WISP2 – A Novel Adipokine Related to Obesity and Insulin Resistance

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Cover illustration: WISP2 decreases the lipid accumulation in mature adipocytes (by John Grünberg)

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“I may not be there yet, but I'm closer than I was yesterday.”

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ABSTRACT

Type 2 diabetes mellitus (T2D) is increasing worldwide at an epidemic rate and is expected to reach 592 million inflicted individuals by 2035 as compared to 382 million in 2013. Obesity is a major risk factor for insulin resistance, defined as an impaired cellular effect of insulin, and this precedes the development of T2D. Around 85% of subjects with T2D are overweight or obese. However, the obesity-associated insulin resistance is not a direct consequence of an increased fat mass per se but rather a reduced ability to recruit new subcutaneous adipose cells following weight gain and the associated dysregulated, inflamed and insulin-resistant adipose tissue characterized by enlarged adipose cells (hypertrophic obesity).

The adipogenic potential of human pre-adipocytes differs between donors and this is related to cell size and maintained activation of WNT-signaling in precursor cells. The canonical WNT pathway allows the mesenchymal stem cells to proliferate and prevents them from committing to the adipocyte linage. We identified a novel secreted "adipokine" induced by WNT activation, WNT1 inducible signaling pathway protein 2 (WISP2). WISP2 is preferentially expressed in mesenchymal precursor cells and links hypertrophic obesity with canonical WNT-signaling. We found transcriptional activation of *WISP2* in the subcutaneous adipose tissue to be a marker of the obesity-associated metabolic complications including degree of insulin resistance, ectopic fat accumulation and hypertrophic obesity. Mechanistically, we found canonical WNT signaling/WISP2 to regulate adipogenic commitment and differentiation in two different ways; - intracellular WISP2 retains the PPARγ transcriptional activator ZFP423 in a cytosolic complex which, when dissociated by BMP4, allows nuclear entry of ZFP423, induction of PPARγ and commitment into to the adipose lineage and; - as a secreted molecule, WISP2 enhances cell proliferation and inhibits

adipocyte differentiation by activating canonical WNT signaling and, thereby, inhibiting PPARγ activation.

To investigate the effect of WISP2 in vivo, we generated a transgenic mouse model overexpressing WISP2 in the adipose tissue under the aP2-promoter. We found WISP2 to be secreted by the adipose tissue and present in serum. The mice had a similar body weight but were characterized by improved insulin sensitivity, increased circulating levels of adiponectin and the novel FAHFA lipids and increased *Glut4* in both adipose tissue and skeletal muscle. They were also characterized by markers of increased mesenchymal stem cell growth and development with a markedly expanded BAT, a ”healthy” hyperplastic subcutaneous adipose tissue and increased lean body mass. Serum from the Tg mice also increased the proliferation of both brown adipose precursor cells and the mesenchymal stem-like CH3T101/2 cells and this was inhibited by adding specific anti-WISP2 monoclonal antibodies to the serum.
Taken together, WISP2 is a novel secreted autocrine/endocrine regulator of mesenchymal stem cell growth and proliferation as well as their adipogenic commitment. There is important cross-talk between WISP2 and BMP4 in the regulation of adipogenic commitment and differentiation and BMP4 is also a regulator of *WISP2* transcriptional activation. WISP2 is a novel target in hypertrophic obesity and the Metabolic Syndrome.

Keywords: Adipose tissue; BMP4; Canonical WNT pathway; Insulin Resistance; Obesity; PPARγ; Type 2 Diabetes; WISP2

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SAMMANFATTNING PÅ SVENSKA

Typ 2 diabetes ökar med epidemisk hastighet världen över och förväntas nå 592 miljoner drabbade år 2035, jämfört med dagens 382 miljoner (6,4 % av den svenska befolkningen).
Insulinresistens är ett förstadium till typ 2 diabetes och innebär en nedsatt förmåga att svara på insulin som till exempel att ta upp socker från blodet. Insulinresistens orsakas av ett samspel mellan genetiska och omgivningsfaktorer såsom övervikt, ohälsosam livsstil och rökning.
En starkt bidragande orsak till insulinresistens och typ 2 diabetes är en allt mer utbredd övervikt hos befolkningen. Idag är ca hälften av alla vuxna män, en tredjedel av alla kvinnor och vart femte barn överviktiga eller obesa. Många upplever övervikt som ett problem och det ökar även risken för flertalet sjukdomar såsom typ 2 diabetes . Ca 85 % av alla som drabbas av typ 2 diabetes är överviktiga eller obesa, men ca 30 % av obesa individer är metaboliskt friska. Orsaken till varför vissa överviktiga individer, men inte alla, drabbas av metabola komplikationer är bland annat kopplat till en nedsatt mognad och funktion av fettvävnaden varvid det bildas få, men stora fettceller, så kallad hypertrofisk fettansamling. Detta leder i sin tur till att fett ansamlas på platser i kroppen som normalt inte lagrar fett, dvs ektopisk fettansamling, vilket inkluderar fettansamling i buken, levern, skelettmuskel, hjärtat och runt blodkärlen. Detta orsakar lipotoxicitet med flera metabola komplikationer, inklusive insulinresistens.

För att motverka hypertrofisk fettansamling krävs nybildning av fettceller när behovet att lagra fett ökar. Detta sker från så kallade mesenkymala stamceller som kan ge upphov till både fettceller, muskelceller, benceller och broskceller. Första steget kallas ”comittment”, och innebär att stamcellerna bara kan utvecklas till en av dessa typer av celler. Nästa steg inom utvecklingen av fettceller innebär att de omogna cellerna genomgår differentiering och utvecklas till mogna fettceller med förmåga att lagra fettsyror och utsöndra olika proteiner, så kallade adipokiner. Dessa processer är komplicerade och strikt reglerade genom ett samspel av flera olika molekylära signaleringsvägar. WNT signalen är en signaleringsväg av stor betydelse för fettcellernas differentiering och som måste stängas av för att tillåta att mesenkymala stamceller mognar ut till fettceller.

Vi har nyligen identifierat ett nytt sekretoriskt protein, WISP2, som framför allt finns och utsöndras av mesenkymala stamceller och som aktiveras av WNT-signaleringen. Det faktum att WISP2 utsöndras till blodet gör att det kan vara en viktig kommunikatör mellan fettväven och andra vävnader och därmed är extra intressant. Vi har visat att förekomsten av WISP2 i fettväven kan kopplas samman med flera riskfaktorer för typ 2 diabetes, att dess förekomst ökar i stora ”hypertrofa” fettceller samt är relaterat till nedsatt insulinkänslighet.
Förmågan att differentiera human fettceller skiljer sig åt mellan individer och detta är försämrat vid förekomsten av ”hypertrof” fetma och insulinresistens. Vi har nu visat att en bibehållen aktivering av WNT-signalen och en ökad förekomst av WISP2 är kopplat till en nedsatt mognadsgrad av fettcellerna. Vidare har vi visat att WISP2 kan

förhindra fettcellsutmognaden och på så sätt bidra till den nedsatta fettvävsfunktion som är kopplad till metabola komplikationer. Vi har funnit att det är två underliggande molekylära mekanismer som regleras av WISP2 och som involverar reglering av såväl ”comittmentsteget” som differentieringen via bland annat de regulatoriska proteinerna ZNF432 och BMP4.

För att ytterligare studera effekten skapade vi en transgen musmodell med ett fettvävsspecifikt överuttryck av WISP2. De transgena mössen visade sig vara skyddade mot de negativa metabola förändringar som en högfett-diet med påföljande fetma normalt leder till. Flera faktorer visade sig vara inblandade i den skyddande effekten av WISP2, vilka till stor del verkar vara kopplade till en ökad förmåga hos de transgena mössen att rekrytera och differentiera mesenkymala stamceller. Förutom en ökad muskelmassa hade de transgena mössen väsentligt mer brunt, energibrännande fett och en så kallad ”frisk” och väl differentierad vit fettväv med många små celler (hyperplastisk fettväv) istället för den mer ofördelaktiga, hypertrofa fettväven. Vidare fann vi att insulinkänsligheten och glukosupptaget var förbättrat i både fettväven och skelettmuskulaturen och att detta var kopplat till en ökad sekretion av adiponectin, ett protein som frisätts av fettväven och som tidigare visats vara associerat med förbättrad insulinkänslighet, samt ett ökat uttryck av glukostransportören GLUT4. Ytterligare ett intressant fynd som kunde påvisas var att de transgena mössen uppvisade ökade nivåer av de nyligen identifierade gynnsamma fettsyrorna FAHFA som både förbättrar insulinsekretion och insulinkänslighet och som är anti-inflammatoriska.

Merparten av de förändringar som dokumenteras i de transgena mössen kan förklaras genom en ökad förmåga att rekrytera mesenkymala stam celler. Denna hypotes kunde vi bekräfta genom att serum från de transgena mössen ledde till en ökad tillväxt av mesenkymala celler *in vitro* och att effekten försvann då vi tillsatte blockerande, specifika WISP2 antikroppar.

Sammantaget, presenteras i denna avhandling bevis för att WISP2 är en ny adipokin som utsöndras från fettväven och som har möjlighet att påverka celler i dess omgivning med huvudmål att påverka mesenkymala stamcellers tillväxt och utmognad. Detta leder också till en förbättrad och insulinkänslighet och metabolism. WISP2s förmåga att reglera mesenkymala stamceller är en intressant upptäckt som också kan leda till ny läkemedelsutveckling mot 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. Hammarstedt A, Hedjazifar S, Jenndahl L, Gogg S, Grünberg JR, Gustafson B, Klimcakova E, Stich V, Langin D, Laakso M, Smith U. **WISP2 regulates preadipocyte commitment and PPARγ activation by BMP4**
Proceedings of the National Academy of Sciences of the United States of America 2013; 110(7): 2563-2568

II. Grünberg JR, Hammarstedt A, Hedjazifar S, Smith U. **The novel secreted adipokine WNT1-inducible signaling pathway protein 2 (WISP2) is a mesenchymal cell activator of canonical WNT**
Journal of Biological Chemistry 2014; 289(10), 6899-6907

III. Grünberg JR, Hoffmann JM, Hedjazifar S, Nerstedt A, Jenndahl L, Castellot J, Wei L, Movérare Skrtic S, Bäckhed F, Syed I, Saghetelian A, Kahn B, Hammarstedt A, Smith U. **Increased brown fat and insulin sensitivity in obese mice overexpressing WISP2 in the adipose tissue**
Manuscript

CONTENT

ABBREVIATIONS

2DOG	2[^{14}C]deoxyglucose
2DOG-6P	2[^{14}C]deoxyglucose-6-phosphate
aP2	Adipocyte protein 2
APC	Adenomatous polyposis coli
α-SMA	α -smooth muscle actin
BAT	Brown adipose tissue
BCA	Body composition analysis
BMI	Body mass index
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
CCN	CTGF, Cyr61, Nov family
cDNA	Complementary DNA
cEBP	C/CAAT enhancer-binding protein
ChREBP	Carbohydrate-responsive-element-binding protein
CTGF	Connective tissue growth factor
Cyr61	Cysteine-rich angiogenic inducer 61
DEXA	Dual energy X-ray absorptiometry
DKK1	Dickkopf 1
DNL	De novo lipogenesis
DVL	Dishevelled
EDL	Extensor digitorum longus muscle
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
eWAT	Epididymal white adipose tissue
FABP4	Fatty acid binding protein 4
FAHFA	Fatty acid esters of hydroxy fatty acids
FDR	First-degree relative
FFA	Free fatty acids
FZD	Frizzled
GIR	Glucose infusion rate
GLUT4	Glucose transporter type 4
GSK3β	Glycogen synthase kinase 3 beta
GTT	Glucose tolerance test
HFD	High fat diet
HIF	Hypoxia inducible factor
hMSC	Human mesenchyme stem cells
IBMX	Isobutylmethylxanthine
IHC	Immunohistochemistry
IP	Immunoprecipitation
ITT	Insulin tolerance test

