Bone Morphogenetic Protein 4 regulates white, beige and brown adipose tissue

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ABSTRACT

Obesity and its associated complications, including Type 2 diabetes, are increasing at an epidemic rate globally. Adipose tissue exerts different functions and is central in energy homeostasis, and it consists of white, beige or brown adipocytes. White adipocytes (in white adipose tissue, WAT) store and release lipids while brown adipocytes (in brown adipose tissue, BAT), oxidize lipids and generate heat. Beige adipocytes reside in WAT, appear white but can oxidize lipids upon stimulation (browning). The aim of this thesis was to investigate the role of Bone Morphogenetic Protein 4 (BMP4) in white, beige and brown fat. In Paper I, we used human WAT biopsies and precursor cells. In Paper II and III, we gave adult mice adeno-associated viral (AAV) vectors to increase circulating BMP4. Endogenous BMP4 is increased in WAT in obesity, and so are BMP antagonists, resulting in reduced BMP4 signalling. WAT browning was enhanced by increasing BMP4 signalling in human precursor cells and in WAT of lean mice. Surprisingly, BAT activity was inhibited in the mice, but whole-body energy expenditure was increased which protected from obesity. However, AAV BMP4 did not enhance browning of WAT in initially obese mice, likely due to the cellular BMP4 resistance. Additionally, all AAV BMP4-treated mice had increased insulin sensitivity. In summary, BMP4 is an important regulator of white, beige and brown fat. BMP4 increases in WAT in obesity but its positive effects are antagonized by the BMP antagonists. However, increasing BMP4 signalling can prevent obesity by browning WAT and also increase insulin sensitivity making it an interesting novel therapeutic target.

Keywords: Obesity, BMP4, browning, WAT, BAT, insulin sensitivity

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SAMMANFATTNING PÅ SVENSKA

Fetma ökar i världen som en konsekvens av att vi rör oss mindre och får i oss mer kalorier. Fetma är associerat med negativa hälsoeffekter såsom typ 2 diabetes, hjärt-kärl sjukdom mm. Fettvävnaden är viktig då det är där vi ska lagra energiöverskott. Underhudsfettet är det primära området att expandera, om vi inte kan expandera det kommer fett lagras i buken, i levern och i muskler, vilket är associerat med negativa hälsoeffekter. En ytterligare central aspekt är att alla fettceller inte är lika. Det finns tre typer fettceller; vita fettceller lagrar/frisätter fett, bruna fettceller förbränner fett och producerar värme och beigea fettceller finns i den vita fettvävnaden men kan börja förbränna fett vid aktivering. Aktivering av beigea fettceller har visat sig ha stor potential i musstudier där det skyddar mot viktökning.

Den här avhandlingen är fokuserad på proteinet Bone Morphogenetic Protein 4 (BMP4) och hur det reglerar vitt, beige och brunt fett. I den första artikeln använde vi humana fettbiopsier och i de två sista artiklarna använde vi möss som fick BMP4-genterapi. De viktigaste fynden är:

- Ökad BMP4 signalering gör vita fettceller beigea med ökad förbränning, både i humana celler samt i smala möss. När vi sen matade mössen med foder med högt fettinnehåll var de skyddade mot viktökning.
- Fetma är associerat med förhöjda nivåer av eget BMP4 protein, både hos möss och människor, och även ökade nivåer av BMP antagonister (som förhindrar signalen) i den vita fettvävnaden. Detta resulterar i att effekten av eget BMP4 försämras, d.v.s. en BMP4-resistens.
- BMP4 ökar insulinkänsligheten hos möss, som vanligtvis är försämrad vid fetma. Denna effekt är oberoende av att fettcellerna blir mer beigea.

Sammantaget så visar vi att genom att öka BMP4 signalen i vitt fett så kan vi göra fettceller beigea och därmed öka förbränningen, samt förbättra insulinkänsligheten. Ökad förståelse av BMP4-signalering i fett kan leda till nya terapeutiska möjligheter vid fetma och typ 2 diabetes.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Gustafson B, Hammarstedt A, Hedjazifar S, Hoffmann JM, Svensson PA, Grimsby J, Rondinone C, Smith U
BMP4 and BMP antagonists regulate human white and beige adipogenesis
Diabetes 2015 May; 64 (5): 1670-81

II. Hoffmann JM, Grünberg JR, Church C, Elias I, Palsdottir V, Jansson J-O, Bosch F, Hammarstedt A, Hedjazifar S, Smith U
BMP4 gene therapy in mature mice reduces BAT activation but protects from obesity by browning subcutaneous adipose tissue
Manuscript under revision

III. Hoffmann JM, Hammarstedt A, Grünberg JR, Elias I, Palsdottir V, Bosch F, Hedjazifar S, Smith U
BMP4 gene therapy improves insulin resistance in obese mice without effects on adipose tissue browning or body weight
Manuscript

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ABBREVIATIONS

AAV	Adeno associated virus
ATGL	Adipose triacylglycerol lipase
BAT	Brown adipose tissue
BMI	Body mass index
BMP	Bone morphogenetic protein
cAMP	Cyclic adenosine monophosphate
CD	Control diet
cDNA	Complementary DNA
C/EBP	CCAAT/enhancer-binding protein
CVD	Cardio vascular disease
DEXA	Dual energy X-ray absorptiometry
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPI	Epididymal
FABP	Fatty-acid binding protein
FDR	First degree relative (of individual with Type 2 diabetes)
FFA	Free fatty acids
GLUT	Glucose Transporter
GTT	Glucose tolerance test
HFD	High fat diet

HSL	Hormone-sensitive lipase
IHC	Immunohistochemistry
IL	Interleukin
I.P.	Intraperitoneal
IR	Insulin resistance
ITT	Insulin tolerance test
MetS	Metabolic Syndrome
MSC	Mesenchymal stem cell
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptor
qRT-PCR	Quantitative real-time PCR
RNA	Ribonucleic acid
SNS	Sympathetic nervous system
SubQ	Subcutaneous
SVF	Stromal vascular fraction
TAG	Triacylglycerol
TEM	Transmission electron microscopy
T2D	Type 2 diabetes
UCP1	Uncoupling protein 1
WAT	White adipose tissue
WT	Wild type
ZFP	Zinc-finger protein

1 INTRODUCTION

1.1 Prevalence of obesity

Obesity is, in the vast majority of cases, caused by the imbalance between calories consumed and calories expended. The prevalence of obesity is increasing due to an increased intake of energy-dense foods and an increase in physical inactivity. Worldwide, obesity has more than doubled since 1980 according to the World Health Organization (WHO). For adults, WHO defines; overweight as a BMI greater than or equal to 25 and obesity as a BMI greater than or equal to 30. According to the WHO, 39% of adults aged 18 years and over were overweight in 2014 and 13% were obese.

1.2 Obesity, the Metabolic syndrome and Type 2 diabetes

Obesity is tightly associated with development of the MetS, which is a constellation of related metabolic risk factors that increase the risk for CVD and T2D, and some forms of cancer [1, 2]. The most widely recognized metabolic risk factors are elevated blood glucose, lipids (dyslipidemia) and elevated blood pressure, and patients usually have increased circulating levels of pro-inflammatory markers [1]. The underlying risk factors, driving the development of these metabolic risk factors, appear to be IR and ectopic fat accumulation in different tissues [1]. The association with fat distribution will be discussed in detail below.

Compared to non-obese, overweight/obese individuals have a 3.5-4.6/10.0-11.2 relative risk of developing T2D over a 10-year period [2]. Other etiological risk factors are age, family history of T2D, and physical inactivity, but populations with the highest prevalence of T2D also have the highest prevalence for obesity [3]. Briefly, T2D can develop following IR, and IR is a state of impaired insulin sensitivity. Insulin sensitivity is the capacity of target cells to respond to insulin and lower blood glucose. The major insulin-sensitive tissues are fat, skeletal muscle and liver [4], and the effects of insulin are summarized in Figure 1. Insulin activation of signalling leads to suppression of hepatic glucose production, and increased hepatic lipogenesis and glycogen synthesis. In skeletal muscle, insulin signalling leads to increased glucose uptake and glycogen synthesis. In fat, insulin signalling increases glucose uptake and lipogenesis, and reduces lipolysis. The specific actions in fat are discussed below. Obesity and dyslipidemia may lead to lipids accumulating

ectopically in skeletal muscle and liver, which leads to reduced insulin sensitivity in these target cells. The pancreatic β-cells, which sense glucose levels, will produce more insulin to compensate for the peripheral tissue IR and T2D develops when the β-cells fail to produce enough insulin [5, 6].

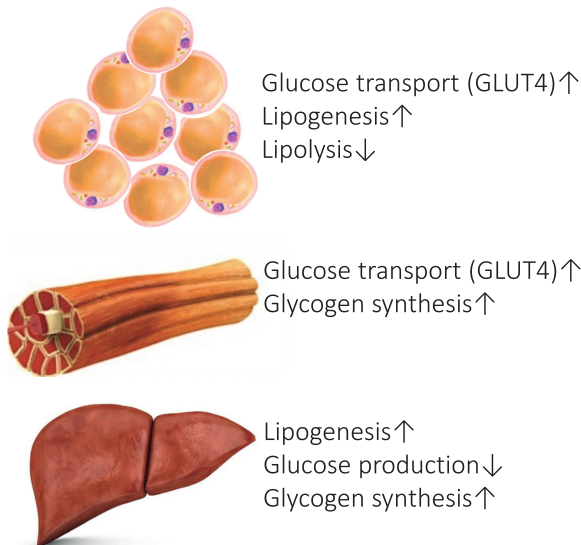

Figure 1. Overview of insulin actions in target tissues.

