy mice. 1-month and 15-month old mice lost bodyweight upon treatment, leading to a significantly lower BMI compared to control animal. Vessel number was reduced significantly in all age groups; however, middle-aged mice displayed a rather moderate effect. Based on these findings, we concluded that ATs of middle-aged mice are less dependent on VEGF signaling and therefore somewhat resistant to anti-VEGF and anti-VEGFR2 treatment. Next, we asked the question if these age-related changes in adipose vasculature have systemic effects on metabolic parameters such as lipolysis and insulin sensitivity. Therefore, blood lipids, fasting glucose and insulin were determined. While only slight alterations in triglycerides, non-esterified fatty acids, glycerol, cholesterol and fasting glucose could be detected in the blood of healthy mice at different ages, we could monitor a drastic increase in circulating insulin levels in the 15-month old group, resulting in substantially reduced insulin sensitivity. HOMA-IR levels were 3-fold higher in 15-month old

mice compared to young and middle-aged mice, suggesting insulin resistance. We further analyzed these metabolic parameters in anti-VEGF treated animals of different ages. Interestingly, anti-VEGF treatment resulted in a decrease of fasting glucose levels in all age groups and a reduction in fasting insulin in older animals, improving HOMA-IR drastically and almost completely reversing the insulin resistance observed in the 15-month control animals. To further evaluate these findings, insulin tolerance tests (ITTs) were performed for all age groups. In accordance with previous results, adolescent and old mice could improve their glucose clearance upon VEGF blockade; middle-aged mice, on the contrary, showed no effect upon treatment. This finding further strengthens the observation that middle-aged animals are somewhat resistant to VEGF treatment and related metabolic effects.

Considering the fact that insulin resistance is not only strongly linked to age, but also body weight, we generated DIO mice. Middle-aged DIO mice were treated with anti-VEGF at 7-month of age and were analyzed for histological as well as metabolic parameters. In contrast to lean mice, HFD fed middle-aged mice showed high sensitivity to anti-VEGF treatment leading to a drastic reduction in vessel numbers. Body weight, fat mass and fasting glucose levels were drastically reduced, and insulin sensitivity significantly increased. To determine the underlying cause of the increased responsiveness to anti-VEGF treatment and the increase in insulin sensitivity in DIO mice, we investigated macrophage number and cytokine production. In obese mice, macrophage number was increased, and anti-VEGF treatment further induced the expression of *Il6* and *Tnfα*. It is, however, unlikely that increase of macrophage number and cytokine production would contribute to anti-VEGF induced insulin sensitivity, because induction of inflammation and the upregulation of both, IL-6 and TNF-α have previously been linked to the development of insulin resistance in DIO[159,160]. Therefore, we conclude that further investigations are necessary to pinpoint the mechanism that makes middle-aged DIO mice more sensitivity to anti-angiogenic treatment and insulin induced glucose uptake.

Taking together, our data demonstrate age-related alterations in vessel number and metabolic activity, which results in altered sensitivity to anti-angiogenic treatment. Importantly, anti-VEGF treatment leads to increased insulin sensitivity and glucose clearance in young and old mice, as well as middle-aged HFD fed animals. These findings give additional insight on how angiogenesis modulating agents can be used to treat obesity and T2DM. Further studies are however needed to clarify if increased or decreased vessel numbers in the AT are more beneficial for long-term treatment of obesity and related diseases.

4 CONCLUSION AND PROSPECTIVE

Activation of BAT increases energy expenditure and might be a treatment option to reverse obesity and related diseases. If the activation of BAT alone is sufficient to reach this goal, is however still under debate. Beside BAT activation, additional WAT browning might be necessary to successfully increase whole-body energy expenditure to a clinically relevant level. In my thesis projects we aimed to: 1) activate WATs to take on a brown-like phenotype; 2) make visWAT more susceptible to browning; 3) characterize the function of blood vessels in AT browning and aging; 4) define secretory factors that alter AC metabolism; and 5) identify regulatory mechanisms that alter the secretion of these factors. Altered proliferation and gene expression patterns in non-ACs including vessel associated cells and inflammatory cells have been described in both WATs and BAT[67]. We believe that a deep understanding of the cross talk and interaction between cells in the AT and their consequences on whole-body metabolism will be essential to develop pharmaceuticals that can reverse metabolic disease and thereby help to generate a healthier population.