JNK	c-Jun N-terminal kinases
KRM	Kremen 1/2
LFD	Low fat diet
LRP5/6	Low-density lipoprotein-receptor-related protein-5 or -6
MAPK	Mitogen-activated protein kinases
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOV	Nephroblastoma overexpressed
PDGF	Platelet-derived growth factor
PPARγ	Peroxisome proliferator activator receptor gamma
PTT	Pyruvate tolerance test
qRT-PCR	Quantitative real-time polymerase chain reaction
RQ	Respiratory exchange quotient
SAT	Subcutaneous adipose tissue
SDS	Sodium dodecyl sulfate
sFRP	Secreted Frizzled-related protein
SREBP-1	Sterol regulatory element-binding protein 1
SVF	Stromal vascular fraction
sWAT	Subcutaneous white adipose tissue
T2D	Type 2 diabetes mellitus
TBX1	T-box protein 1
TCF/LEF	T-cell factor/lymphoid enhancer factor
TCP7L2	Transcription factor 7-like 2
Tg	Transgenic mice
TGFβ	Transforming growth factor beta
TMEM26	Transmembrane protein 26
TNFα	Tumor necrosis factor alpha
TSP1	Thrombospondin-1
TZD	Thiazolidinedione
UCP-1	Uncoupling protein-1
UPR	Unfolded protein response
VCO_2	Carbon dioxide production
VO_2	Oxygen consumption
VSMC	Vascular smooth muscle cells
VWC	von Willebrand factor type C domain
WAT	White adipose tissue
WIF1	WNT inhibitory factor 1
WISP	WNT1 inducible signaling pathway protein
WNT	Wingless-type MMTV integration site family members
wt	Wildtype mice
ZFP423	Zinc-finger protein 423

1 INTRODUCTION

1.1 Prevalence of type 2 diabetes

Type 2 diabetes mellitus (T2D) is increasing worldwide at an epidemic rate and is expected to reach 592 million inflicted individuals by 2035 as compared to 382 million in 2013 (6,4% of the Swedish population) and where the vast majority lives in low- and middle-income countries. The health cost for diabetes was expected in 2010 to be 12% of the world's total health expenditure and in Sweden the cost per person with diabetes was in 2010 predicted to be more than 4000 USD. The global health expenditures for diabetes in 2030 will be 30-34% larger than those of 2010. These results show that this epidemic imposes a major economical burden on the society worldwide and prevention efforts are needed (1,2).

1.2 Obesity, type 2 diabetes mellitus and insulin resistance

Obesity in both men and women is associated with a greater risk of developing chronic diseases like T2D, hypertension, cancer and heart disease and it is increased with increasing body mass index (BMI). World Health Organization has categorized BMI as: underweight ≤18.5, healthy weight range ≤ 24.9, overweight ≤ 29.9 and obese as ≥ 30. Compared with healthy weight, overweight/obese men and women have a 3.5-4.6/10.0-11.2 relative risk of developing diabetes over a 10-year period and more severe obesity with a BMI > 35.0 has a 17.0-23.4 risk (3).

About 85% of patients with T2D are overweight or obese (4). However, about 30% of obese individuals are metabolically healthy and, conversely, around 20-30% of normal weight individuals develop these metabolic abnormalities (5,6).

Insulin resistance is an essential component of T2D. Basically, this means reduced insulin sensitivity, i.e., effect of insulin to lower plasma glucose by suppressing hepatic glucose production and stimulating glucose uptake/utilization in peripheral tissues. Insulin resistance leads to higher glucose levels in the blood (hyperglycemia), which makes the glucose sensing β-cells in the pancreas secrete more insulin to compensate for the imbalance (hyperinsulinemia). Enhanced insulin secretion can compensate for insulin resistance and enhanced insulin sensitivity can compensate insulin secretory defect (7).

Obesity is associated with toxic cellular effects such as increased inflammation, ER-stress, production of reactive oxygen species, mitochondrial dysfunction, ectopic accumulation of lipids/triglycerides and activation of serine-threonine kinases.
The inflammation is a low-grade chronic inflammation that can be considered as an abnormal immune reaction triggered by nutrients or other intrinsic cues. Usually it is referred to as meta-inflammation or para-inflammation and it occurs in metabolically important organs such as liver and adipose tissue.
Together, these responses contribute to the insulin resistance in the liver, skeletal muscle, adipose tissue and β-cells. Obesity-induced metabolic impairments then lead to a vicious cycle where excess nutrients trigger an inflammatory response that enhances insulin resistance, placing a greater demand on the β-cells. Eventually, this and other factors promote β-cell dysfunction leading to insufficient insulin secretion and hyperglycemia (8-10).

1.3 Metabolic syndrome & abdominal obesity

The metabolic syndrome is a collection of risk factors that together are associated with a higher risk for cardiovascular diseases and T2D. These risk factors are elevated plasma glucose, dyslipidemia, hypertension, a prothrombotic profile, and a state of inflammation. Elevated fasting or postprandial plasma glucose fall under the range of either pre-diabetes or diabetes. Dyslipidemia includes elevated very low-density lipoprotein-triglycerides and decreased high-density lipoproteins. A prothrombotic profile suggests impairments in procoagulant factors, anti-fibrinolytic factors, platelet abnormalities and endothelial dysfunction. An inflammatory state is illustrated by increased circulating cytokines and acute phase reactants (11,12).
The major underlying risk factors of the metabolic syndrome are obesity and insulin resistance. Risk association with obesity is measured as waist circumference, and not primarily BMI, to assess visceral/abdominal obesity. Therefore, it is not only degree of obesity that influences the risk of metabolic disturbances but also where the fat is accumulated. Excess visceral adipose tissue, reflected as increased abdominal girth or waist-to-hip ratio, is an important factor for the correlation between metabolic aberrations and obesity rather than the amount of subcutaneous abdominal fat (7,8,12,13). This has raised the question about the difference between different adipose tissue depots.

1.4 Adipose tissue distribution and metabolic complications

Human adipocytes can expand about 20 fold in diameter and several 1000-times in volume (5) and the subcutaneous cell size can differ markedly between individuals with the same BMI and amount of fat. The number of cells in the adipose tissue is set after puberty with a 10% annual replacement and the turnover rate is lower in individuals with enlarged cells (14-16). The subcutaneous adipose tissue has a limited expandability and when the subcutaneous adipose tissue expands, due to excess energy intake, it can be accomplished in two principally different ways; either by expanding the existing adipocytes (hypertrophy) or by recruiting new cells (hyperplasia).
Hypertrophic obesity is associated with local inflammation and a dysregulated and insulin- resistant adipose tissue. When the subcutaneous adipose tissue storage capacity is exceeded, lipids will accumulate in non-adipose organs, i.e., the so-called ectopic fat accumulation. This includes intra-abdominal and visceral areas, liver, skeletal muscle, heart and around vessels (5,12,16,17). Importantly, a genetic predisposition for T2D, defined as being a first-degree relative (FDR) to individuals with T2D, is associated with inappropriate hypertrophy of abdominal subcutaneous adipose cells even in non-obese individuals indicating an impaired subcutaneous adipogenesis (18).

The increased size of the adipose cells triggers release of stress signals and hypoxia can occur when vascularization is insufficient for the growing adipose tissue. ER-stress is triggered by hypoxia or excess nutrients, leading to an unfolded protein response (UPR). In the ER, proteins are translated, folded and checked for quality before they are released. These functions are decreased during ER-stress, and the number of misfolded proteins increases. This triggers the UPR, which activates genes involved in producing, folding, modifying and degrading proteins to decrease the ER-stress. UPR also triggers activation of stress and inflammatory pathways and production of cytokines that alters the insulin-signaling pathway (5,9). Stressed and enlarged adipocytes also attract different immune cells including macrophages. This leads to a positive feedback loop where infiltrating macrophages recruit more immune cells and introduce a chronic state of inflammation, the meta-inflammation pathway. Dysfunctional, hypertrophic adipose tissue produces more inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-6. Some of these cytokines contribute to the insulin resistance and defect adipose tissue function (5,9,10,12,19).

Dysfunctional fat in hypertrophic obesity is also associated with increased fibrosis of the adipose tissue, which also causes activation of stress-related pathways, inflammation and ectopic lipid accumulation (20).
The visceral fat is less insulin-sensitive and lipolytically more active than subcutaneous fat and is therefore considered to release more free fatty acids (FFA). Elevated FFAs in the peripheral circulation can interfere with the insulin signaling pathway in target tissues leading to increased insulin resistance (7,12,13). However, it is probably not the FFA per se that enhance insulin resistance but rather their metabolites such as long-chain acyl-coenzyme A, diacylglycerol and ceramides (5).

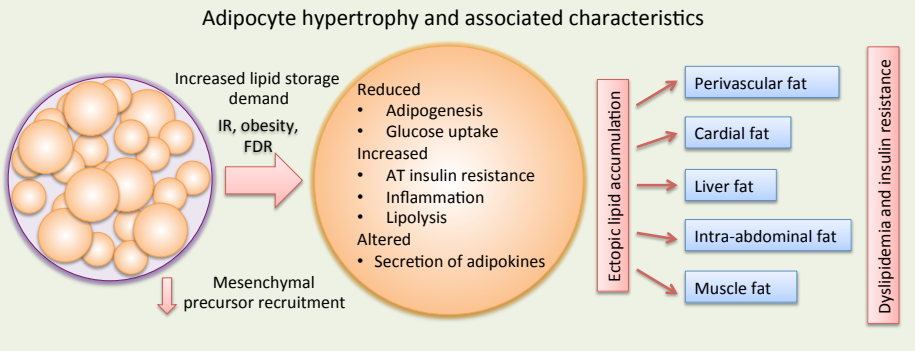

Figure 1. Adipocyte expansion with a dysregulated subcutaneous adipose tissue (SAT) promotes ectopic fat accumulation and the Metabolic Syndrome. Adipocyte hypertrophy characterizes the SAT of insulin-resistant (IR) obesity and first-degree relatives (FDR) of individuals with type 2 diabetes (Modified from(21)).

1.5 Adipose tissue

1.5.1 Precursor cells in the adipose tissue

The adipose tissue in mammals mainly consists of 2 types of fat: white adipose tissue (WAT) and brown adipose tissue (BAT). There are also mixed areas known as brown in white "brite" or beige adipose tissue. WAT and BAT displays many similarities but WAT mainly stores excess energy, whereas BAT generates heat through mitochondrial uncoupling of oxidation (10,20,22).
The adipose tissue does not only consist of adipocytes and its precursor preadipocytes, but also mesenchymal stem/precursor cells, immune cells, fibroblasts and vascular cells. Adipocytes develop from multipotent mesodermal stem cells residing in the adipose tissue. This process, i.e.

adipogenesis, can be divided into two related steps. Firstly, during determination/commitment the mesenchymal stem cells loose their ability to differentiate into other mesenchymal linages and become committed to preadipocytes. They are now no longer able to transform into osteoblasts, myocytes or chondrocytes. Secondly, the preadipocytes differentiate to become mature adipocytes, acquiring lipids droplets and gain the ability to respond to hormones such as insulin and catecholamines (20,23,24).