There is evidence for the contribution of genetics in the risk of developing T2D. A number of genetic risk variants have been shown to contribute to T2D risk [7]. However, the individual effect of these variants is modest and, together, they can account for approximately 10% of the disease prediction [3]. Yet, we and others have demonstrated that genetic predisposition for T2D, defined as being a FDR, is associated with reduced insulin sensitivity also in lean adults [8, 9], and that FDRs exhibit a dysfunctional WAT associated with impaired insulin sensitivity [8]. Furthermore, around 30% of obese individuals are metabolically healthy [10] and, on the contrary, around the same percentage of lean individuals are metabolically unhealthy with reduced insulin sensitivity [11]. Thus, BMI per se is not a sufficiently sensitive marker of individual risk for developing T2D. WAT distribution has emerged as an additional important measure and this is further discussed below.

1.3 WAT and white adipocytes

Adipose tissue can be broadly divided into two distinct types, WAT and BAT. Additionally, in the last couple of years, a third type called beige/brite/recruitable brown adipocytes have emerged as a separate type. The characteristics of the different tissues/cells are discussed in the following sections.

1.3.1 WAT distribution

The SubQ WAT is the largest fat depot in man and is also the preferable site to store excess fat. Expansion of the visceral WAT depot has a clear association to MetS. Although increased BMI itself is a risk factor for IR and T2D, for any given amount of total body-fat, an individual with increased visceral fat is at greater risk for developing IR and its consequences [12]. The SubQ WAT does not show this association, and some studies have actually shown it to be inversely correlated with MetS disease risk [13].

The SubQ WAT has a limited expandability. When the SubQ WAT storage capacity is exceeded, fat is stored in ectopic sites like the visceral depot, skeletal muscle and liver, driving IR. Safe storage of lipids in the SubQ depot has been demonstrated in several mouse models. Over-expression of adiponectin in fat of obese mice leads to healthy expansion of the SubQ fat with complete rescue of the diabetic phenotype, demonstrating the clear benefits of "shunting" lipid storage to the SubQ depot over ectopic storage [14]. The limit for when the SubQ fat storage capacity is reached varies greatly between individuals and is likely determined by both genetic and environmental factors [15]. There is also a gender difference in the capacity to expand the SubQ WAT as females maintain a greater capacity to recruit new adipocytes in the lower-body region as adults [16] and obese women have reduced ectopic visceral fat compared to obese men [17]. Visceral obesity correlates strongly with IR and individuals with IR, not necessarily obese, commonly have increased visceral fat [12, 18]. Taken together, fat distribution is an important determinant for the risk of developing MetS.

1.3.2 Hypertrophic adipose cell expansion

WAT can expand through two separate events, either through enlargement of existing adipocytes (hypertrophic expansion) or by differentiation of new adipocytes from precursor cells (hyperplastic expansion). Hypertrophic, rather than hyperplastic expansion, is associated with IR and dyslipidemia for a given BMI [19, 20]. Reduced ability to recruit new adipocytes leads to an inappropriate, hypertrophic expansion of existing adipocytes and this is a

typical trait of high-risk individuals with a family history of T2D; FDRs [21]. This expansion is associated with adipose tissue fibrosis, infiltration and activation of pro-inflammatory immune cells, local and systemic IR, increased lipolysis and altered adipokine secretion [22, 23]. Furthermore, hypertrophic obesity is closely associated with expansion of the visceral fat depot, IR and T2D [24]. These events are summarized in Figure 2.

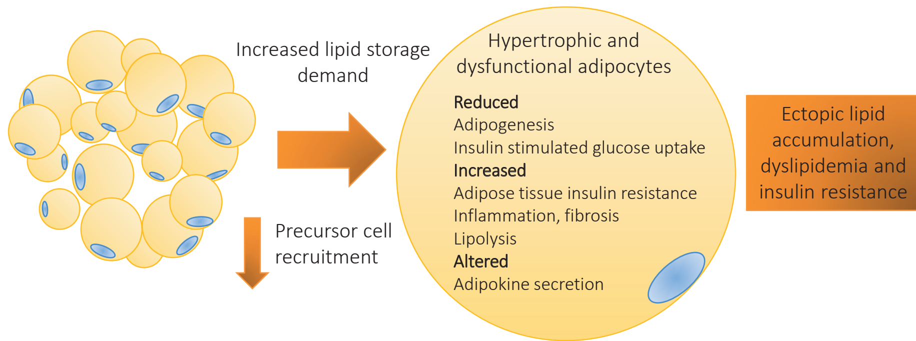

Figure 2. Characteristics of adipocyte hypertrophy. Adipocyte expansion with dysregulated subcutaneous (SubQ) fat promotes ectopic fat accumulation and the MetS. Adipocyte hypertrophy characterizes the SubQ fat of insulin-resistant obesity. Adapted from [31].

1.3.3 White adipogenesis

White adipocytes have a MSC origin, and MSCs provide a reservoir for adipose precursor cells, which can proliferate and differentiate into mature adipocytes [25]. Commitment and differentiation of white adipocytes is a complex process and all events are not yet known. One of the earliest events in the commitment of MSCs into the adipocyte lineage is repression of ZFP521 [26]. ZFP521 acts upstream of the PPARg transcriptional activator ZFP423 and inhibits its expression by repressing early B-cell factor 1 (EBF1) [27]. BMP4 is an important commitment factor into the white adipose lineage [25, 28], and in Paper I we show that BMP4 is secreted by mature adipocytes, probably as a positive feed-back regulator to recruit new adipocytes. ZFP423 is translocated to the nucleus following BMP4 activation and signalling [29]. Also EBF1 is localized to the nucleus and, together, EBF1 and ZFP423 activate PPARg, the master regulator of adipogenesis [29, 30]. This event commits the cells to the adipose lineage. Following PPARg activation, several transcription

factors are activated such as C/EBPb, d and a and several genes inhibiting adipogenesis are repressed [31]. Once expressed, PPARg and C/EBPa act in a positive feedback loop to maintain expression and drive terminal differentiation, including adipocyte-specific genes such as Fabp4, Glut4 and adiponectin, and induce cellular insulin sensitivity and lipid accumulation [31].

1.3.4 Lipid storage and release

Main functions of the white adipocytes are storage of energy, as TAG, and release of energy, as FFAs. In the fed state, adipocytes take up FFAs from the circulation via fatty acid transporters and glucose is taken up through GLUTs [32, 33]. TAG synthesis requires both glucose and FFAs and synthetized TAG is stored in lipid droplets [34, 35]. When energy substrates are needed, TAGs are hydrolyzed to glycerol and FFAs (lipolysis) to allow FFAs to reach other tissues [33]. Catecholamines (e.g. epinephrine) stimulate lipolysis through β-adrenergic signalling which increases cAMP and Protein Kinase A activity leading to increased lipolysis [33]. The three major lipolytic lipases are ATGL (the main TAG lipase), HSL (the main diacylglycerol lipase) and monoacylglycerol lipase [33]. Insulin, on the other hand, inhibits lipolysis, acting at several different levels [33]. Insulin-resistant adipocytes have increased lipolysis, resulting in increased circulating FFA levels, which contribute to ectopic lipid accumulation and IR in other tissues as discussed above.

1.3.5 Adipokine secretion

Another important function of the white adipocytes is secretion of adipokines with a wide range of functions that signal locally or systemically. Two of the best characterized adipokines are leptin and adiponectin. Leptin was discovered in 1994 and its discovery gave WAT a role also as an endocrine organ as circulating leptin signals centrally to reduce food intake [36, 37]. Leptin levels are increased in obesity and correspond to body fat mass [37]. Adiponectin is also secreted by mature adipocytes but is negatively correlated with fat mass. It is a well-established marker of insulin sensitivity and it exerts several different effects promoting metabolic health [36, 37]. Obese, adiponectin over-expressing mice have an improved diabetic phenotype, and reduced fat- and systemic inflammation (due to the healthy expansion of the SubQ WAT) [14]. A large class of adipokines are the immunoregulatory proteins. In the obese, hypertrophic state, adipocytes secrete pro-inflammatory cytokines like Tumor necrosis factor (TNF)a and IL6, promoting a local proinflammatory state, recruitment of proinflammatory immune cells and secretion of proinflammatory factors into the circulation [23]. In addition, TNFa and IL6 can directly induce IR in adipocytes [38].

1.3.6 Insulin-stimulated glucose uptake

The insulin signalling pathway that mediates glucose uptake is schematically depicted in Figure 3. Insulin receptor signalling is initiated by the binding of insulin to the extracellular α-subunits of the insulin receptor, which leads to auto-phosphorylation of the β-subunits and subsequent phosphorylation of Insulin receptor substrates (IRSs) [39]. IRS1 and 2 function as docking proteins recruiting and activating phosphoinositide 3-kinase (PI3K), leading to the generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) [39]. Membrane-bound PIP3 recruits and activates 3-phosphoinositide-dependent protein kinase 1 (PDK1), which phosphorylates and activates Protein Kinase B (PKB/AKT) [39]. However PIP3 can also directly activate AKT [4, 39]. AKT phosphorylates TBC1 Domain Family Member 1 (TBC1D1) and AS160/TBC1D4 which mediate glucose uptake through increased GLUT4 translocation to the membrane [39, 40].