Along with metabolic activation, vessel density and vessel-associated cell numbers are markedly increased in all ATs upon adrenergic stimulation[62,161]. This effect was originally thought to be a consequence of the increased energy needs of AC due to mitochondrial uncoupling[162]. Recent studies from our laboratory, however, demonstrate that vessel numbers even increase in *Ucp1* KO mice treated with cold or adrenergic agonists, suggesting activation of angiogenesis independent of uncoupling[62,163]. We could further demonstrate that angiogenesis in the AT is VEGF dependent and time-course experiments indicated that as early as 2 day after induction of browning, VEGF levels are significantly increased[62-64]. Microarray analysis of 3d CL treated SVFs in the visWAT confirmed this finding and suggested the involvement of other growth factor families, such as FGF and PDGF, in AT browning[63]. These data exclude the possibility that mitochondrial uncoupling in ACs activates angiogenesis during early browning and suggest that blood vessel changes precede metabolic activation. Therefore, we speculated that in-depth analysis and modulation of these growth factors in a browning setting might aid in increasing metabolic activity in ATs and might further be responsible for an increased susceptibility of visWAT for browning, which in general is less responsive to adrenergic stimulation compared to scWAT. Indeed, two of the identified factors, PDGF-CC and FGF10, could induce WAT browning and loss of either of these factors resulted in diminished AC activation. Cell type specific expression patterns suggest further, that both of these growth factors act on PDGFR-α^+ preadipocytes to drive them into beige ACs differentiation. Our data strongly suggest that angiogenesis is a prerequisite that is essential to induce beige adipocyte differentiation under adrenergic stimulation.

Although we could see clear functional effects *in vivo* and strong increase in protein levels, microarray and qPCR analysis resulted in rather moderate changes in mRNA levels for some growth factors in whole tissue homogenates. Possible explanations are: 1) limited local but rather systemic production of these growth factors and delivery to the AT through the

vasculature, 2) alterations in protein stability, or 3) post-transcriptional control through miRNAs. MiRNA array analysis revealed that miRNA levels are indeed significantly altered in the SVF of 3d CL treated visWAT. MiRNA target analysis further confirmed that many of these miRNAs have predicted target sites in the 3'UTR of VEGF, PDGF and FGF family members. The strong reverse correlation between FGF10 and miR-327 expression in browning AT together with the fact that FGF10 is essential for WAT development[127] warranted us to ask if this miRNA has a regulatory function in WAT browning. We could demonstrate that miRNAs can play a role in WAT browning by controlling preadipocyte differentiation. In recent years, several RNAi-based therapeutics have been developed and the first miRNA-targeting drugs entered clinical trials (reviewed in[164]). Poor delivery and toxicity are, however, current challenges. Only future will tell if such obstacles can be resolved to bring miRNA targeting drugs to patients for the treatment of various diseases, including obesity.

While the function of VEGF-VEGFR2 interaction is clearly defined in AT angiogenesis, binding and activation of VEGFR1 by VEGF, VEGF-B and PlGF is less well understood. In our studies we found that VEGFR1 is upregulated in middle-aged mice, which might explain the phenotype that ATs are less vascularized in this age group. Furthermore, we found that EC specific VEGFR1 KO is sufficient to induce VEGF-VEGFR2 dependent angiogenesis and to create a browning phenotype in WATs as well as a resistance against DIO. A related study conducted in Dr. Alitalo's laboratory demonstrated that overexpression of VEGF-B produces a similar phenotype. This phenomenon is explained by the fact that VEGF binds with higher affinity to VEGFR1 than to VEGFR2, therefore, increased VEGF-B levels resulted in a higher number of VEGF-B-VEGFR1 complexes. This further allows VEGF to bind to VEGFR2 instead of VEGFR1[107]. Our work presented in this thesis and Dr. Alitalo's study contradict however Dr. Jain's study from 2009, which concluded that VEGFR1 blockade does not affect body weight in HFD fed mice[151]. Another early study utilized PlGF KO mice, which showed reduced vessel numbers in AT and resistance to DIO[165]. We conclude that further studies on ligand specific differences in VEGFR1 downstream signaling are needed to fully explain VEGFR1 function in ATs.

While increase in vessel numbers is crucial for AT browning, controversial results have been described in DIO models. VEGF TG[166], VEGF-B overexpressing[107] or VEGFR1 KO[64] mice all have an increased amount of blood vessels in ATs and are resistant to DIO and fatty liver disease. On the contrary, blockade of VEGF[167] or depletion of PlGF[165] results in decreased vessel numbers, however, similar improvements in body weight and insulin sensitivity in DIO models could be measured. Other factors such as hypoxia, inflammation, age and genetic background might be of importance to explain why gain- and loss- of blood vessels in the AT can protect against DIO and further studies are needed for clarification.

So, where do we currently stand and where is the future heading? Findings presented in our works give important insides in the communication between blood vessel associated cells and ACs and demonstrate a strong link between PDGF, FGF and VEGF signaling

components and AT function. This work suggests several novel strategies for the treatment of obesity and metabolic diseases, including posttranscriptional modulation by miRNAs. We hope that some of the novel targets provided through our research will lead to the delevopment of therapies that improve the quality of life for patients suffering from obesity and related secondary diseases.

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