1.5.2 Adipogenesis

In order to commit mesenchymal stem cells to the adipocyte linage, the bone morphogenetic protein (BMP) family member 4 plays a key role (25-27).
BMP is part of the transforming growth factor beta (TGFβ) superfamily and signals through BMP receptors 1 and 2, which phosphorylate SMAD1/5/8. Phosphorylated SMAD1/5/8 forms a complex with SMAD4 that translocates to the nucleus and activates specific genes. BMPs have many cellular antagonists and, for instance, Noggin can block differentiation to the adipocytic linage by binding to BMP4 and prevent receptor activation (22,25,28). BMP4 induces adipogenic commitment by binding to zinc-finger protein 423 (ZFP423), a transcriptional activator of peroxisome proliferator activator receptor gamma (PPARγ), via a SMAD-binding domain (29).
Committed preadipocytes have the potential to be terminally differentiated to mature lipid-accumulating adipocytes. This process involves a series of well-characterized steps which have been extensively studied *in vitro*. This includes mitogenic clonal expansion followed by a well-coordinated activation of several transcription factors and where cEBPβ and cEBPδ are upregulated followed by PPARγ together with C/CAAT enhancer-binding protein alpha (cEBPα). PPARγ activates the promoter of cEBPα, and vice versa, which creates a positive feedback loop in order to maintain the differentiated state. This is followed by expression of genes that characterize the mature adipose phenotype and are involved in insulin sensitivity, lipogenesis and lipolysis such as lipoprotein lipase, adipocyte protein 2 (aP2) / fatty acid binding protein 4 (FABP4), the insulin receptor and glucose transporter type 4 (GLUT4). Sterol regulatory element-binding protein 1 (SREBP-1) is also activated by cEBPβ and cEBPδ, which regulates lipogenic genes and can activate PPARγ by enhancing expression as well as promoting production of endogenous ligand. However, the identification of a definitive endogenous PPARγ agonist has not yet been successful (22,30-33).
Some inhibitors of adipogenesis are proinflammatory molecules such as TNF-α (10,28,31), growth factors such as platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF) (31,34). Many of these inhibitory effects are mediated through mitogen-activated protein kinases

(MAPK) including extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases. ERK1 is necessary for the proliferative phase of differentiation but needs to be downregulated during the terminal differentiation stage (30,31). MAPK are also regulated by the canonical wingless-type MMTV integration site family members (WNT) signaling (35).

1.5.3 Brown adipose tissue

BAT consists of highly specialized cells which waste energy through heat production in the mitochondria. BAT contains many tightly packed mitochondria where the BAT-specific protein, uncoupling protein-1 (UCP-1), catalyzes a proton leak from the inner membrane which uncouples substrate oxidation from ATP-synthesis.
The most powerful and physiological stimulus to activate BAT is cold exposure or hormonal stimuli, like β-adrenergic agonists. (36-38).
BAT is present in all placental mammals and human babies have brown fat depots which gradually reduce in size with aging. However, recent data showed that classical BAT with UCP-1 positive cells does exist in the supraclavicular and spinal regions of adult human. Human BAT consists of both brown and white fat cells and data indicates that the brown adipose tissue found in adult man has the molecular markers resembling murine beige fat more closely than classical brown fat (37,39-41).

1.5.4 Beige adipose tissue

Within the white adipose tissue, a new type of adipose cells has been found. They have been named beige or brite (brown in white) cells and have a brown-like phenotype but probably a common origin with the white adipose cells. Beige adipose cells have the characteristics of dissipating energy through generation of heat like BAT, when stimulated. Beige cells are similar to, but not identical to BAT cells, with lower levels of BAT genes such as UCP-1, low uncoupled respiration and larger/unilocular lipid morphology comparable to white adipose cells (20,41,42). Inducible browning effect seems to be reversible; beige adipose cells can switch from being energy storing to become energy-dissipating and back again, depending on environmental conditions or stimuli (42-44).

1.6 Canonical WNT

1.6.1 Canonical WNT signaling

The canonical WNT pathway inhibits mesenchymal stem cells from committing to the adipocyte lineage and terminal differentiation. WNT signaling also restrains adipocyte differentiation by inhibiting the expression of PPARγ and cEBPα. These transcription factors are induced directly by cEBPβ and cEBPδ in response to adipogenic stimuli, which also serve to switch off the canonical WNT pathway (23,45-47).
Canonical WNT signaling regulates mesenchymal stem cell fate. Activation of WNT signaling promotes entry of mesenchymal precursor cells into the myocyte and osteocyte lineages while suppressing commitment to the adipocytic lineage and terminal differentiation (23,45,46,48).

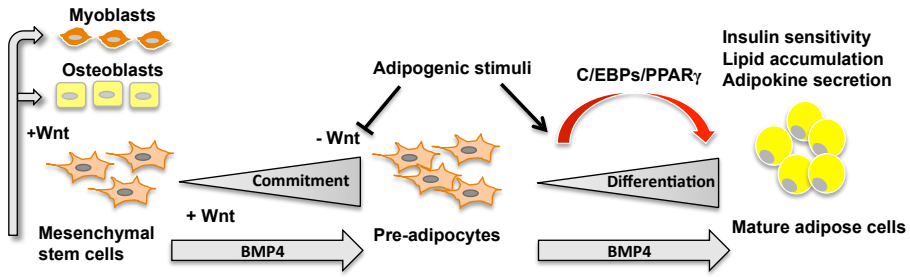

Figure 2. Schematic figure over the regulation of adipogenesis from mesenchymal stem cells and other uncommitted precursor cells. WNT silencing and BMP4 activation are required for adipogenic commitment and differentiation of preadipocytes into mature adipose cells by activation of transcription factors of the C/EBP family and the key regulator of adipogenesis, PPARγ (Modified from (16)).

The name WNT comes from a discovery in Drosophila where it was found that the polarity gene Wingless and the proto-oncogene Int-1 had a common origin, the WNT signaling pathway. The WNT signaling cascade controls a multitude of biological processes during development and adult life, especially stem cell biology. An abnormal WNT signaling underlies a wide range of pathologies in humans; for example cancer, osteoporosis and metabolic diseases. Single nuclear polymorphisms in WNT5B, WNT10B and transcription factor 7-like 2 (TCF7L2, formerly called TCF4) are all linked to an increased risk for T2D; TCF7L2 plays an important role in downstream signals of the canonical WNT pathway (49,50).
In the mammalian WNT family, there are nineteen members who are expressed in both embryos and adults. The WNT proteins are about 40 kDa

and are cytosine-rich glycoproteins that in their active form bind locally to cellular receptors. WNT proteins are lipid-modified in order to be secreted from the cell and bind to a receptor; one of these is palmitoleic acid, a mono-unsaturated fatty acid attached to a conserved serine (49,51). WNT molecules affect cell proliferation, survival, fate and behavior by signaling through different "canonical" and "non-canonical" pathways. Cytosolic β-catenin is fundamental for the canonical pathway and, therefore, it is often referred to as "WNT/ β-catenin dependent" and non-canonical pathway as "WNT/ β-catenin independent". In this thesis, only the canonical pathway will be discussed.

Cells can release or present WNT proteins in an autocrine or paracrine manner by binding to the cell-surface receptors of the Frizzled (FZD) family and the low-density lipoprotein-receptor-related protein-5 or -6 (LRP5/6) co-receptors. In the absence of WNT molecules, cytoplasmic β-catenin is recruited to a destruction complex consisting of Axin, adenomatous polyposis coli (APC) protein and glycogen synthase kinase 3 beta (GSK3β). This results in ubiquitination of cytosolic β-catenin followed by proteosomal degradation.
Normally, the cellular β-catenin levels are low but when WNT proteins bind to the cell-surface receptors of the FZD family and LRP5/6, Axin is recruited to the phosphorylated cytoplasmic tail of LRP6. This phosphorylation is regulated by GSK3 and CK1γ. Another cytoplasmic protein that is activated is Dishevelled (DVL), which interacts with the cytoplasmic part of FZD and Axin, facilitating an interaction between the LRP tail, Axin and FZD. This leads to inhibition of the Axin/GSK/APC complex, also called β-catenin destruction complex, resulting in higher concentrations of stabilized cytoplasmic β-catenin and its nuclear translocation. In the nucleus, β-catenin interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and promotes specific gene expression to regulate the transcription of WNT target genes, many of which are associated with cell proliferation and cell fate decision (23,35,47,49).
WNT-signaling can be inhibited by a number of extracellular antagonists acting in different ways. Secreted Frizzled-related protein (sFRP) 1 and 2, and WNT inhibitory factor 1 (WIF1) bind to the WNT proteins and, thereby, inhibit activation. Dickkopf 1 (DKK1) prevents the formation of the LRP/FZD receptor complex by binding with high affinity to LRP5/6 and Kremen 1/2 (KRM). This leads to specific inhibition of canonical WNT signaling by endocytosis and removal of the WNT receptors (52). WNT signaling is highly activated in precursor cells and needs to be downregulated for activation of adipogenesis (24,53,54). Inappropriate WNT activation is related to a poor adipogenesis and seen in obese patients with hypertrophic obesity (26).

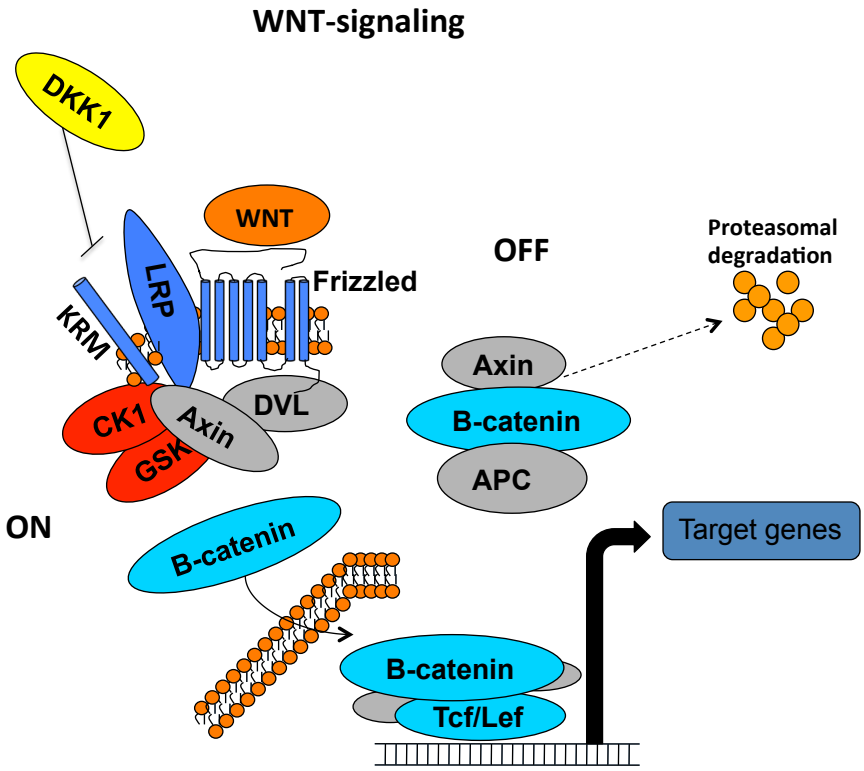

Figure 3. Canonical WNT-signaling.