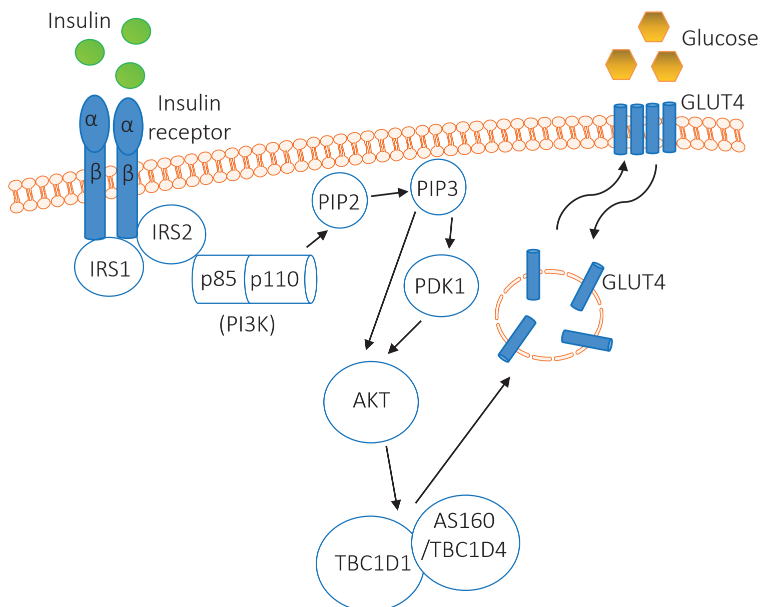

Figure 3. Overview of the insulin signalling pathway. Insulin receptor substrate 1, 2 (IRS1, IRS2), phosphoinositide 3-kinase (PI3K), phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylinositol 3,4,5-trisphosphate (PIP3), 3-phosphoinositide-dependent protein kinase 1 (PDK1), protein kinase B (PKB/AKT), TBC1 domain family member 1, 4 (TBC1D1, AS160/TBC1D4), glucose transporter 4 (GLUT4).

1.4 BAT and brown adipocytes

BAT consists of specialized, brown adipocytes which greatly differ from white adipocytes and are derived from stem cells able to differentiate into both brown adipose cells and skeletal muscles [41]. Brown adipocytes have a multilocular phenotype, contain numerous mitochondria, and have the capacity to burn glucose and fat to produce heat. Figure 4a and b show the different appearances of white (a) and brown (b) adipocytes. Figure 4c and d show beige adipocytes, which will be described in the next section.

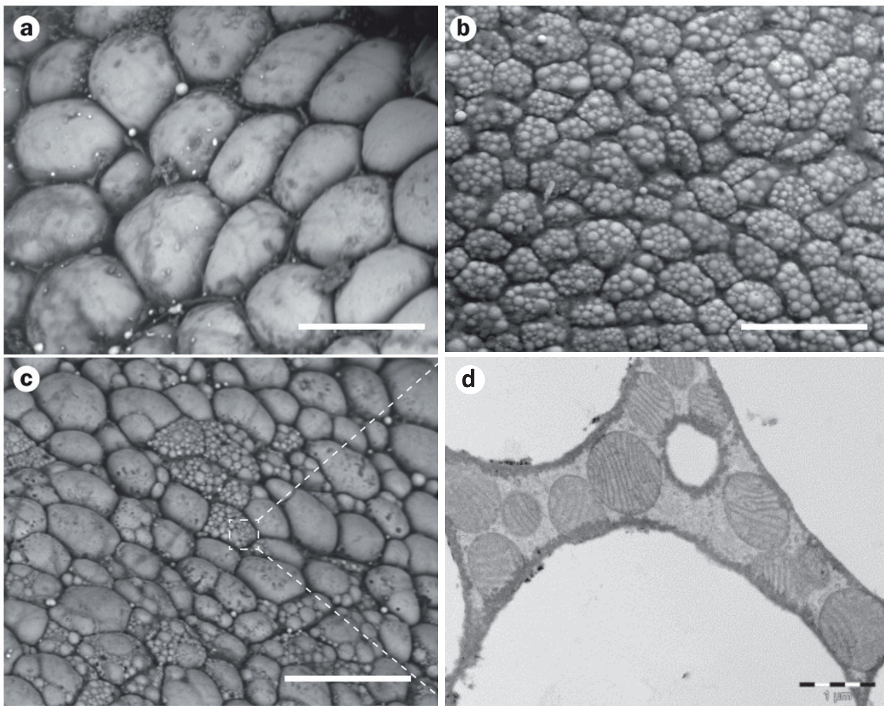

Figure 4. Electron micrographs demonstrate the characteristic appearances of different mouse adipose tissue depots. White adipocytes in subcutaneous (SubQ) white adipose tissue (WAT) contain a single lipid droplet (a). Brown adipocytes in brown adipose tissue contain multilocular lipid droplets (b). Browning leads to formation of islets of multilocular beige adipocytes within SubQ WAT (c), with increased mitochondrial content (d). Scale bars: a–c, 50 µm; d, 1µm. a-c are adapted from [66], d: SubQ WAT from AAV BMP4 mice, Cohort 2, Paper II.

The thermogenic function of brown adipocytes is mediated by the key protein UCP1, which allows uncoupled oxidation meaning that, in addition to generating adenosine triphosphate (ATP), UCP1 allows the proton gradient to flow back over the inner mitochondrial membrane resulting in heat generation [42]. BAT is present only in mammals, and in rodents, BAT is an essential organ throughout life [43]. Rodent BAT is located in distinct depots in the neck region. Humans are also born with substantial amounts of BAT in the neck-region but, commonly, BAT is lost with age. Rodent BAT and the BAT that humans are born with is often referred to as classical BAT (as compared to beige fat or recruitable BAT that is described below). It was not until 2009 that evidence emerged for the presence of active, UCP1-positive BAT also in adult humans [44-46] in the neck- and spinal region of patients examined with Positron Emission Tomography (PET) scans, albeit this BAT seems to differ from the classical BAT (further discussed below). Regardless, as BAT has the capacity to oxidize lipids instead of storing them, the potential of increasing numbers or activity of brown adipocytes is of great interest. The presence of BAT in adult man was found to be inversely correlated with age, BMI and circulating glucose levels suggesting the potential metabolic relevance of BAT in adults [44].

1.5 Beige adipocytes

In addition to white and brown adipocytes, a separate third variant, the beige/brite or recruitable brown adipocyte, has been described in the last couple of years in rodents [42, 47, 48]. It has been known for many years that some rodent WAT depots contain cells with a brown adipocyte appearance (UCP1 expression and multilocular phenotype) following cold-stimulation or pathways that increase cAMP [49]. The beige adipocytes appear white under basal conditions, have low basal UCP1 expression but have the capacity to induce UCP1 upon stimulation and oxidize lipids and drive thermogenesis [47, 48]. As the activity of beige adipocytes is inducible, and a number of recent mouse models have shown that induction of beige adipocytes are linked to improved metabolic health [50-52], beige adipocytes are gaining a lot of research interest. Figure 4 shows beige adipocytes in SubQ WAT (c-d) compared to white (a) and brown (b) adipocytes in mice.

1.5.1 *De novo* differentiation or transdifferentiation?

As opposed to white or classical brown adipocytes, the origin of beige adipocytes is debated in the literature. Generally, beige adipocytes are assumed not to share the Myogenic factor 5 (Myf5)-developmental lineage with classical, rodent brown adipocytes [41]. However recent results indicate that

there are some adipocytes also in WAT depots that have the Myf5-developmental origin [53]. Furthermore, the debate concerns whether beige adipocytes emerge following *de novo* differentiation, i.e. that they are a unique, committed cell type that undergo beige differentiation following stimuli, or if they are transdifferentiated, mature white adipocytes. Both ideas are supported in the literature.

In support of the *de novo* differentiation hypothesis, it was shown in 2012 that beige adipocytes arise from a separate population of isolated and cloned WAT-derived precursor cells [47]. These cells had unique marker genes distinguishing them from typical white or brown adipocytes and only the beige WAT-derived clones could induced UCP1. Moreover, the brown preadipocyte marker *Ebf2* has been found in a specific subset of SubQ WAT-derived precursor cells, and only this subset underwent browning following stimulation [54]. Furthermore, experiments using genetic adiponectin-driven labelling of adipocytes *in vivo*, showed that cold- or β-adrenergic agonist induced browning occurs to a great extent through *de novo* differentiation of (beige) precursor cells rather than transdifferentiation of mature white adipocytes [55].

On the other hand, several groups have provided evidence in favor of the transdifferentiation hypothesis. Also by using adiponectin-labelling of adipocytes *in vivo*, it was shown that cold exposure drives browning in SubQ WAT from mature white adipocytes through transdifferentiation [56]. Additionally, it has been shown that there is no or only a small change in DNA content or adipocyte number during browning of WAT, and that most beige adipocytes are derived from non-dividing cells in fat which makes the mature adipocytes the most probable source [57-60].

Altogether, the literature provides evidence that both sources of cells may contribute to browning of WAT. Interestingly, it has been shown that beige adipocytes lose their UCP1 expression, but remain in WAT during warm adaptation with a white-like morphology. In a second cold exposure, these cells can again induce UCP1 expression [61]. This study may suggest that the extent of beige cell recruitment through transdifferentiation or *de novo* differentiation may be dependent on the environmental history and/or age of the mouse.

1.5.2 Human brown/beige fat

Human infants are born with BAT that shares morphological and molecular characteristics of the classical, rodent BAT [62]. In adults, however, BAT is generally composed of both white and brown adipocytes and the gene expression pattern resembles more the rodent beige adipocytes than the classical, rodent brown adipocytes [47, 63, 64], although some studies have shown that adults do have classical BAT in some regions [65, 66]. In adults who initially lacked detectable BAT, prolonged cold exposure increases BAT activity in the neck region, which was associated with a decrease in body fat mass [67]. Given the inducible nature of rodent beige fat, it is likely that what we today call human BAT is more beige than brown.

1.6 Bone Morphogenetic Proteins

1.6.1 BMPs in development

BMPs are members of the Transforming growth factor beta (TGFb)-superfamily. Today, there are over 20 known BMPs [68] and the protein family was originally named after its ability to induce ectopic bone formation. BMPs have crucial and wide roles during development, e.g. dorsoventral patterning during embryogenesis involves incremental levels of BMP-2/4 and of BMP antagonists, which restrict cells to specific developmental lineages [25]. Also at later stages, BMPs are involved in tissue development and maintenance [69].

1.6.2 BMP signalling

BMPs are synthesized as precursor proteins with an N-terminal signal peptide, a prodomain for folding and secretion and a C-terminal mature protein. BMPs are cleaved to generate the mature proteins, which dimerize and interact with the receptors [69, 70]. BMPs can signal both through Smad-dependent and Smad-independent pathways. In the Smad-dependent pathway, BMPs bind cell surface receptors aggregated in a heterotetrameric complex, comprised of two type 1 receptor subunits and two type 2 receptor subunits [69]. When BMP ligands bind the receptor complex, the constitutively active type 2 receptors phosphorylate the intracellular domain of the type 1 receptors which will then phosphorylate the downstream substrate proteins; the receptor-regulated Smads (R-Smads). The R-Smads involved in BMP signalling are R-Smad1, 5 and 8. R-Smads associate with the co-mediator Smad (co-Smad) 4, and the complex translocates to the nucleus and regulates transcription [69, 71].

Ligand binding to BMP Receptor 1a (BMPR1a) is also able to initiate Smad-independent pathways, such as the p38 mitogen-activated protein kinases

(MAPK) signalling pathway. Briefly, the adapter protein X-linked inhibitor of apoptosis protein (XIAP) binds the receptor and allows binding of the TGFb activated kinase 1 (TAK1) and TGFb activated binding protein (TAB1), allowing subsequent activation of p38 MAPK and regulation of gene expression [68, 69, 71]. Mammalian cells have four different p38 MAPKs, initially described as transducers of stress responses (such as inflammation), but have since then been shown to be activated also following various non-stress stimuli [72]. The Smad-dependent and the Smad-independent pathways are summarized in Figure 5.

BMPs signal through three different type 1 receptors: BMPR1a (ALK3), BMPR1b (ALK6) and type 1A activing receptor (ActR1A or ALK2), and three different type 2 receptors: BMPR2, type 2 activin receptor A and B (ActR2A, 2B) [69]. The mechanism of receptor complex formation is intricate and varies with the BMP ligands, and a number of co-receptors and antagonists are also regulating the signal [68, 69].

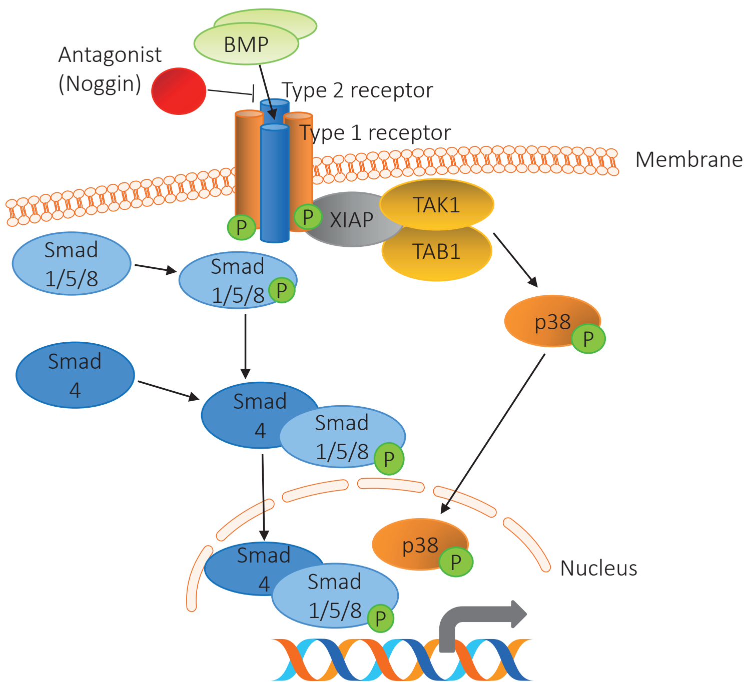

Figure 5. Overview of Smad dependent and Smad independent Bone Morphogenetic Protein (BMP) signalling pathways. X-linked inhibitor of apoptosis protein (XIAP), TGF-b activated binding protein (TAB1), TGF-b activated kinase (TAK1), p38 mitogen-activated protein kinase (p38). Adapted from [71].

1.6.3 BMP4 in adipogenesis

BMP4 is crucial for white adipogenesis. It is a key regulator of commitment of precursor cells into the white adipocyte lineage [28, 73, 74] by allowing nuclear localization of ZFP423 and *Pparg* activation [29]. BMP4 treatment of WAT SVF cells leads to a robust increase in adipocyte gene expression demonstrating their adipogenic commitment, shown in Paper I and in earlier work from our lab [74]. Moreover, in hypertrophic WAT, characterized by an impaired recruitment of precursor cells, adding BMP4 to the cultured precursor cells rescues adipogenesis [74]. In Paper I and in our earlier work, we have also shown that BMP4 is expressed and secreted by mature white adipocytes [74], likely as a signal to uncommitted cells to undergo adipogenic differentiation, thereby enhancing lipid storage capacity and preventing the development of a dysregulated hypertrophic expansion.

In addition to driving white adipogenesis, there is a growing body of evidence that BMP4 is also involved in the development of beige adipocytes. In a mouse MSC line, BMP4-treatment during adipogenic differentiation induced a beige phenotype and implantation of BMP4-treated MSCs into WAT in mice gave rise to UCP1-positive adipocytes [75]. Furthermore, transgenic mice, over-expressing BMP4 in the adipose tissue, had increased browning of WAT [50]. Likewise, in Paper II of this thesis, we show that lean mice undergoing BMP4 gene therapy had increased browning of WAT, increased energy expenditure and were protected against obesity. Also, in Paper I, we show that adding BMP4 and/or silencing the BMP antagonist Gremlin1 in human adipose precursor cells increases UCP1 and mitochondria. Similar effects of BMP4 on human adipose precursor cells have also been shown elsewhere [76].

Additionally, it was recently shown that BMP4 treatment of differentiating mouse brown preadipocytes *in vitro* led to increased lipid accumulation and a decreased brown fat-phenotype, and this was further confirmed *in vivo* with impaired BAT function following adenovirus BMP4-treatment [77]. The negative effect of BMP4 on BAT function and lipolysis was also seen in the BMP4 gene therapy-treated mice in Paper II.

1.6.4 BMP4 in obesity

Elevated circulating BMP4 levels are seen in obesity, and appear to be further increased in individuals with impaired glucose tolerance and T2D [77]. This study also shows increased circulating BMP4 in individuals with hypertrophic obesity, in agreement with our findings in Paper I. Specific genetic variants of the BMP receptors BMPR1A and BMPR2 are associated with obesity in man [78-80]. In this thesis, we introduce the concept of BMP4 resistance in obesity.

In Paper I, we found BMP4 secretion to be increased by differentiated hypertrophic human adipocytes. However, the effect of BMP4 was regulated by the secreted BMP antagonist Gremlin1 which was also increased. The findings in Paper I are in agreement with our findings in mice in Papers II and III where endogenous *Bmp4* is increased in WAT of obese mice. However, we show that in mice, it is the BMP antagonist Noggin that is increased with obesity (rather than Gremlin1 in human cells), and both the expression of *Bmp4* and Noggin were associated with markers of IR.

1.6.5 p38 MAPK in adipogenesis

Increased p38 MAPK signalling following BMP-stimulation has been implied to play a role in adipogenesis in MSCs and in 3T3-L1 cells [50, 81]. However, it is currently not clear if increased p38 MAPK signalling is beneficial or not for adipogenesis. p38 MAPK activity is important for proper 3T3-L1 differentiation and *Pparg* activation is impaired when p38 MAPK is inhibited [82, 83]. In other reports, using isolated mouse preadipocytes, the inhibition of p38 MAPK was beneficial for adipogenesis as p38 MAPK inhibits *C/ebpb* and *Pparg* expression in those cells [84]. In brown adipocytes from mice, cAMP-induced activation of p38 MAPK was shown to induce PGC1a and UCP1 and p38 MAPK activation was actually suggested to be necessary for the cold-induction of UCP1 [85]. Increased p38 MAPK signalling was also the proposed mechanism behind the increased browning of WAT in the fat-specific BMP4 over-expressing mice [50]. In Paper III, we show increased p38 MAPK signalling in WAT following BMP4 gene therapy, together with a phenotype of enhanced adipose cell terminal differentiation and insulin signalling. However, short-term incubation of adipose cells with BMP4 did not increase p38 MAPK. Thus, we cannot conclude that the phenotype is a result of increased p38 MAPK signalling by BMP4.

1.7 Adeno-associated viral vector mediated gene therapy

WT AAVs are naturally occurring viruses but until recently they have not been a topic of interest due to the lack of pathogenicity without a helper virus [86]. However, the understanding of AAVs potential in gene therapy has sparked much interest. WT AAVs integrate in the genome, however the recombinant *AAV vectors* are designed to lack the gene responsible for integration and are, therefore, unable to integrate in the host genome and remain inside the cell preferentially as episomes [87]. They are also replication deficient, and other attractive traits of the AAV vectors are their structural simplicity, low inflammatory response and their capacity to deliver transgenes into targeted

post-mitotic host cells and to drive this transgene expression for an extended period of time [88]. AAV vectors exist in 11 different serotypes, with different tropism. Serotype two is the best characterized and serotype eight has the greatest liver transduction efficiency [89]. In Papers II and III we used AAV vectors of serotype eight to target the liver of the mice. The effectiveness of AAV vector gene therapy is becoming more and more well-characterized [90-92] and they are also used in a number of clinical trials. Briefly, AAV vectors have been used in humans to transduce cells in the retina [93], the central nervous system [94, 95], the liver [96], skeletal- [97] and cardiac muscle [98].

2 AIMS

The over-all aim of this thesis was to investigate the effect of BMP4 on white, beige and brown fat and the effect of BMP4 gene therapy on phenotype and metabolism in mature mouse models *in vivo*.