1.6.2 WISP2

One of the many genes activated by the canonical WNT signaling is the WNT1 inducible signaling pathway protein 2 (WISP2 also known as CCN5) (55,56). *Wisp2* has been shown to only be activated by the canonical WNT and not non-canonical WNT signaling. WISP2 has a molecular weight of 27.5 kDa and the homology between mouse and human WISP2 is 73% (57,58).

1.6.3 CCN-family & structure

Human and mouse WISP2 are homologues to the rat gene *rCop1* and belong to the CCN family of growth factors. The family consists of 6 members;

CTGF or CCN1, cysteine-rich angiogenic inducer 61 (Cyr61 or CCN2), nephroblastoma overexpressed (NOV or CCN3), and WISP 1-3 (CCN4-6).
The CCN family of proteins is essential for embryonic development and plays important roles in inflammation, wound healing, and injury repair in the adult. Many are considered to be involved in the pathogenesis of fibrosis, artherosclerosis and cancers (57,59-65).

The CCN family of proteins contains, apart from WISP2, of 4 conserved cysteine-rich domains which display homology to conserved regions in a variety of extracellular proteins. All CCN proteins have an N-terminal signaling peptide, important for secretion (66). Module 1 is an insulin-like growth factor-binding domain and Module 2 is a von Willebrand factor type C domain (VWC) that may participate in protein complex formation. Module 3; the thrombospondin-1 domain (TSP1), involved in the binding to sulfated glycosaminoglycans either on the cell surface or in the extracellular matrix. Module 4 does not exist in WISP2 but is a cysteine-knot-containing module recognized by many growth hormones and may participate in dimerization and receptor binding. Between the VWC domain (Module 2) and the TPS1 domain (Module 3) there is a non-conserved central hinge-region, suggesting possible translational processing by proteolytic digestion. Considering the structure of CCN proteins, it not much of a surprise that they are involved in many essential biological functions (57,66-68).

1.6.4 WISP2 in human disease

WISP2 is a secreted protein (69) and has been reported to have various effects in different cancers. It has both growth-promoting and growth-arresting properties depending on cell types and environment of the cells.
WISP2 has also been suggested to have potential tumor-suppressive properties in colorectal cancer, breast cancer and bone metastases (70-75). *Wisp2* expression is unaltered during the osteogenic and chondrogenic differentiation of mesenchymal stem cells, but downregulated during adipogenic differentiation (74,76).
Many of the studies of WISP2 have been focused on osteoblasts, myocytes or chondrocytes. However, mRNA expression of both human and mouse tissues showed that *WISP2/Wisp2* is by far most highly expressed in the adipose tissue (77) and activation of canonical WNT inhibits adipogenic differentiation and upregulates *Wisp2* (55,56). The secretome of the human adipose tissue showed that WISP2 is an adipokine, i.e.; secreted by the adipose tissue. Furthermore, *WISP2* expression is increased in obesity and, in particular, in the subcutaneous adipose tissue (78).

2 AIM

The overall aim of this thesis is to characterize effects and molecular mechanisms for the novel "adipokine" WISP2 in the regulation of mesenchymal precursor/stem cell growth and adipogenic commitment and the association with hypertrophic obesity and its metabolic consequences, i.e.; insulin resistance and Type 2 diabetes.

The specific aims:

Paper I. To investigate WISP2 in human hypertrophic adipose tissue and its involvement in the regulation of adipogenic commitment by BMP4.

Paper II. To characterize the signaling mechanisms for WISP2 and its effects on adipogenesis and adipocyte differentiation.

Paper III. To evaluate the effects of WISP2 activation in the adipose tissue *in vivo* using a transgenic mouse model.

3 METHODS

3.1 Ethical statement

Informed consent was obtained from all subjects after the purpose and the potential risks of the study were explained. The study protocols were approved by the Ethics Committees of the University of Gothenburg, Charles University (Prague), and the University of Kuopio and were in accordance with the Declaration of Helsinki.

All animal experiments were performed after prior approval from the local Ethics Committee for Animal Studies at the Administrative Court of Appeals in Gothenburg, Sweden.

3.2 Subjects and samples

Nondiabetic subjects
Thirty-six healthy, nondiabetic subjects (Gothenburg cohort) were recruited. Inclusion criteria were two first-degree relatives with type-2 diabetes or one first-degree relative and two second-degree relatives, normal glucose tolerance, fasting triglyceride concentration < 2.0mM, and no evidence of hypertension, endocrine disease or metabolic disease. Subcutaneous adipose tissue biopsies were obtained by needle aspiration from lower part of the abdomen after local dermal anesthesia with lidocaine. Biopsies were transferred to the laboratory for immediate processing.

Subcutaneous and Visceral Adipose Tissue Arrays of Subjects
Individuals scheduled to have abdominal surgery (Prague cohort) were monitored and 53 women [age 21–66 y, body mass index (BMI) 17.3–48.5 kg/m2] were included. According to BMI and presence or absence of the metabolic syndrome evaluated according to the International Diabetes Federation criteria (79) participants were assigned into one of the four groups (lean, overweight, obese, or obese with metabolic syndrome). A clinical investigation was performed 7–14d before the surgery. Anthropometric measurements and euglycemic–hyperinsulinemic clamps (80) were performed at rest after an overnight fast. Body composition was evaluated using bioelectrical impedance. Visceral and subcutaneous fat areas were derived from computed tomography scans at the level of L4–5. During the surgical procedures, paired samples of subcutaneous abdominal and visceral

adipose tissue biopsies were obtained and processed immediately. The samples were stored at−80 °C until analyzed.

Type-2 diabetic subjects
Ten drug-naive type-2 diabetic patients (Kuopio cohort) with mild diabetes (fasting plasma glucose ≤ 8.0 mmol/L), four men and six postmenopausal women, were recruited. Exclusion criteria were evidence of peripheral vascular disease or heart disease, blood pressure ≥160/85 mm Hg, and treatment with calcium channel blockers or nonsteroidal anti-inflammatory drugs on a regular basis. No prior anti-diabetic treatment was allowed. Subcutaneous adipose tissue biopsies were obtained by needle aspiration from lower part of the abdomen. The biopsies were stored at−80 °C until analyzed.

3.3 Isolation of adipocytes

The adipose tissue from human (*Paper I*) or mice (*Paper III*) biopsies were handled according to referenced method. Briefly, adipose tissue were cut into smaller pieces and digested with collagenase type II in Hank's balanced salts medium (pH 7.4) complemented with 4% BSA for 45-60 min at 37 °C in a shaking water bath.
The cells were then filtered through a 250 μm nylon mesh and washed 4 times in Hank's medium (without glucose when glucose uptake was measured (*Paper III*) and average cell diameter measured. Remaining isolated adipocytes where either; snap frozen in liquid N_2 or stimulated for 15 min with or without insulin (1000μU/ml) and stored in lysate buffer for protein extraction or used for glucose uptake measurements.

3.4 Cell culture experiments

Cells
To study the effect of WISP2 on commitment to the adipogenic phenotype *in vitro*, NIH-3T3 fibroblasts, which do not have activated endogenous PPARγ, were used (81). The well-characterized preadipocyte cell line 3T3-L1 was used to study the effect of WISP2 on adipocyte differentiation and mature adipocytes. 3T3 L1 cells are an immortalized subclone of mouse 3T3 fibroblasts (82). Both cell lines where cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 1 % antibiotics.
Human mesenchyme stem cells (hMSC) were also used and were cultured in Mesenchymal Stem Cell Growth Medium.

Human stromal vascular cells were extracted from abdominal subcutaneous adipose tissue biopsies. The first non-floating cell fraction from the isolation procedure contains the stromal vascular fraction (SVF). SVF were washed and centrifuged to remove red blood cells and cultured in DMEM/F12 media supplemented with 10% FBS, 2mM glutamine, 1% antibiotics until confluence. Inflammatory cells (CD14- and CD45-positive), and early progenitor cells (CD 133-positive) were removed by magnetic immune separation.

Cell transfection

The main purpose of cellular transfection is to study the function of a gene by overexpression/inhibition or to produce recombinant proteins (83). Foreign nucleic acids can be introduced in the cell either by stable (long-term) or transient (short-term) methods. To introduce the foreign DNA/RNA, liposome-mediated transfection was used. In this thesis we transfected 3T3-L1 preadipocytes stably with a vector carrying *Wisp2* shRNA (*Paper I*) to conditionally study the effect of WISP2 depletion during adipocyte differentiation. For short-term experiments, we used siRNA or different overexpressing plasmids. Detailed information is presented in *Papers I, II*.

Cell differentiation

NIH3T3 fibroblasts and 3T3-L1 preadipocytes were grown to confluence and remained quiescent for 48h before induction of differentiation. To differentiate these cell lines, an adipogenic differentiation cocktail was added containing DMEM with a combination of insulin, dexamethasone and isobutylmethylxanthine (IBMX). Insulin increases the number of lipid droplets (84), the glucocorticoid, dexamethasone, induces the expression of C/EBPδ and inhibits the antagonist of adipogenesis, Pref-1 (85), and IBMX, a phosphodiesterase, enhances differentiation through increased cAMP levels. To activate PPARγ in the NIH-3T3 fibroblasts, rosiglitazone, a thiazolidinedione (TZD), was added to the cocktail. After 8 days, the preadipocytes had become mature adipocytes with lipid droplets.
hMSC were grown until confluence after which three cycles of induction/maintenance were performed according to manufacture's instructions. rhWISP2 or WNT3a where added to supplemented adipogenic induction medium/adipogenic maintenance medium.

Oil-Red O staining

To determine lipid accumulation, cells were fixed with paraformaldehyde, stained with Oil Red O and washed with PBS. Quantification of optical density was determined by dissolving the Oil Red O stained cells in 2-propanol and measured at λ 510 nm.

Immunofluorescence staining
Cells were grown on glass slides and treated with respective agents. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Samples were blocked with 20% FCS or goat serum and incubated with specific antibodies. After washing in PBS, samples where incubated with a secondary antibody conjugated with a fluorophore to visualize the proteins and DAPI staining was used to visualize nuclei. Samples were analyzed with a Leica SP5 confocal microscope.

Proliferation assay
To evaluate cell proliferation, a bromdeoxyuridine (BrdU) proliferation assay kit was used. BrdU incorporates in newly synthesized DNA by replacing thymidine. Briefly, C3H10 T1/2 mesenchymal cells and brown adipose precursor cells were cultured with mouse serum from Tg and wt mice and with or without anti-WISP2 antibody (58) for 48h. Cells were then fixed and stained with BrdU antibody according to the manufacturer's instructions.

3.5 Animal experiments

Generation and genotyping of transgenic mice overexpressing aP2-Wisp2
The aP2-Wisp2 transgenic mice were generated as stated in *Paper* III created in the laboratory of Fatima Bosch (Universitat Autònoma de Barcelona, Spain) using microinjection of oocytes from C57Bl6/SJL mice (86). WISP2 transgenic (Tg) founders were then bred to generate F1 Tg mice and subjected to PCR analysis and Southern blot to check the transgene expression. The Tg F2 offspring was generated by backcrossing the F1 Tg mice with wild type (wt) C57BL/6NTac mice (Taconic) for 10 generations and then inbred for 4-7 generations (B6N.SJL/J-Tg(aP2-Wisp2)92Fbos ; N10,F4-F7).