In Paper I we aimed to characterize the effect of BMP4 in regulating white and beige adipogenesis in human WAT precursor cells *in vitro* and its relation to obesity and adipose cell size of the donors.

In Paper II we aimed to examine the effects of BMP4 gene therapy in mature mouse models including effects on development of obesity, whole-body insulin sensitivity, energy expenditure and browning of WAT.

In Paper III we aimed to examine the possible effect of BMP4 gene therapy in treating, rather than preventing, obesity in mice.

3 METHODS

3.1 Ethical statement

The Ethical Committee of the University of Gothenburg approved the study design and written informed consent was received from study participants prior to inclusion in the study. Likewise, approval from the local Ethics Committee for Animal Studies at the Administrative Court of Appeals in Gothenburg was obtained prior to performing any animal experiments.

3.2 Tissue donors and samples

The human material used in Paper I was biopsies, isolated adipocytes and precursor cells from SubQ WAT. The material was obtained through needle biopsies from the abdominal SubQ depot of the study participants, except for one biopsy that was obtained through bariatric surgery. All subjects had normal glucose levels and had no known chronic disease. In Papers II and III, male C57BL6/N mice were purchased from Taconic. They were group caged, maintained on a 12 hour light-dark cycle in a temperature-controlled facility (+21°C), and had ad libitum access to food and water. During studies, the mice were fed either HFD (45 kcal% fat) or nutrient-matched CD (10 kcal% fat).

3.3 The AAV BMP4 mouse model

3.3.1 AAV vector-mediated, liver-specific BMP4 over-expression

In this mouse model, which was used in Paper II and III, the goal was to deliver the *Bmp4* transgene to the liver to have BMP4 protein secreted and reach target tissues. We used recombinant AAV vectors of serotype eight and the transgene expression was under control of the human Alpha 1-Antitrypsin (*hAAT1)* promoter, a combination described previously [90]. The AAV vectors were produced in the laboratory of Fatima Bosch, with the method described briefly in Papers II and III and in referenced literature [99]. For the control mice, an empty AAV vector was produced. The AAV vectors were mainly delivered intravenously and subsequently reached the liver. However, the AAV vectors were injected retro-orbitally in Cohort 1 in Paper II but all other animals had tail vein-injections. All mice received 5 x 10^{11} viral particles/200 ul saline/mouse. Upon termination, we verified that the vectors induced BMP4 in the liver and increased circulating BMP4 levels. Thus, the BMP4 over-expression was stable for the full duration of the study.

3.3.2 Metabolic phenotyping

The mice in Paper II and III underwent weighing and food intake was recorded. Metabolic phenotyping was performed as described below. Upon termination, the mice were euthanized using isofluorane. Blood was collected through heart puncture for analysis of metabolites. Tissues were weighed and either snap frozen in liquid nitrogen, fixed in 4% formaldehyde or incubated in sterile Hank's balanced salts medium prior to digestion (WAT only, method described below).

Glucose- and insulin tolerance tests

A GTT was performed to assess the systemic clearance of glucose (determined by insulin secretion and action). Briefly, a bolus dose of glucose is given by I.P. injection, and blood glucose and insulin levels are monitored before and repeatedly during the test [100]. During the ITT, a bolus dose of insulin is given by I.P. injection and blood glucose levels are monitored before and repeatedly during the test. The decrease of blood glucose during the ITT shows the systemic insulin sensitivity [100]. Both tests were performed following 4 hours fasting. More specific details of the tests are given in Paper II and III.

Body composition

Analysis of body composition, through measurement of total body fat, lean body mass and bone mineral density and –content was performed using a DEXA [101] (Paper II).

Indirect calorimetry

O_2 consumption, CO_2 production and activity were assessed through an Indirect Calorimetry analysis using the SOMEDIC Metabolic System [102]. Briefly, O_2 consumption and CO_2 production were calculated by measuring the difference in chamber inlet and outlet of gasses. Indirect calorimetry was performed on mice in Papers II and III and the values were normalized to body weight. A MiniMitter Probe was inserted surgically in the peritoneal cavity of the mice, which allowed concurrent monitoring of activity and body temperature. The measurements were performed both during the light- and dark-phase of the day. More details can be found in Papers II and III.

3.4 *In vitro* experiments

3.4.1 Isolation of adipocytes, precursor cells and their differentiation

WAT biopsies, both human and mouse, were treated as described in detail in Paper I, II and III. Adipocyte cell size was measured as described, and SVF cells following WAT digestion were seeded and expanded [38, 74, 103] or harvested for gene expression analysis. We performed various treatments and transfections. SVF cells were induced to differentiate at passages three to four with an adipocyte differentiation cocktail as described [38]. After three days, media was replaced with adipocyte maintenance media, renewed every third day throughout the differentiation period, and cells were cultured for up to 21 days for full differentiation.

3.4.2 Cell respiration

In Paper II, we measured cell respiration in live cells using an extracellular flux analyzer (Seahorse Bioscience; Agilent Technologies). Prior to the analysis, primary mouse SubQ WAT preadipocytes were differentiated for seven days with and without BMP4 and with and without 24 hours adrenergic stimulation with isoproterenol. The cellular respiration analysis yielded data on basal respiration, ATP production, uncoupled respiration and maximum respiration of the cultured cells. More details can be found in Paper II.

3.4.3 mRNA analysis

RNA was isolated from cells or homogenized tissues using commercially available kits optimized for the various starting materials. The isolated RNA was used to synthesize cDNA for the subsequent qRT-PCR reaction. In all Papers, the TaqMan system for qRT-PCR gene expression analysis was used. Gene expression of the ribosomal housekeeping gene 18S rRNA was used to normalize the data.

3.4.4 Protein analysis

Cell and tissue proteins

Isolated adipocytes were separated from the media by centrifugation through dinonyl phthalate oil. Protein lysis buffer was added to cells and tissues, which were then rocked at 4°C for 2h. The samples were centrifuged and the supernatant, containing the protein lysate, separated and protein concentration determined. Specific proteins were separated and analyzed with and without

prior immunoprecipitation using conventional immunoblotting procedures in all three Papers.

ELISA

Serum concentrations of Insulin, Adiponectin and Serum Amyloid A isoform 3 (SAA3) (Paper II and III), and secreted levels of BMP4 and Gremlin1 during preadipocyte differentiation (Paper I) were quantified using ELISA techniques as described in the Papers.

Histology and IHC

Histology and IHC are commonly used techniques to visualize tissue morphology and tissue/cell proteins. The techniques are usually performed on fixed samples, commonly using paraformaldehyde which crosslinks proteins resulting in increased rigidity of the samples. We used hematoxylin-eosin morphology staining in Paper II and III [104]. Additional stainings used were Mitotracker staining of mitochondria (Paper I), LipidTOX Red staining of lipids (Paper II) and Picosirius Red staining of collagen (Paper II). IHC was used for visualization of specific proteins in cells or tissues. Details of the IHC-techniques used are described in Papers I and II. In Paper II, TEM was performed to visualize mitochondria in SubQ WAT, essentially as described [105].

3.5 Statistical analyses

The experimental data are presented as means ± the standard error of the mean (SEM). Student's t-test or Mann-Whitney non-parametric U-test were used for comparisons of two groups and ANOVA was used for comparisons of several groups. In Paper I, Students t-test or one-way ANOVA (with Tukeys post-hoc test) were used. In Paper II, Students t-test, non-parametric Mann-Whitney U-test or one-way ANOVA (with Bonferronis post-hoc test) were used where indicated. Additionally, Spearman's nonparametric correlation coefficient was used to measure dependence between variables. In Paper III, Students t-test or non-parametric Mann-Whitney U-test were used where indicated. Statistics were calculated using IBM SPSS Statistics version 20, GraphPad Prism version 6.03 for Windows, or Microsoft Excel. $P<0.05$ was considered statistically significant.

4 RESULTS

In this section, the main results from Paper I, II and III are summarized. Details of the results can be found in the separate Papers.

4.1 BMP4 and BMP antagonists regulate human white and beige adipogenesis (Paper I)

In Paper I, we characterized the effect of BMP4 and BMP antagonists in regulating white and beige adipogenesis in human WAT in relation to obesity. We found BMP4 to be induced during adipose cell differentiation, and also secreted by differentiated mature adipocytes. Hypertrophic obesity is characterized by impaired precursor cell recruitment and, as BMP4 is an adipogenic commitment factor, our initial hypothesis was that hypertrophic adipose tissue had reduced BMP4 levels. In contrast, BMP4 secretion was increased by hypertrophic adipose tissue and correlated positively with adipocyte size of the donors. However, the precursor cells exhibited a resistance to BMP4 due to increased expression and secretion of the BMP antagonist Gremlin1. This was also increased in hypertrophic obesity and correlated positively with adipocyte size. Gremlin1 is rapidly down-regulated when adipose cell differentiation is initiated in precursor cells and follows the same time course as *PPARg* induction.

Addition of BMP4, and/or silencing (si)Gremlin1, increased *PPARg* and *ZFP423* mRNA, thus indicating a role of Gremlin1 in regulating the proadipogenic effects of BMP4. Furthermore, siGremlin1 promoted preadipocytes to assume an oxidative beige/brown adipose phenotype with increased mitochondrial biogenesis and UCP1. Enhancing white adipose cell differentiation inhibited the beige/brown markers but siGremlin1 and/or adding BMP4 prior to the white adipose cell differentiation re-activated the beige/brown phenotype markers.

In summary: BMP4 is secreted by mature white adipose cells, likely part of a feed-back mechanism to recruit new adipocytes. BMP4 secretion is increased in hypertrophic obesity similar to the BMP antagonist Gremlin1, resulting in a BMP4 resistance in the adipose precursor cells. BMP4 regulates both white and beige adipogenesis. Enhanced BMP4 signalling, either by increased BMP4 itself and/or by inhibiting the BMP antagonist Gremlin1, promotes

beige/brown differentiation. A schematic view of the findings in Paper I are summarized in Figure 6.

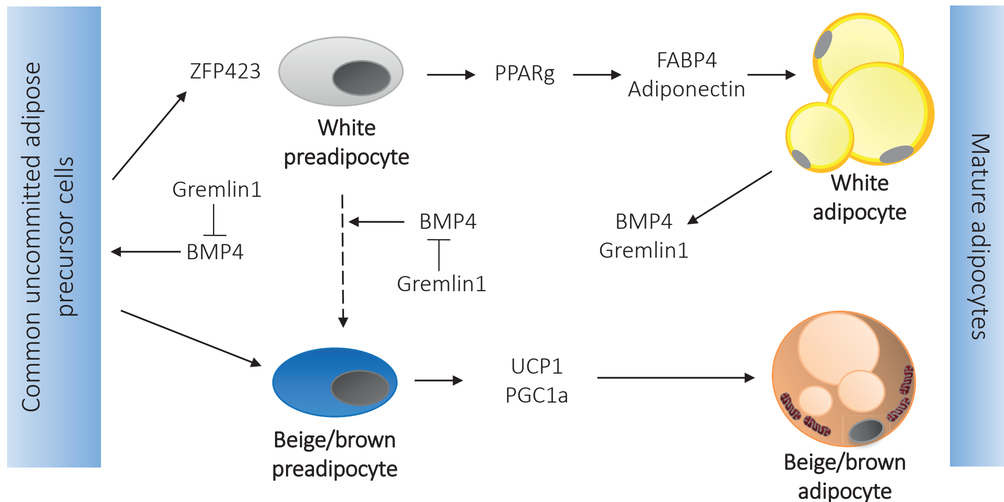

Figure 6. Schematic view of the findings in Paper I, on the regulation of commitment and differentiation of human subcutaneous adipose precursor cells by BMP4 and Gremlin1. BMP4 increases during differentiation and exerts effects as a secreted molecule. This effect of BMP4 enhances commitment and differentiation of new preadipocytes as a positive feed-back mechanism to prevent adipose cell hypertrophy. The effect of BMP4 is regulated by the endogenous BMP inhibitors, and in human adipose cells, Gremlin1 regulates the effect of BMP4 on both white and beige/brown adipocyte differentiation. Bone Morphogenetic Protein 4 (BMP4), Zinc finger protein 423 (ZFP423), Peroxisome proliferator-activated receptor gamma (PPARg), Fatty-acid binding protein 4 (FABP4), Uncoupling Protein 1 (UCP1), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a).

4.2 BMP4 gene therapy in mature mice reduces BAT activation but protects from obesity by browning subcutaneous adipose tissue (Paper II)

In Paper II, we investigated if we could influence the browning of WAT in adult, lean mice through BMP4 gene therapy using AAV BMP4 vectors (serotype eight). The vectors targeted the liver and increased hepatic and circulating BMP4 levels. Following vector administration, the mice were fed CD or HFD for several weeks before phenotyping.

AAV BMP4 treatment induced pronounced browning of the SubQ WAT, with increased number and size of mitochondria, increased UCP1 expression, increased whole-body energy expenditure and the mice were protected from HFD-induced obesity. BMP4-treated mice fed CD had unchanged body weight but, like the HFD-treated mice, improved insulin sensitivity. Furthermore, we confirmed our findings of increased browning by BMP4 in differentiating mouse SubQ WAT preadipocytes *in vitro* together with increased respiration. There was no change in adipocyte numbers in WAT of the AAV BMP4-treated mice, and no increase in endothelial cell markers, supporting that browning of WAT following AAV BMP4 treatment is likely a result of transdifferentiation of mature adipocytes.

Unexpectedly, the AAV BMP4 mice had increased lipid accumulation, decreased UCP1 and increased expression of white adipose cell genes in BAT suggesting that BMP4 treatment impairs BAT function. However, regardless of these negative effects in BAT, the AAV BMP4 mice had increased whole-body energy expenditure which prevented obesity.

Similar to the results in Paper I, endogenous *Bmp4* was increased in WAT of obese mice. This was accompanied by increased expression of the BMP antagonist Noggin (rather than Gremlin1 as in human WAT). Both *Bmp4* and Noggin mRNA levels were associated with measures of degree of insulin sensitivity supporting BMP4 resistance also in WAT of obese mice.

In summary: Increased circulating levels of BMP4 in adult, initially lean mice prevent HFD-induced obesity and IR through increased browning of SubQ WAT combined with increased energy expenditure. In contrast, BMP4 reduces BAT activation showing the importance of browning the large SubQ WAT in preventing HFD-induced obesity in these mice. Lastly, WAT in obese mice is

characterized by BMP4 resistance, similar to what was found in human hypertrophic obesity (Paper I) but Noggin, rather than Gremlin1, was increased in obese mice. Figure 7 shows a schematic view of the findings in Paper II.

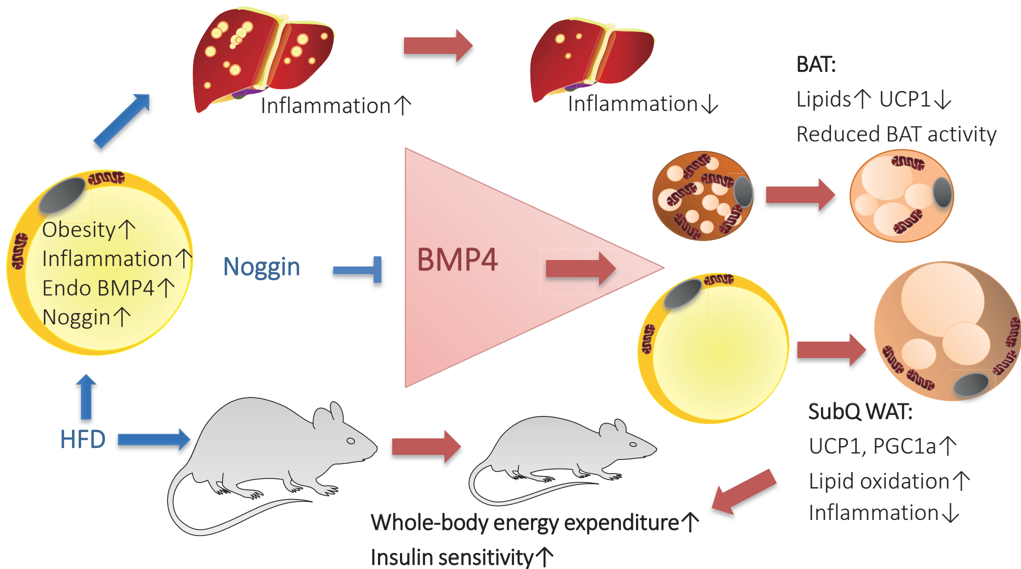

Figure 7. Schematic view of the findings in Paper II. Obesity leads to BMP4 resistance also in mice with increased expression of endogenous BMP4, and of the BMP antagonist Noggin. By increasing BMP4 signalling, the subcutaneous (SubQ) white adipose tissue (WAT) undergoes browning, whereas the brown adipose tissue (BAT) has increased lipid accumulation and reduced activity. Despite the effects in BAT, whole-body energy expenditure is increased, which is protective against obesity and associated consequences. Endogenous (Endo), High fat diet (HFD), Bone Morphogenetic Protein 4 (BMP4), Uncoupling Protein 1 (UCP1), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a).

4.3 BMP4 gene therapy improves insulin resistance in obese mice without effects on adipose tissue browning or body weight (Paper III)

Based on our findings in Paper II, we examined if AAV BMP4 gene therapy in mature mice reduces already established obesity. In this study, we used the same AAV BMP4 vectors (serotype eight), in older, HFD-induced obese mice who continued on HFD following vector administration. As before, AAV BMP4 treatment resulted in increased hepatic and circulating BMP4 levels.

Interestingly, the positive effects of AAV BMP4 on browning SubQ WAT in initially lean mice was not seen in initially obese mice. BMP4 gene therapy did not reverse established obesity or increase whole-body energy expenditure. However, whole-body insulin sensitivity and glucose tolerance were improved despite the lack of effect on body weight. Adipocyte insulin signalling was enhanced in the AAV BMP4-treated mice with increased IRS1 protein levels and insulin-stimulated pAKT activation. Circulating adiponectin levels were also increased which, combined with increased SubQ WAT adiponectin and G-protein coupled receptor 120 (*Gpr120*) mRNA levels, support that BMP4 enhanced terminal differentiation of the adipocytes. p38 MAPK signalling was also increased in SubQ WAT of BMP4-treated mice but this was not reproduced by short-term BMP4 incubations *in vitro*.

BAT lipid accumulation was increased in both obese groups and there was no further increase in lipid accumulation following BMP4 therapy and UCP1 levels were also unaltered. Thus, obesity by itself produced a similar effect in BAT as seen in the lean mice treated with BMP4 in Paper II. mRNA levels of endogenous *Bmp4* and the BMP antagonist Noggin were increased in both SubQ WAT and BAT in the obese mice and pSMAD1/5/8 signalling was reduced. Together, these findings further support the concept of adipose tissue BMP4 resistance in obesity and that this also includes BAT.

In summary: Obesity is a state of adipose tissue BMP4 resistance, likely due to increased Noggin in mice. There was no change in body weight, browning of SubQ WAT or in energy expenditure in initially obese mice receiving AAV BMP4 gene therapy. However, BMP4 therapy did increase whole-body insulin sensitivity and improved adipose cell insulin signalling. This positive effect of BMP4 is apparently not resistant in the SubQ WAT and may be a consequence of p38 MAPK activation and/or enhanced adipose cell terminal differentiation. The findings in Paper III are summarized in Figure 8.