Animals
Only male mice were used for phenotyping. Animals were weaned at 3 weeks of age and housed 2–5/cage in a temperature-controlled (21°C) facility with a 12-h light-dark cycle with free access to chow food and water. From the age of 6 weeks, age-matched male transgenic mice and wild-type littermates were fed either pelleted high-fat diet, HFD (45 kcal% fat), pelleted control low fat diet, LFD (10 kcal% fat) or kept on chow diet, CD (16% protein). The HFD had the same amount of proteins (20 kcal%) and minerals as the LFD. They only differ in the ratio carbohydrates/fat; HFD 35/45 and HFD 70/10. Chow

diet contained 22% calories from proteins, 12% from fat and 66% from carbohydrates.

Body weight and blood sampling
Total body weight was recorded weekly during the period of either 11 weeks on HFD or 17 weeks on HFD or LFD. Fasting glucose (4h food withdrawal) was measured using an Accu-Check glucometer and blood sampling was performed every 4th week. Blood for measuring glucose was taken from the tip of the tail vein using a scalpel and blood for measuring metabolites was taken from the submandibular vein using Goldenrod Animal Lancet (87). Taking submandibular blood has the advantage of getting a large amount very fast as well as reducing the stress on the animals. When small amounts of blood is needed (fasting glucose) or repeated blood sampling is needed (GTT/ITT/PTT); tail vein blood sampling is preferred.
Blood was stored in serum tubes until centrifuged. The supernatant serum was stored in -80 for further analyses. Saline solution (9 mg/ml) was given as fluid replacement.

Necropsy
The mice were euthanized using 5% isofluorane with a mixture of air. Blood was collected by heart puncture for analysis of metabolites. Tissues and organs were weighed and stored. Epididymal white adipose tissue (eWAT), subcutaneous white adipose tissue (sWAT) and brown adipose tissue (BAT) were placed in 37°C sterile Hank's balanced salts medium without collagenase II (pH 7.4), snap-frozen in liquid nitrogen and stored in -80 for further analyses or stored in 4% paraformaldehyde (PFA) for 2 days and then in 70% ethanol.
EDL muscle for glucose uptake was placed in 37°C sterile Hank's medium without collagenase II and glucose (See Glucose uptake).

Open field activity test
Activity test was performed to study locomotor activity and food intake. The test lasted 23h; 11 h consisted of daylight (150 lux, 10:00–19:00 and 07:00–09:00) and 12 h of nightlight (20 lux, 19:00–07:00). The equipment consists of an opaque box (50×50×22.5 cm) that has a lower and a higher row of infrared sensors built into the walls connected to a control unit for tracking of the mouse. The mouse was placed in the center of the box, and the test was performed for 23 h, which allowed the mouse to acquaint itself with the open field test chamber for the first 3 h. Locomotion (increased by 1 every time the animal breaks a new beam) was recorded. The amount of food was measured before and after the test and daily food intake per animal was calculated.

Indirect calorimetry

Respiratory exchange quotients (RQ) were assessed by a non-invasive in vivo technique; The SOMEDIC Metabolic system (INCA®) which provides a high-resolution calorimetric evaluation of energy expenditure using measurements of oxygen consumption and carbon dioxide production. The difference in gas concentrations between chamber inlet and outlet is used as the basis for calculation of the RQ (CO_2 produced divided by O_2 consumed).
Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were recorded every 2 min for 23 h, 11h light and 12h dark. Animals had *ad libitum* access to food and water during the measurements. The data for the first hour were discarded to account for animal acclimatization to the testing conditions. In all calculations, $W^{0.75}$ has been used to correct for body size according to Kleiber's law (88).

Body composition

Analysis of total body fat and lean body mass can be performed by dual energy X-ray absorptiometry (DEXA) or minispec body composition analysis (BCA). DEXA is not as accurate as the BCA, but it also evaluates bone mineral density. In this thesis, DEXA was performed using Lunar PIXImus II Densitometer (version 2.10.041), under anesthesia with isofluorane (89). Computed tomography scans were performed with the peripheral quantitative computed tomography (pQCT) XCT RESEARCH M (version 4.5B, Norland) operating at a resolution of 70 μm, as described previously (90).

Glucose tolerance tests (GTTs), insulin tolerance tests (ITTs) and pyruvate tolerance test (PTTs)

Before the tolerance tests, mice were fasted for 4h . For GTT, glucose (1 g/kg) was injected intraperitoneally and repeated blood samples are taken for determination of glucose and insulin levels according to *Paper III*. GTT evaluates the disposal of a glucose load and the result is determined by insulin secretion, insulin action and effectiveness of glucose (91).
During an ITT, the glucose concentration is monitored after an intraperitoneal injection of human recombinant insulin (0.8 U/kg). The response in blood glucose concentration to a bolus dose of insulin is indicative of whole-body insulin action. Fasting glucose levels reflect hepatic gluconeogenesis, but also reflect many other systemic parameters (91).
A pyruvate tolerance tests (PTT) investigates more specifically the process of gluconeogenesis as pyruvate is converted to glucose through hepatic gluconeogenesis. Sodium pyruvate (2 g/kg) was injected intraperitoneally (ipPTT) and the response in blood glucose concentration followed.
Saline (9 mg/ml), insulin (0.8 U/kg) or glucose (1 g/kg), was given as fluid replacement or to compensate for high/low plasma glucose at the end of the examination.

Euglycemic-hyperinsulinemic clamp studies in conscious mice

To measure metabolic insulin sensitivity directly, the "golden standard" method is the hyperinsulinemic euglycemic clamp technique (80). Briefly, insulin is intravenously infused at a constant rate whereas glucose is simultaneously infused at a variable rate (GIR). At steady-state i.e.; when the plasma insulin, blood glucose and GIR are stable over a period of time, GIR provides a measurement of the whole-body insulin action (92-94).

Mice on a HFD (45 kcal% fat) for 10 weeks were anesthetized and were given analgesia and fluid replacement subcutaneously. A catheter was inserted in the left jugular vein and tunnulated behind the head where it was connected to a Vascular Access Button anchored in an incision behind the head. Post-surgery, mice where individually housed in cages with free access to water and HFD. At least 3 days of recovery were given before the experiments.

Mice were fasted for 4 hours and placed in individual plastic containers for tail vein sampling. A fasting blood sample was taken to determine fasting insulin levels and a priming dose of insulin (67 mU/kg) was injected.

Insulin (7.5 mU/min/kg) and glucose (30%) were infused; GIR was guided by measuring glucose concentrations every 5 min. The mean glucose infusion rate, normalized to body weight, was calculated at steady-state as an index of insulin sensitivity. At steady state, 2[^{14}C]deoxyglucose (3 µCi) was injected and repeated blood samples were collected for determining 2[^{14}C]deoxyglucose (2DOG) radioactivity and plasma insulin.

The mice were euthanized and tissue and blood were handled according to *Paper III*. Briefly; 2DOG is converted to 2[^{14}C]deoxyglucose-6-phosphate (2DOG-6P) in the tissue and trapped since Glucose 6-phosphatase is only present in liver. The disappearance of 2DOG from the blood and conversation to 2DOG-6P in tissues is measured and, thus, the rate of glucose uptake can be calculated.

Blood chemistry

The plasma concentration of insulin was determined using the Ultrasensitive Mouse Insulin ELISA kit and plasma concentration of adiponectin using Mouse Adiponectin/Acrp30 Quantikine ELISA Kit.

Serum triglycerides and cholesterol were measured in whole plasma using colorimetric enzyme assays, Infinity Triglyceride and Infinity Cholesterol kit.

To analyze the blood concentration of PAHSAs, serum from Tg and wt mice on HFD and LFD was used. Lipidomic analysis was performed with MS as previously described (95). Briefly, PAHSA Levels = ((PAHSA peak abundance/13C-9-PAHSA peak abundance)/(Vol. of the serum in ml))*(Concentration of the internal standard).

Histological analysis and immunohistochemistry
Immunohistochemistry (IHC) is using antibodies to detect antigens in cells or tissue, the range of IHC is much broader than Western blots and ELISA, all from amino acids and proteins to infectious agent and specific cell populations (96).
Liver and BAT were immediately after dissection fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. 6mm sections were stained with hematoxylin and eosin. BAT sections were incubated with anti-UCP-1 antibody followed by anti-rabbit secondary biotinylated antibody and diaminobenzidine staining and counterstained with hematoxylin solution.

Glucose uptake
Isolated adipocytes from eWAT and sWAT (97) and intact extensor digitorum longus muscle (EDL) (98) where transferred to vials with glucose - free Hank's medium 199 supplemented with 4% BSA, 0.15 μM adenosine, pH 7.4,. Insulin (10 ηM for adipocytes, 100 ηM for EDL) was added for 15 min followed by D-[U-^{14}C)-glucose (0.26 mCi/l, 0.86 μM) for 45 min. Isolated adipocytes where separated from the medium through centrifugation and transferred to scintillation tubes to measure incorporated radioactivity. EDL muscles were immediately washed with cold PBS 3 times and digested in 0.5 M NaOH at 50C^{o} for 30 min. Cleared protein lysates were used to measure incorporated radioactivity.

3.6 Quantitative validation of mRNA and proteins

PCR and Southern blotting
The PCR method allows rapid amplification of DNA sequences. The PCR is based on cycles of heating and cooling through a defined series of temperature steps, for DNA melting and enzymatic replication of the DNA region of interest.
PCR analysis was used in *Paper III* to determine the genotype of the mice from mouse-tail DNA. HotStarTaq Master Mix Kit was used with the following primer pairs: forward, 5′-CCAGGGAGAACCAAAGTTGA-3′; reverse, 5′-AAGATGGATCAGTGGGTTGC -3′.
The PCR product was mixed with a loading dye and placed on an agarose gel. A DNA ladder with different defined molecular-size DNA fragments is used to determine the size of the PCR product. A DNA staining agent is mixed in the gel to visualize the DNA fragment by UV excitation.

RNA isolation
RNA is purified from cells or homogenized tissue samples, using commercially available kits. In this thesis, kit specific for fibrous tissues or adipose tissue was used when appropriate (*Paper I-III*). The extracted RNA is synthesized to complementary DNA (cDNA) by reverse transcriptase and DNA polymerase. The synthesized cDNA is more stable than RNA and is used in qRT-PCR as template for expression analysis.

Quantitative real-time PCR (qRT-PCR)
TaqMan-based detection (ABI prism 7900HT) also uses a fluorogenic reporter probe which is specific for the gene of interest. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter probe over the background. The cycle number, at which enough amplified product accumulates to yield a detectible fluorescent signal that is higher than the minimal detection level (99), is the threshold cycle. To adjust for dilution and pipetting errors, 18S rRNA was used as a housekeeping gene to normalize the data. Detailed information how the quotient of expression for each gene is calculated is described in each of the papers.

Protein extraction
Cultured cells and homogenized tissue (*Paper I-III*) were carefully washed and lysed on ice in lysis buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 10 mM NaF, 0.2 mM leupeptin, 1 mM benzamidine, and 0.1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride), centrifuged in 4^{o}C (10000g, 10 min) and the supernatant, containing the lysate, stored at -80^{o}C.
Isolated adipocytes (*Paper I and III*) were separated from medium by centrifugation (12000 rpm, 10 s) through dinonylphtalate, lysis buffer was added and samples were rocked for 2h in 4^{o}C. Detergent-insoluble material was sedimented by centrifugation in 4^{o}C (12000 rpm, 10 min) and supernatant stored at -80^{o}C.
The protein concentration was measured using the BCA protein assay kit in duplicates.