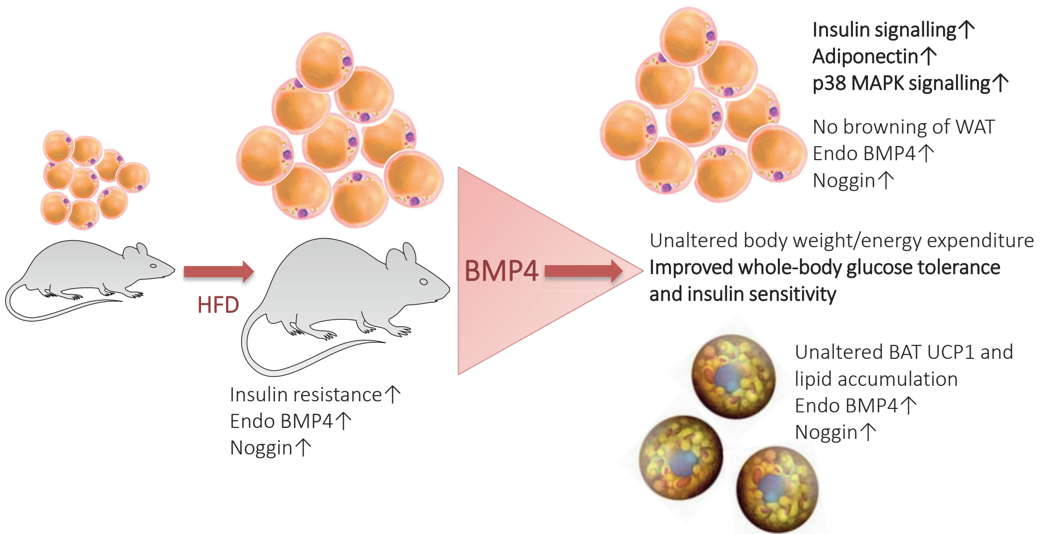

Figure 8. Schematic view of the findings in Paper III. Obesity leads to insulin resistance and BMP4 resistance with increased expression of endogenous BMP4, and of the BMP antagonist Noggin. BMP4 gene therapy given to obese mice does not drive browning of the white adipose tissue (WAT), but whole-body glucose tolerance and insulin sensitivity are improved. Adiponectin and adipocyte insulin signalling are also increased, and so is p38 mitogen-activated protein kinases (p38 MAPK) signalling in WAT, suggestive of additional actions of BMP4, independent of browning. Endogenous (Endo), High fat diet (HFD), Bone Morphogenetic Protein 4 (BMP4), Uncoupling Protein 1 (UCP1).

5 DISCUSSION

Obesity and its associated complications are increasing at an epidemic rate, which imposes a major economic burden on society and vast impacts on quality of life for afflicted individuals. Therefore, increased knowledge to develop new therapeutic strategies is warranted. In this thesis, we have studied BMP4 and its effects in adipose tissue and the main results are comprehensively discussed below.

5.1 BMP4-induced browning of WAT

Probably the most important finding in this thesis is the AAV BMP4-induced browning of SubQ WAT in lean, mature mice, which increases whole-body energy expenditure and is protective against diet-induced obesity. We and others have previously shown that BMP4 is an important commitment factor for mesenchymal precursor cells into the white adipose lineage and induction of PPARg [25, 28, 29, 73, 74]. In this thesis, we show the importance of maintained BMP4 signalling under adipogenic differentiation and, in the mature SubQ WAT, to direct the adipose cells towards a beige adipocyte phenotype.

WAT browning in rodents occurs through two different mechanisms; either by transdifferentiation of existing adipocytes or by *de novo* recruitment of committed adipocytes [42, 55, 61]. When increasing BMP4 signalling during *in vitro* differentiation of human or mouse preadipocytes in Papers I and II, or in WAT in adult mice in Paper II, adipocyte development is directed towards a beige and oxidative phenotype. Our data show the flexibility during SubQ adipocyte differentiation and in the mature SubQ WAT. We do not provide firm proof of classical transdifferentiation in the mature mouse models; i.e.; BMP4-induced browning of mature adipocytes, since we did not genetically label the adipose cells initially. However, we do provide evidence that BMP4 re-directs preadipocytes towards a beige phenotype *in vitro* and in mature WAT in lean mice with minimal fat expansion when BMP4 is provided long after the pool of SubQ adipose precursor cells is established [55]. Moreover, we did not find an increased adipocyte number in WAT following browning and endothelial/precursor cell markers (indicative of proliferation of precursor cells) were unaltered. Taken together, our data strongly support that BMP4 drives transdifferentiation of white adipocytes to induce browning. Surprisingly, when we gave AAV BMP4 vectors to obese mice, we did not induce WAT browning or weight loss. Endogenous BMP4 antagonists are

increased in the adipose tissue in obesity, which inhibit the effects of BMP4, and this is further discussed below.

Regarding the mechanisms for the AAV BMP4-induced browning, sympathetic input was apparently unaltered in WAT (and BAT) following AAV BMP4 treatment, which supports a local rather than a central effect of BMP4, consistent with recent findings by Modica et al in BAT [77]. Importantly, a recent study demonstrated that ZFP423 acts as a molecular brake on adipocyte thermogenesis by inhibiting the brown-fat transcription factors *Ebf2* and PR domain containing 16 (*Prdm16*) in white adipocytes [106]. It was shown that deletion of *Zfp423* increased EBF2-mediated browning *in vivo* in mature adipocytes and mice. Additionally, it was shown *in vitro* that ZFP423 and EBF2 form a complex in white adipocytes, which is disrupted by BMP7 and BMP4, allowing EBF2 to drive the thermogenic gene program [106]. This is a likely and attractive mechanism also in the SubQ WAT of our AAV BMP4 mice and experiments to test this hypothesis are currently being carried out.

Today, many mouse models of WAT browning have been described. There are three lines of evidence that emphasize that beige adipocytes in rodents do contribute to energy expenditure and can protect against weight gain. Firstly, a strong correlation between WAT browning potential and susceptibility to diet-induced obesity has been identified by using different inbred mouse strains [107, 108]. UCP1 levels in classical BAT did not differ between the strains, supporting the potential of WAT browning. Secondly, induction of beige adipocytes contributes to non-shivering thermogenesis upon ablation of BAT, indicating that beige adipocytes compensate when BAT is diminished [109]. Lastly, several mouse models with increased browning of WAT are protected against diet-induced obesity by activating various signalling pathways. The classical model is cold-exposure, where norepinephrine is released from the SNS which increases β-adrenergic signalling [42]. Other models include transcriptional activators of the brown adipose gene program (PRDM16 [110] and Forkhead box C2 [105]), immune pathways (IL-4 [111] and IL-33 [112]) and other secreted factors such as fibroblast growth factor 21 [52], and BMP4 in our AAV BMP4 gene therapy mice.

Targeting white adipocytes pharmacologically in man to induce browning is an intriguing concept. However, the absolute majority of research on WAT browning is done in rodents. Human BAT has been proposed to have more in common with beige fat, rather than classical BAT, and the presence of human BAT is diminished with increasing age and BMI [44, 46, 47, 113]. Browning of WAT in man has been described in severe medical conditions and strains

such as cancer cachexia [114], following severe burns leading to prolonged adrenergic stress [115], and in hyperthyroidism and thyroid hormone treatment [116]. It has been shown that subjects who initially lacked active BAT could recruit BAT upon moderate, daily cold-exposure, or by ingestion or capsinoids (also activating the SNS), and the BAT recruitment was associated with decreased body fat mass [67]. Moreover, in Paper I we show that increasing BMP4 signalling in human preadipocytes drives a beige phenotype, thus confirming that targeting this pathway may become a future therapeutic also in man. Importantly, the finding that BMP4 controls browning through direct effects on adipose cells, without increased adrenergic stimulation, is beneficial from a future therapeutic point-of-view as β-adrenergic mediated weight loss in man has proven to be a problematic route [117].

5.2 BMP4-induced "whitening" of BAT

Until recently, BAT was not a known target of BMP4. However, as we and others have discovered lately, BMP4 does target BAT as well as WAT. BMP4 signals through a specific set of receptor isoforms [118] and there is only a small difference in their expression in white compared to brown preadipocytes [119]. Despite the evidence that BMP4 drives browning of WAT, BMP4 actually impairs the development of the classical brown phenotype of mouse cells *in vitro* and *in vivo* by reducing HSL, increasing lipid accumulation and disrupting the thermogenic capacity, which results in reduced BAT activity and energy expenditure [50, 77]. Interestingly, it was also found that when BMP4 was administered systemically, energy expenditure was increased [77]. When comparing those results to our mice in Paper II, the phenotypes are similar as we found whole-body energy expenditure to be increased following AAV BMP4 treatment due to browning of WAT, despite BAT being impaired with increased lipids and reduced UCP1. These findings point out two important things. Firstly, the effect of BMP4 is cell-type dependent in terms of increasing or decreasing the oxidative capacity in primary white or brown adipocytes. Secondly, these findings underscore the metabolic potential of browning WAT and are in line with the previously mentioned mouse model with compensatory browning of WAT upon ablation of BAT [109]. These results are important since the majority of adult humans have very little BAT and it is largely of a beige rather than classical brown phenotype [47, 64].

The opposite effects of BMP4 on WAT and BAT are interesting in light of the increased circulating BMP4 levels seen in human obesity as shown in Paper I and by others [77, 120]. As BAT is inversely correlated with age and BMI [44], increased circulating BMP4 may drive "whitening/beigeing" of BAT during

ageing or obesity. This is similar to what we see in BAT from lean AAV BMP4-treated mice in Paper II, where increased circulating BMP4 levels induce an "obese/beige phenotype" in BAT with increased lipids and decreased UCP1. However, as we saw in obese mice in Paper III, increased BMP antagonists in obesity may also induce BMP4 resistance in BAT which reduces BMP4-mediated "whitening/beigeing" compared to what we saw in lean AAV BMP4-treated mice.