Western blot and Immunoprecipitation
Western blot separates molecules according to their size by electrophoresis. Small proteins move faster than large proteins through a SDS-polyacrylamide gel, allowing separation by molecular weight. The proteins are then transferred to nitrocellulose membranes to make them accessible to the antibodies. The membranes are blocked, to avoid unspecific binding, and incubated with primary antibodies and secondary antibody. The bands were visualized with an Immun-Star HRP Chemiluminescent Kit for quantification

with ChemiDocTM XRS System. For quantification details and data normalization, see respective *Papers*.

Immunoprecipitation (IP) is used to isolate and concentrate a specific protein from samples by using a specific antibody. In *Paper II,* IP was used to identify WISP2 (58) in conditioned media where there is an abundance of proteins that interferes with the detection under normal conditions.
Co- immunoprecipitation can be used to identify protein complexes and protein-protein interactions. By targeting a known member of the protein complex (WISP2 *Paper I*), there is a possibility that both the whole complex and the known single member are precipitated by the specific antibody. After denaturation of the complex, both the known member and the interaction member will be present in the sample loaded on the SDS-polyacrylamide gel. A specific antibody can then identify unknown members of the complex.

3.7 Statistical Analyses

The experimental data are presented as means ± SEM. To compare clinical parameters (*Paper I*) between the groups of subjects from the Prague cohort, log-transformed data were analyzed by one-way ANOVA with a Bonferroni post hoc analysis using SPSS 17.0 statistical software. For the Gothenburg and Kuopio cohorts, data (*Paper I*) analyses were carried out with StatView (SAS Institute) or PASWstatistics (SPSS) programs for Macintosh. Pearson's correlation was used for samples with normal distribution, for non-normal distribution Spearman's correlation was applied.
A non-parametric Mann-Whitney unpaired test (*Paper I, III)* is used to compare two independent samples or unpaired samples. Non-parametric Wilcoxon paired test (*Paper I, II)* is used when comparing two related samples or paired samples. Both tests can be used when non-normal distribution or a mixture of normal distribution is present. These tests were used to compare samples for $n \geq 6$; otherwise Student's t-test was used. $p<0.05$ was considered statistically significant.

4 SUMMARY OF RESULTS

Details of the results can be found in *Paper I-III* at the end of the thesis.

4.1 Paper I

WISP2 regulates preadipocyte commitment and PPARγ activation by BMP4

WISP2 expression and other markers of *WNT* activation were found to be increased in human abdominal subcutaneous adipose tissue characterized by hypertrophic obesity together with increased visceral fat accumulation and insulin resistance. *WISP2* activation in the subcutaneous adipose tissue, but not in visceral fat, identified the presence or not of the Metabolic Syndrome in equally obese individuals.

WISP2 is a novel adipokine, highly expressed and secreted by adipose precursor cells. Silencing WISP2 induced spontaneous differentiation of 3T3-L1 preadipocytes and allowed NIH 3T3 fibroblasts to become committed to the adipose lineage by BMP4.
Mechanistically, we found that WISP2 forms a cytosolic complex with the PPARγ transcriptional activator ZFP423 and this complex is dissociated by BMP4 in a SMAD-dependent manner, thereby allowing ZFP423 to enter the nucleus and activate PPARγ.

However, extracellular canonical WNT/WISP2 signaling also needs to be inhibited to induce full adipogenic differentiation and PPARγ activation. This requires the inhibition of secreted WNT/WISP2 by inhibitors like DKK1. Thus, adipogenic commitment and differentiation is regulated by the cross-talk between BMP4 and canonical WNT signaling and where WISP2 plays a key role. These results also link canonical WNT activation and WISP2 with hypertrophic obesity and the Metabolic Syndrome as well as with insulin resistance and visceral fat accumulation.

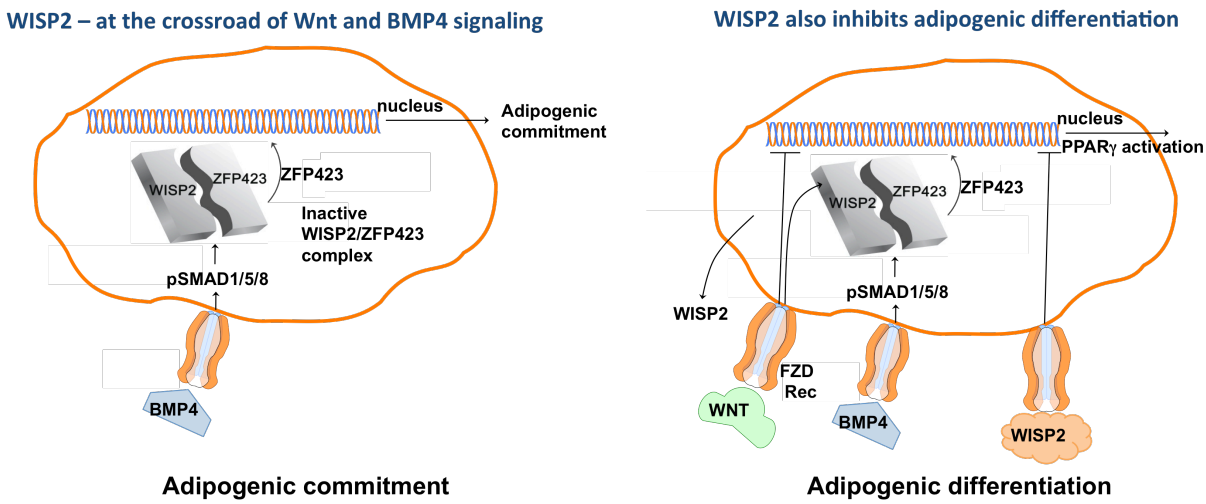

Figure 4. *BMP4 can target ZFP423 to the nucleus under WNT/WISP2 activation and allow the initial step of commitment. However, PPARγ does not become activated unless extracellular WNT/WISP2 also is inhibited. This allows transcriptional activation of PPARγ by ZFP423 (From Paper I).*

In summary: Our results show that canonical WNT signaling inhibits adipogenic commitment and differentiation through WISP2 which retains ZFP423 in the cytosol and, as a secreted molecule, WISP2 also elicits an intracellular signaling pathway that inhibits PPARγ activation. Furthermore, as a secreted molecule, WISP2 can exert both autocrine and paracrine (and possibly endocrine) regulation and be an important adipokine mediating cross-talk between the adipose tissue and other cells.

4.2 Paper II

The Novel Secreted Adipokine WNT1-Inducible-Signaling Pathway Protein2/WISP2 is a Mesenchymal Cell Activator of Canonical WNT

We here address the signaling pathway of secreted WISP2 (full-length) compared with intracellular WISP2 (truncated in N-terminal), which cannot be secreted.
We found that full-length, but not intracellular (truncated and not secreted), WISP2 increases β-catenin expression and nuclear-targeting serine phosphorylation. Full-length WISP2, but not truncated, increased pS LRP5/6 phosphorylation. In line with these results, recombinant WISP2 as well as the full-length protein activated the nuclear receptor for β-catenin in a *Tcf/Lef*

reporter assay. WISP2 also inhibited *Pparg* activation and the WNT antagonist DKK1 reversed the effect of secreted WISP2 further supporting its interaction with the canonical WNT signaling pathway.
WISP2 maintains the mesenchymal stem cells undifferentiated by activating canonical WNT. However, WISP2 differs from conventional WNT ligands in that it does not have to be acylated/palmitoylated to be secreted. In previous work (48), the canonical WNT ligand, WNT3a was found to also target mature adipocytes through inhibition of differentiation and lipid accumulation. And, indeed, secreted WISP2 was also able to partially de-differentiate mature adipose cells with loss of lipid accumulation and significant inhibition of several PPARγ -regulated genes.
Thus, WISP2 activates canonical WNT with induction of β-catenin and phosphorylation of the LRP5/6 receptor and, like other WNT ligands, also promotes a transition towards a myofibroblast phenotype with induction of α-smooth muscle actin (α–SMA). These data indicate that WISP2 can be considered an endogenous and secreted ligand of the canonical WNT pathway. In addition, WISP2 activates the MAPK's pathway and exhibits a mitogenic effect in preadipocytes.

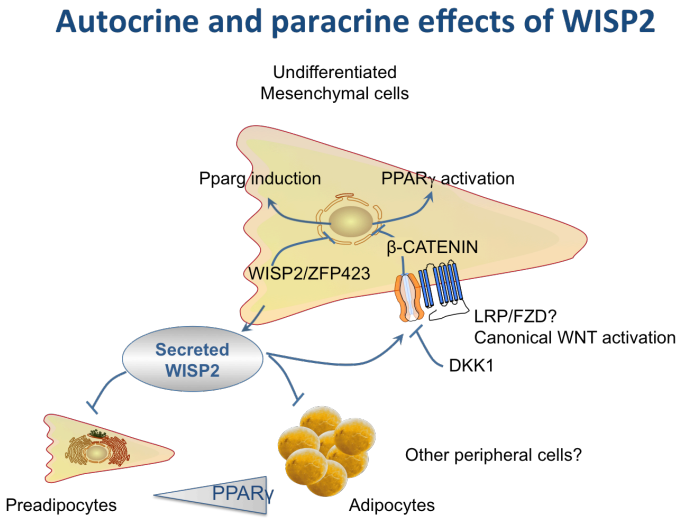

Figure 5. *WISP2 is both an intracellular and a secreted protein by mesenchymal precursor cells. Intracellular WISP2 retains ZFP423, the transcriptional activator of Pparg, from entering the nucleus and initiates adipogenic commitment of the precursor cells. Secreted WISP2, in an autocrine manner, activates the canonical WNT pathway through an unknown receptor and cellular signaling pathway involving LRP5/6. This prevents PPARγ activation and maintains the precursor cells in an undifferentiated state. This effect of WISP2 is antagonized by the canonical WNT inhibitor DKK1 (From Paper II).*

In summary: These results show that the secreted adipokine WISP2, which is highly expressed in mesenchymal stem cells and early precursor cells, is a regulator of adipogenesis and PPARγ activation as a canonical WNT ligand, thereby keeping the cells in an undifferentiated state. WISP2 plays an important role in adipogenesis by promoting precursor cell mitogenesis and their commitment in response to BMP4 as well as the subsequent differentiation and PPARγ activation.

4.3 Paper III

Increased brown fat and insulin sensitivity in obese mice overexpressing WISP2 in the adipose tissue

One important experimental tool in conducting research on adipose tissue *in vivo* has been the adipose-specific transgenic or knockout mouse models. The fatty acid binding protein adipocyte P2 (aP2) promoter has been used by the research community for the past decades. aP2 is primarily, but not exclusively, expressed in the adipose tissue and its expression is highly regulated during adipocyte differentiation. A novel mouse model was generated that overexpresses WISP2 under the aP2-promoter, aP2-Wisp2.
The transgenic mice (Tg) and the wildtype (wt) mice were challenged with a high fat diet (HFD) or a low fat diet (LFD) for 17 weeks. No major differences were found between wt and Tg mice on LFD.

The Tg mice on HFD had lower fasting glucose and insulin levels compared to wt in spite of the same body weight. Glucose and insulin tolerance tests were performed to further characterize their glucose tolerance and insulin sensitivity. Data revealed that the HFD Tg mice had significantly improved glucose tolerance and were more insulin-sensitive compared to their wt littermates. This was further confirmed in hyperinsulinemic euglycemic clamp examinations. These findings also where supported by increased insulin-dependent glucose uptake in epididymal fat and EDL muscle *ex vivo*. Pyruvate tolerance test showed low gluconeogenesis in HFD Tg, similar to that seen in lean mice. Furthermore, HFD Tg mice had high levels of circulating adiponectin, increased *Glut4* skeletal and WAT and upregulation of markers of adipose tissue lipogenesis. Serum levels of the novel fatty acids-hydroxy fatty acids (FAHFAs) were also elevated in Tg HFD mice which can account for their increased insulin sensitivity.
While there was no difference in total body weight, the Tg mice had more lean body mass and significantly less percent fat although both epididymal and, in particular, brown adipose tissue were increased in weight.

Interestingly, subcutaneous adipose tissue did not differ in weight but consisted of a greater number of smaller cells compared to the control mice, implicating hyperplastic adipose tissue expansion.
Indirect calorimetry data demonstrated that Tg mice had higher energy consumption, which is in line with an increased lean and brown adipose tissue mass and a reduced adipose tissue.
Taken together, these findings suggested that WISP2 increases the proliferation of mesenchymal stem/precursor cells leading to an increase in lean body mass and a metabolically healthy hyperplastic expansion of the adipose tissue when combined with a nutritional overload. This concept was supported by the finding that serum from Tg mice increases the proliferation of brown and white adipose tissue precursor cells, a response that was inhibited by adding monoclonal anti-WISP2 antibodies. Another important coupling between BMP4 and WISP2 was the finding that *Wisp2* mRNA is rapidly inhibited by BMP4 in mesenchymal stem cells while this was not found for *Wnt10b,* another important canonical WNT ligand in adipose precursor cells.

In summary:
Our data provide evidence for the concept that WISP2 is an endogenous and secreted canonical WNT ligand by mesenchymal precursor cells, also targeting these cells in an autocrine fashion to increase their proliferation and tissue growth. WISP2 must be timely activated and silenced to enable mesenchymal tissue growth and development. Thus, WISP2 plays an important role in the development of metabolically important tissues and its expression in human subcutaneous adipose tissue is also associated with obesity- related metabolic complications.

5 DISCUSSION

Development of T2D, like obesity, is associated with a genetic predisposition. An important association between T2D and adipogenesis is our recent finding that a genetic predisposition for T2D, but not for obesity, is associated with an inappropriate expansion of the subcutaneous adipose cells for a given BMI (26). This suggests that a genetic predisposition for T2D is associated with an impaired ability to recruit new subcutaneous adipose precursor cells, thereby enhancing ectopic lipid accumulation. This is an important concept since it is well established that the subcutaneous adipose tissue has a limited capacity to expand (17) and that this is under genetic regulation.

Activation of the canonical WNT pathway promotes commitment of mesenchymal stem cells into the myogenic and osteogenic linage while suppressing commitment and terminal differentiation of the adipogenic linage. Inappropriate activation of canonical WNT in the adipose tissue can then be a driving force towards the development of hypertrophic adipose cells and ectopic fat accumulation through the inhibition of recruitment and commitment of precursor cells into the adipose lineage (26).

5.1 WISP2 is associated with markers of Metabolic Syndrome

WISP2 is known to be activated by the canonical WNT signaling pathway (57) and was previously found by our laboratory to be one of the genes upregulated in the adipose tissue of FDR in microarrays. In *Paper I,* we found the expression of *WISP2* to be associated with WNT-regulated genes such as *CYCLIND1* and markers of hypertrophic obesity i.e.; increased subcutaneous cell size and waist circumference in non-diabetic individuals. It was also positively correlated with markers of ectopic fat accumulation and negatively correlated with insulin sensitivity. These data provide evidence for increased activation of canonical WNT in hypertrophic obesity. *WISP2* is highly expressed in mesenchymal stem cells and preadipocytes and WISP2 protein is not found in isolated mature adipocytes. During differentiation of both human preadipocytes and 3T3-L1 preadipocytes, *WISP2*/*Wisp2* is rapidly downregulated. However, it remains elevated in the adipose tissue in hypertrophic obesity.

Inappropriate hypertrophic expansion of the subcutaneous adipose tissue is associated with insulin resistance and the Metabolic Syndrome. Positive

energy balance leads to accumulation of lipids in the subcutaneous adipose tissue but this tissue has a limited expandability and, when exceeded, lipids accumulate ectopically in visceral depots, liver, around the heart and other places (5,12,17). Experimental studies have shown that this can then be prevented by a hyperplastic response as seen, for instance, in mice overexpressing adiponectin in the adipose tissue which leads to an extremely overweight, but metabolic healthy, phenotype with small and insulin-sensitive cells (100). Conversely, lipoatrophy leads to insulin resistance and ectopic fat accumulation which can be reversed by adipose tissue transplantation (101).
As shown in *Paper I, WISP2* transcriptional activation is higher in subcutaneous adipose tissue compared to visceral tissue and also higher in equally obese individuals fulfilling the criteria for the Metabolic Syndrome. *WISP2* mRNA levels are also associated with other markers of the Metabolic Syndrome, such as intra-abdominal fat mass and negatively correlated with insulin sensitivity. This indicates that inappropriate WNT/WISP2 activation in the subcutaneous fat is associated with a dysfunctional and hypertrophic tissue expansion, ectopic fat accumulation, insulin resistance and its metabolic complications.

5.2 WISP2 and adipogenesis

Recent studies have shown that other members of the CCN-family, NOV and WISP1 (63,64) also are associated with metabolic disorders such as obesity and inflammation. Both these proteins are secreted from the adipose tissue but their effect on adipose tissue commitment/differentiation was not investigated (NOV) or seen (WISP1). In *Paper I,* we show that WISP2 has profound effects on both adipogenic commitment and differentiation. Like other CCN proteins (66), WISP2 is both present in the cytosol and secreted and prevents adipogenic commitment and PPARγ-induced differentiation through two different mechanisms. Cytosolic WISP2 forms a complex with the PPARγ transcriptional activator ZFP423 (29). This prevents the ZFP423 from entering the nucleus and activating transcriptional programs that allow the cell to enter commitment to the adipocyte lineage. The WISP2/ZFP423-complex is dissociated by BMP4 through the SMAD binding domain on ZFP423, which then allows entry into the nucleus. This is an important mechanism for adipogenic commitment as shown by the induction of adipogenic markers when WISP2 is silenced in the presence of BMP4. The presence of a BMP4 inhibitor, such as Noggin (26), prevents the ability of BMP4 to dissociate the WISP2/ZFP423-complex and, as a consequence, also adipogenic differentiation. In a recent study, it was shown that hypertrophic

obesity is associated with an increased activation/secretion of the endogenous BMP4 inhibitor Gremlin1 while BMP4 protein actually is increased indicating that hypertrophic obesity is a condition of BMP4 resistance (102).

Secreted WISP2 also inhibits adipogenesis and differentiation (*Paper I,II)* and, like the canonical WNT3a ligand (48), it activates the canonical WNT pathway and prevents PPARγ-activation. Secreted WISP2 initiates transcriptional activation of *Tcf/Lef* and directs β-catenin to the nucleus, whereas silencing of *Wisp2* leads to a decrease in β-catenin as well as its nuclear-targeted phosphorylation. The specific receptor for WISP2 is currently unknown but WISP2 activates the WNT coreceptor LRP5/6 via phosphorylation. Thus, it likely that WISP2 signaling is via FZD/LRP5/6 activation.

In *Paper II* WISP2, like WNT3a (48), was found to target mature adipocytes ex vivo by activating the canonical WNT pathway and inhibiting adipogenic genes while MAPK-kinases were activated. A partial dedifferentiation toward the myofibroblast phenotype was also seen with increased expression of *Ctgf*, α-SMA and other markers of WNT activation associated with fibrosis (103,104). Interestingly, fibrosis did not seem to be increased in the aP2-Wisp2 Tg mice in *Paper III*, possibly because of the hyperplastic adipose tissue with smaller adipocytes in the subcutaneous depots. A heart muscle-specific WISP2 overexpressing mouse model, using α-myosine heavy chain as promoter, further supported these findings. WISP2 was shown to protect from cardiac hypertrophy and fibrosis in response to pressure overload when compared to a CTGF-overexpressing model (105). If this is because WISP2 does not directly induce fibrosis or it does not enhance the TGFβ-pathway like CTGF is unknown (105,106). The activation of both the canonical WNT and TGFβ signaling pathways have been shown to be required for induction of fibrosis (107).

5.3 WISP2 signaling

LRP5/6 is a co-receptor for canonical WNT as well as several other pathways including TGFβ, CTGF and PDGFα through physical interaction with PDGFRβ and TGFβR1 (104). In *Paper II,* we show that WISP2 does not need acylation for its secretion while other WNT ligands have to be acylated in order to be secreted and bind to the FZD receptors (49,51). Thus, WISP2 may bind to the LRP5/6 receptor directly and/or activate it through other signaling pathways.

Supporting evidence that WISP2 signals through the LRP5/6 receptor (*Paper II*) is that the WNT inhibitor DKK1 antagonizes the inhibitory effect of WISP2 on *Pparg* and *Fabp4*. DKK1 is a marker and mediator of well-functioning adipogenesis (53) and can rescue the impaired differentiation seen in hypertrophic obesity together with BMP4 (26). How DKK1 and other canonical WNT antagonists are regulated is currently unclear, although PPARγ ligands can increase the secretion of DKK1 during late differentiation of 3T3-L1 cells and also in mature cells (108). However, this later effect is seen when *Pparg* is induced and cannot account for terminating the concurrent WNT activation needed for differentiation. Nevertheless, once PPARγ is activated it can suppress WNT-activation by increasing degradation of β-catenin and thus maintaining the differentiated state (108,109).

Recently, it was shown that WISP2 interacts with the cell surface receptor integrin αvβ3 in vascular smooth muscle cells (VSMC) podosomes, but the downstream signaling effects are unknown. WISP2 does, however, prevent the matrix degradation required for cell migration in podosomes (110). This is further supported by data from Castelot et al. where ectopic expression of WISP2 in a mouse model for restenosis strongly suppresses VSMC migration and proliferation. It was suggested that WISP2 protects against restenosis by blocking the ability of medial VSMC podosomes to degrade matrix, thus preventing migration into the intima (110).
Integrin αvβ3 is promiscuous receptor that binds a wide range of proteins (110). It is possible that WISP2 simultaneously interacts with integrin αvβ3, since stimulation of integrin αvβ3 in mesenchymal stem cells activates p38. However, p38 reduces the levels of ERK and β-catenin, which promotes adipogenesis (111). This is not seen in *Paper II*, where both p38 and ERK are simultaneously activated by WISP2 in mature adipocytes and the activity of canonical WNT signaling and β-catenin levels are high, similar to that seen for WNT3a (48). It is possible that WISP2 acts as a suppressor of integrin αvβ3 since increased levels of integrin αvβ3 binding sites in mesenchymal cells cultured in adipogenic media leads to increased activity of p38 which promotes adipogenic differentiation (111). Further experiments are needed to test this hypothesis.

5.4 WISP2 regulation

Canonical WNT3a and GSK3β inhibition can moderately increase *Wisp2* (2-3 times) in mesenchymal stem/precursor cells (*Paper I*), but the detailed regulation of WISP2 is largely unknown.