5.3 BMP4 resistance in the adipose tissue in obesity

In this thesis we have introduced the concept of BMP4 resistance in obesity. We show that BMP4 is increased in the expanding WAT and it is also increased in circulation [77, 120], likely as a positive feed-back mechanism to promote recruitment of new adipocytes as BMP4 is an important commitment factor for the white adipose lineage [25, 28, 29, 73, 74]. However, we show that endogenous BMP antagonists are also increased in WAT in obesity. In man, Gremlin1 is increased, whereas in mice Noggin is increased, resulting in reduced BMP4 signalling through the Smad1/5/8 pathway. Interestingly, WAT mRNA expression and genetic variants of BMPR1a and BMPR2 are associated with obesity in man [78, 79]. Very little is known about the regulation and functions of the BMP antagonists in mature tissues and in WAT specifically. In Paper I, we provide the first evidence, to the best of our knowledge, of their regulation in WAT upon weight gain. Gremlin1 is somewhat more well-characterized than Noggin as it is involved in other pathways, including inflammation and fibrosis [121, 122], and in regulating angiogenesis in a BMP-independent manner [123].

In human WAT precursor cells and in WAT in lean mice, maintaining functional BMP4 signalling is sufficient to promote browning of WAT. This is further supported by the positive effects of silencing Gremlin1 in the human WAT precursor cells. To further characterize the effect of AAV BMP4 gene therapy, we also treated obese mice but did not see the same effects on browning WAT and this was associated with BMP4 resistance defined as reduced activation of pSMAD1/5/8 signalling. This is likely explained by the increased Noggin levels in both obese groups which antagonize BMP4. Additionally, the impaired BAT phenotype that we saw in the BMP4-treated non-obese cohort was less different between the obese treated and non-treated mice in Paper III. Like in WAT, Noggin levels were increased in BAT in both obese groups of mice, suggesting a BMP4 resistance also in BAT in obesity. However, it should be emphasized that the BMP4 resistance following BMP4

treatment/obesity is relative and pronounced activation of SNS by cold or massive β3-adrenergic stimulation clearly activated BAT and reduced the lipids. Further studies are required to elucidate if BMP4 regulates peripheral adrenergic sensitivity in BAT cells but we did not see any evidence of impaired activation of lipolysis by isoproterenol in white adipocytes in Paper II.

p38 MAPK is another putative down-stream mediator of BMP4 signalling [50]. Surprisingly, p38 MAPK signalling was not resistant in obesity, as p38 MAPK was increased in WAT following AAV BMP4 treatment of obese mice. Noggin antagonizes BMP signalling by inhibiting BMP- and receptor-interaction [124]. However, structural alterations in the BMP receptor complex formation allow Smad1/5/8- or p38 MAPK signalling [125], which suggests that Noggin acts at the level of inhibiting the Smad1/5/8 pathway specifically and allowing p38 MAPK signalling.

Based on these findings, we suggest that as a future therapeutic to increase WAT browning and energy expenditure in obesity, it is more strategic to target and reduce Gremlin1 in WAT, rather than increasing overall BMP4 signalling. Moreover, p38 MAPK signalling is not resistant and increased BMP4 is sufficient to induce the specific insulin-sensitizing effects also in obese mice.

5.4 BMP- and insulin signalling

Throughout all the cohorts, the AAV BMP4 mice have increased insulin sensitivity compared to controls. In the HFD-fed AAV BMP4 mice in Paper II, the large difference in body weight is a major confounder for the improved glucose homeostasis. However, both the CD-fed, lean AAV BMP4 mice in Paper II and the obese mice in Paper III have increased whole-body insulin sensitivity, despite similar body weights as their controls. This indicates that the improved insulin sensitivity is independent of, and not secondary to, weight loss following browning of WAT. The increased insulin sensitivity was also observed in HFD-fed transgenic mice over-expressing BMP4 in the adipose tissue and with similar body weights as the WT control mice [50]. In the lean, AAV BMP4-treated mice in Paper II, and in the transgenic BMP4-mice [50], one can speculate that the improved insulin sensitivity is caused by increased glucose uptake by beige or brown adipocytes. Cold-activation increases glucose uptake in BAT [126], which may also be the case upon browning of WAT. However, the obese mice in Paper III do not have increased browning, yet the mice have a clearly improved whole-body insulin sensitivity and glucose tolerance indicating other insulin sensitizing pathway(s). Furthermore, the obese AAV BMP4 mice had improved insulin signalling in adipocytes, increased markers of adipocyte differentiation, and increased circulating

adiponectin, an established marker of insulin sensitivity and metabolic health [127]. It should be added that we also did not see any differences in GLUT4 expression in cells from mice treated with BMP4.

Additional evidence for crosstalk between the BMP- and insulin signalling pathways has been reported. BMP7-treatment of MSCs leads to a coordinated regulation of several components of the insulin signalling pathway [128]. Recently, it was shown that ablation of BMPR1a in fat improves age-related insulin resistance in mice [129]. These data are somewhat contradictory to ours, as ablation of BMPR1a decreases BMP signalling, whereas we increased BMP4 signalling in our mice. However, ablation of BMPR1a caused a BMP-mediated change in immune cell infiltration in fat promoting the insulin sensitive phenotype [129] while we did not see any differences in markers of the immune-phenotype in fat in our mice.

p38 MAPK is a downstream target of BMP4 and p38 MAPK has also been proposed as a target in IR. Increasing p38 MAPK in skeletal muscle has positive effects on insulin-stimulated glucose uptake [130, 131], and activation of p38 MAPK in the liver of obese mice enhances blood glucose regulation [132], supporting a role of p38 MAPK in antagonizing IR. However, we cannot conclude that the increased insulin sensitivity is a direct result of increased BMP4/p38 MAPK signalling. We did not see any direct effects by short-term BMP4 incubations *in vitro* on the p38 MAPK- or insulin signalling pathways, but this may require longer exposure times and/or different BMP4 concentrations. p38 MAPK has also been implicated in adipogenesis, both as a positive and negative factor depending on context [82-84]. One can speculate that long-term BMP4 stimulation *in vivo* targets p38 MAPK improving adipocyte terminal differentiation, which is reduced in obesity and associated with IR [38]. This is supported by our finding of increased expression of other markers of terminal differentiation of the adipose cells including adiponectin and *Gpr120*. Taken together, our results support a crosstalk between BMP4- and insulin signalling, either directly or via enhanced terminal adipocyte differentiation, and these observations warrant further investigations.

5.5 Other BMPs also regulate metabolism

Also other BMPs than BMP4 are involved in regulating adipose tissue and metabolism. BMP2 is, similar to BMP4, a commitment factor for the white adipose lineage [133]. BMP8B increases BAT thermogenesis both locally and when administered centrally and knock-out mice have reduced metabolic rate which causes weight gain [134]. BMP7 regulates brown adipogenesis and increases energy expenditure [119]. Recently, also BMP3b and BMP9 have

been implied in browning of WAT [135, 136]. In addition to regulating brown adipogenesis directly, BMP7 has central effects in suppressing food intake [137]. However, in the same experimental setting, it was shown that BMP2, which in many ways resembles BMP4, did not have a direct effect on appetite-regulation [137]. The lean, AAV BMP4-treated mice in Paper II had increased food intake. However, we strongly favor this to be secondary to the increased energy expenditure. When the browning-phenotype is lost in the obese AAV BMP4 mice in Paper III, food intake is also unaltered supporting the important role of the increased energy expenditure. This is also in line with the above-mentioned data regarding BMP2 [137].

5.6 AAV-mediated gene therapy

We here used a gene therapy approach, with AAV vectors that increase circulating BMP4 in adult mice. AAV-mediated gene therapy is efficient in long-term gene transfer in animal models [90-92] and it has also been used in clinical trials for human diseases [93-98]. Regarding animal models in research, AAV-mediated gene therapy has several benefits. Firstly, vector administration is easy, and it is less time- and mouse-consuming compared to the use of transgenic mice. Secondly, by targeting a specific tissue in adult mice, one reduces the risk of confounding results at several levels. The vectors are administered at a specific time-point, which gives clear results and avoids early developmental effects. Our AAV BMP4 mice received vectors as adult mice, making the observed phenotype also more interesting from a therapeutic point-of-view compared to genetically manipulated models. Additionally, gene expression of the AAV vector delivered transgene is controlled by a cell- or tissue- specific promotor, which provides additional control of the transgene expression. We think that the gene therapy models we have developed here, using mature animals, provide important insight into the potentials of BMP4 signalling and action as targets for therapy in human obesity and T2D.

6 CONCLUSION

Taken together, we show that BMP4 and its antagonists are important regulators of beige, white and brown adipose tissue. BMP4 secretion is increased in WAT in obesity but its effect is antagonized by the induction of endogenous BMP inhibitors; Gremlin1 in man and Noggin in mice. These inhibitors are increased in hypertrophic obesity and may contribute to this development by inhibiting commitment of adipogenic precursor cells. BMP4 gene therapy in adult, initially lean mice leads to increased circulating levels of BMP4, browning of WAT, increased whole-body energy expenditure and prevention of HFD-induced obesity. However, obesity is a state of adipose tissue BMP4 resistance, and BMP4 gene therapy does not enhance browning of WAT when given to initially obese mice. Yet, WAT terminal differentiation may be enhanced since adiponectin levels are increased. Importantly, we also found that BAT activation is antagonized by BMP4 leading to a more beige, less metabolically active phenotype as also seen in human BAT in obesity. Taken together, the current results suggest the possibility that the association between obesity and impaired BAT activation seen in human studies may be a consequence of the increased BMP4 secretion by WAT leading to the impaired BAT activation and its assumption of a beige phenotype rather than impaired BAT activation leading to obesity in man.

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