The WISP2 promoter contains *TCF*, hypoxia inducible factor (*HIF)* and nuclear factor kappa-light-chain-enhancer of activated B cells (*NFKB*) sequences as well as binding domains for PPARγ and ZFP423. WISP2 is regulated by hypoxia through the HIFα isoforms in low-invasive luminal-like breast cancer cell lines, preferentially HIF2α. WISP2 is also negatively correlated with tumor macrophage invasion in breast cancer samples which could provide an additional marker for a better tumor prognosis (112). WISP2 has also been reported to be directly regulated by estrogen in the human breast cancer cell line MCF-7 and non-transformed human mammary epithelial cells and is more highly expressed in less-aggressive breast cancer cell lines (MCF-7) compared with highly aggressive (MDA-MB-231) (70-73).
Expression data from 79 human and 61 mouse tissues showed that *WISP2*/*Wisp2* is by far most highly expressed in the adipose tissue (upregulated 950 times) (77). Similar to the findings in *Paper I*, the secretome of the human adipose tissue was analyzed and showed that WISP2 is a highly secreted adipokine that is downregulated in the visceral adipose tissue, compared with the subcutaneous adipose tissue, and correlated to obesity (78) Furthermore, *WISP2* expression has been implicated to be a marker of differential number and/or activity of precursor adipocyte cell populations and extracellular matrix remodeling in cattle, which would be a good predictor of intramuscular fat i.e., marbling of the meat that impacts flavor and juiciness (113).

5.5 WISP2 in vivo

Wisp2 transcript begins to be expressed at the early medulla stage (12-16 cells) in embryogenesis and it persists in all three germ layers (endoderm, mesoderm and ectoderm) throughout the embryonic development in mice. The WISP2 protein is present in most cells in early embryos and is not restricted to a particular germ layer in mice and humans. Tissue specificity appears as the embryo develops. In adult rodents, WISP2 is widely distributed in many cell types, both in the cytosol and the nuclei, but WISP2 has not been found in the nucleus of mouse and rat pancreas, liver and spleen (114-117).

To further elucidate the role of WISP2 in the adipose tissue, an overexpression model of WISP2 in mice regulated by the aP2/FABP4 promoter, aP2-Wisp2, was generated (*Paper III*).
The aP2 promoter is a widely used promoter (86,118,119). Transgenic Cre-lines driven by the aP2 promoter demonstrate recombination in fat with

minimal protein expression in most other tissues; still there is some expression in capillaries of the myocardium and in perivascular cells. Expression in other non-fat tissues such as testis and salivary gland may be due to the different sites of transgenic integration (119).
The aP2 protein is a cytosolic fatty acid chaperone protein found mostly in mature adipocytes where it facilitates the utilization of lipids in the metabolic pathway and it has been shown that the aP2 promoter is already active at embryonic day 9.5. At day 17.5, it is co-localized with lipids in the mesenchymal stem cell population and becomes adipose-specific after birth (120). This coincides with the development of the subcutaneous adipose tissue which develops at embryonic day 14-18 (43). Global overexpression and knock-out models under a not identified promoter have shown to be embryonically lethal (121) but since the expression of the aP2 promoter starts at mid-gestation and is not globally expressed, the aP2-Wisp2 mice are viable.

Other *in vivo* models for studying the metabolic consequences of canonical WNT have been published. WNT10b overexpression under the aP2-promoter displayed an obesity-protected phenotype with reduced brown and white adipose tissue, reduced weight and the mice were not insulin resistant (118). Overexpressing activated β-catenin in PPARγ-expressing adipose precursor cells showed a similar lipodystrophic phenotype while using the later aP2-promoter in differentiated cells did not produce a clear phenotype. Moreover, mice overexpressing β-catenin in the precursor cells where found to release unidentified factor(s) that increased glucose uptake in muscles *ex vivo* (122).

In *Paper III*, the aP2-Wisp2 show a completely different phenotype compared with the aP2-Wnt10b mice, although WISP2 activates canonical WNT in mesenchymal precursor cells. Transgenic mice (Tg) on high fat diet (HFD) had similar body weight and were more insulin-sensitive during both non- and steady state conditions and this was also validated *ex vivo*. There were several markers of increased mesenchymal tissue growth such as increased BAT, lean body mass and weight of skeletal muscles/heart. Serum from Tg mice promoted proliferation of mesenchymal precursor cells and this effect was inhibited by WISP2 monoclonal antibodies, verifying the direct effect of elevated levels of WISP2 in the circulation.

During HFD in mice, both subcutaneous (sWAT) and epididymal adipose tissue (eWAT) starts to expand through hypertrophy during an early stage. After prolonged calorie excess (1 month), the eWAT initiates adipogenesis i.e., hyperplasia, which is not seen in the sWAT depots (43). However, sWAT in the Tg mice was hyperplastic and characterized by smaller cells, both by mean cell size and total distribution. This “healthy” adipose tissue

profile can probably account for the finding that the Tg mice had higher circulating adiponectin levels as well as transcriptional activation in the adipose tissue. However, there were no signs of increased beige markers in the white adipose tissues (*Tbx1*, *Tmem26* or *Cd137*) that could dissipate energy or improve insulin sensitivity (41,42,44). The increased BAT mass did not show markers of increased activity, although it was not stimulated since the mice were not exposed to cold-exposure or a β3-agonist. Thus, the improved insulin sensitivity is most likely associated with positive metabolic effects of the increased lean body mass combined with a "healthy" hyperplastic adipose tissue with increased levels adiponectin and adipose tissue glucose metabolism.
The increased levels of *Glut4* in both adipose tissue and skeletal muscle can be mechanistically related to the increased insulin-stimulated glucose uptake. Increased GLUT4 in the adipose tissue is associated with increased whole-body insulin sensitivity (123). Mice overexpressing GLUT4 under the aP2-promoter have recently been shown to also have increased de novo lipogenesis (DNL) regulated by carbohydrate-responsive-element-binding protein (ChREBPβ) (95,123,124). ChREBP is activated by glucose, independent of insulin, and is one of two major transcription factors for DNL. The other is SREBP-1, which is activated by insulin (125,126). Activation of DNL by ChREBPβ in GLUT4 mice leads to increased induction and secretion of lipid species that are metabolically beneficial, called fatty acid esters of hydroxy fatty acids (FAHFAs), by the adipose tissue (95). In *Paper III*, *Chrebp* was increased in both adipose depots as well as other members important of the DNL (95,124). Consequently, we measured several of the novel FAHFAs in serum and found that obesity induced by HFD was associated with lower 13/12- and 5-PAHSA while the levels in the Tg HFD mice were at least as high as in the non-obese mice. This finding can be a likely mechanism for the increased insulin sensitivity in the HFD Tg mice since FAHFAs also increase glucose uptake (95). It is unclear how increased WISP2 leads to increased levels of FAHFAs but the "healthy" and hypercellular adipose tissue is a likely contributing factor.
To what extent WISP2/FAHFAs can be related to the unknown circulating factor(s) mediating the increased glucose uptake seen in mice overexpressing β-catenin in the precursor cells (122) is currently unclear.

The data presented in *Paper III* demonstrate that secreted WISP2, under the control of the aP2-promoter in mice, leads to increased amount of BAT and hyperplastic subcutaneous adipose tissue. This is the completely opposite to the results seen in aP2-Wnt10b or the aP2-activated-β-catenin mice models as discussed. This clearly indicates that the proliferative effect of WISP2, albeit being a canonical WNT activator in the cell studies in *Paper II*, also allows the precursor cells to enter adipogenesis and differentiation. *Paper III* shows

that, in mesenchymal precursor cells, BMP4 can quite rapidly inhibit *Wisp2* transcriptional activation but had no acute effect on *Wnt10b*. We also found that *Bmp4* expression is increased ≈165% in sWAT and eWAT as well as BAT in Tg mice which may be secondary to the increased adipogenesis which increases cellular BMP4 (102). However, this finding adds another dimension on the cross-talk between BMP4 and WISP2, where BMP4 can overcome WISP2 inhibition, but not WNT10b, on mesenchymal adipogenic commitment and differentiation. Recent data also support the concept that BMP4 is secreted by differentiated pre/adipocytes (102) and acts as a feed-back regulator, promoting the entry of mesenchymal precursor cells into adipogenic commitment and differentiation (26,127).

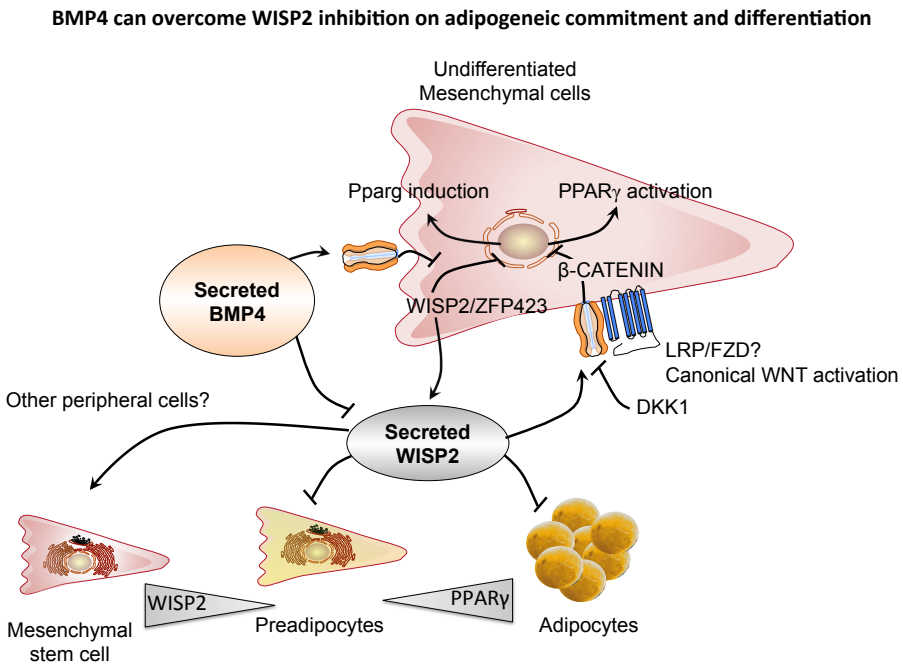

Figure 6. WISP2 is both an intracellular and a secreted protein by mesenchymal precursor cells. Intracellular WISP2 retains ZFP423 from entering the nucleus and initiate adipogenic commitment of the precursor cells. Secreted WISP2 activates the canonical WNT pathway through an unknown cellular signaling pathway involving LRP5/6. The proliferative effect of WISP2, in contrast to that of WNT (10b) ligands, also allows the precursor cells to enter adipogenesis. BMP4 can overcome WISP2 inhibition on mesenchymal adipogenic commitment and differentiation.

6 CONCLUSION

Taken together, WISP2 is an endogenous and secreted auto/paracrine WNT ligand, targeting mesenchymal precursor cells and promoting their growth and expansion.

WISP2 plays several roles in the regulation of adipogenesis by both promoting precursor cell proliferation and tissue growth, by regulating precursor cell commitment in response to BMP4 as well as the subsequent differentiation and PPARγ induction. In addition, as a secreted molecule, WISP2 can exert autocrine, paracrine and also endocrine regulation and be an important adipokine mediating cross-talk between the adipose tissue and other cells. In order to induce adipogenesis, WISP2 has to be inhibited by external signals and where BMP4 seems to be a key regulator.

In conclusion, WISP2 is a novel regulator of mesenchymal tissue growth and development and can, thereby, be an important target for preventing obesity-related metabolic complications.

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