Foxf2 and Foxc2, two transcription factors that regulate adipocyte metabolism

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Abstract

Type 2 diabetes is the most common metabolic disorder today and has reached epidemic proportions in many countries. Diet-induced insulin resistance plays a central role in the development of type 2 diabetes. Studies included in this thesis describe findings regarding two forkhead genes, Foxf2 and Foxc2, and their involvement in the development of insulin resistance. We produced a mouse in which the forkhead factor FOXF2 is overexpressed in an adipose tissue-restricted fashion, such mice display induced insulin secretion in response to an intravenous glucose load. In addition we could demonstrate that adipocytes from FOXF2 transgenic mice have an impaired insulin-mediated glucose uptake. We argue this to be, at least in part, due to lower expression of insulin receptor substrate 1. Mice overexpressing forkhead factor FOXC2 in adipose tissue have previously been shown to be protected from diet-induced obesity and glucose intolerance. In hyperinsulinemic-euglycemic clamp experiments, we demonstrated that FOXC2 transgenic mice are protected from diet-induced insulin resistance in liver and skeletal muscle. Furthermore, on high-fat diet, FOXC2 transgenic mice displayed decreased intramuscular levels of fatty acyl CoA compared with wild-type littermates. Expansion and regression of adipose tissue requires continuous remodelling of the vasculature in order to meet demands of metabolism. We have shown that the adipose tissue of FOXC2 transgenic mice exhibit a higher vascular density and altered pattering of vascular smooth muscle cells and pericytes. Furthermore, we could show this, at least in part, to be dependent on the role of FOXC2 as a direct regulator of Angiopoietin 2.

Keywords: forkhead genes, Foxc2, Foxf2, Irs1, insulin signaling, glucose metabolism, insulin resistance, Ang-2, adipose tissue, angiogenesis.

List of publications

This thesis is based on the following three articles, which are referred to in the text by their roman numerals (I-III). The articles are included at the end of the thesis.

I. **Westergren R**, Nilsson D, Heglind M, Arani Z, Grande M, Cederberg A, Ahrén B, Enerback S. Overexpression of Foxf2 in adipose tissue is associated with lower levels of IRS1 and decreased glucose uptake in vivo. Am J Physiol Endocrinol Metab. 2010 Mar; 298(3): E548-54

II. Kim JK, Kim HJ, Park SY, Cederberg A, **Westergren R,** Nilsson D, Higashimori T, Cho YR, Liu ZX, Dong J, Cline GW, Enerback S, Shulman GI. Development 132:2623-2632. Adipocyte-specific overexpression of FOXC2 prevents diet-induced increases in intramuscular fatty acyl CoA and insulin resistance. Diabetes. 2005 Jun; 54(6): 1657-63

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

Publications not used in this thesis

Virtanen KA, Lidell ME, Orava J, Heglind M, **Westergren R**, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerbäck S, Nuutila P. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009 Apr 9; 360(15): 1518-25.

Vidarsson H, **Westergren R**, Heglind M, Blomqvist SR, Breton S, Enerbäck S. The forkhead transcription factor Foxi1 is a master regulator of vacuolar H-ATPase proton pump subunits in the inner ear, kidney and epididymis. PLoS One. 2009; 4(2): e4471.

Cornier MA, Donahoo WT, Pereira R, Gurevich I, **Westergren R**, Enerback S, Eckel PJ, Goalstone ML, Hill JO, Eckel RH, Draznin B. Insulin sensitivity determines the effectiveness of dietary macronutrient composition on weight loss in obese women. Obes Res. 2005 Apr; 13(4): 703-9.

Di Gregorio GB, **Westergren R**, Enerback S, Lu T, Kern PA. Expression of FOXC2 in adipose and muscle and its association with whole body insulin sensitivity. Am J Physiol Endocrinol Metab. 2004 Oct; 287(4): E799-803.

Yanagisawa K, Hingstrup Larsen L, Andersen G, Drivsholm T, Cederberg A, **Westergren R**, Borch-Johnsen K, Pedersen O, Enerbäck S, Hansen T. The FOXC2 -512C>T variant is associated with hypertriglyceridaemia and increased serum C-peptide in Danish Caucasian glucose-tolerant subjects. Diabetologia. 2003 Nov; 46(11): 1576-80.

Abbreviations

ANG-2	- Angipoietin 2
BAT	- Brown adipose tissue
FAT/CD36	- Fatty acid translocase
FATP1	- Fatty acid transport protein 1
FFAs	- Free fatty acids
GLUT1	- Glucose transporter 1
GLUT4	- Glucose transporter 4
IKK-β	- κB kinase-β
IR	- Insulin receptor
IRS1	- Insulin receptor substrate 1
JNK	- c-Jun amino-terminal kinase
MEFs	- Mouse embryonic fibroblasts
PDK1	- Phosphoinositide-dependent kinase
PI3K	- Phosphatidylinositol 3-kinase
PIP3	- Phosphatidylinositol-3-phosphates
PKB	- Protein kinase B
PKCθ	- Protein kinase C θ
PTP-1B	- Protein-tyrosine phosphatase-1B
T2D	- Type 2 diabetes
TNFα	- Tumor necrosis factor-α
VEGF	- Vascular endothelial growth factor
vSMC	- Vascular smooth muscle cells
WAT	- White adipose tissue

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Background

Forkhead transcription factors

Transcription factors

The phenotypic differences that distinguish the different cell types of a multicellular organism are largely due to cell specific patterns of gene expression. Regulation of transcription is important both in order to distinguish the cell type and to regulate gene activity in response to specific stimuli (D. S. Latchman, 1997). The first step of gene expression is transcription initiation, which could briefly be described as the RNA polymerase II binding to the DNA, initiating transcription in order to produce mRNA. Eukaryotic RNA polymerase, however, will not bind to a "naked" DNA sequence. Rather, it requires the presence of additional proteins known as transcription factors. A defining feature of transcription factors is that they contain one or more DNA-binding domains, capable of binding to *cis*-acting regulatory DNA elements (A. D. Frankel and P. S. Kim, 1991; H. F. Lodish and J. E. Darnell, 2000). Multiple transcription factors often control transcription by binding to different *cis* elements on a single promoter generating a complex transcriptional regulating profile (L. Chen, 1999). The crucial role of transcription factors during development as well as in adult tissues has been repeatedly demonstrated in a number of organisms through analysis of phenotypes resulting from deletions or overexpressions. That transcription factors are important genes is further supported by the fact that as much as 7 % of the total mouse genes encode transcription factors (P. A. Gray et al., 2004).

The forkhead family of transcription factors

Transcription factors are usually classified into families based on sequence similarities of the evolutionarily conserved DNA binding domains. All proteins of the forkhead family of transcription factors share a highly conserved 110 amino acid DNA binding domain. Since the identification of the first member in 1990, more than 100 genes have been classified into this growing gene family (P. J. Wijchers et al., 2006). Due to the inconsistent use of different names and classification systems of the forkhead proteins, a

unifying nomenclature was proposed in 1998 and the name Fox (Forkhead box) was adopted (K. H. Kaestner et al., 2000). Family members are further classified into subclasses based on sequence homology and to date there are 19 defined subclasses (A-S) (P. J. Wijchers et al., 2006). In mammals, over 40 forkhead genes have been identified. Using targeted inactivation, many of these genes have proven to be essential for proper embryonic development and survival (review in (P. Carlsson and M. Mahlapuu, 2002)). Forkhead genes have been demonstrated to play crucial roles in a variety of processes such as regulation of the immune system (P. J. Coffer and B. M. Burgering, 2004; H. Jonsson and S. L. Peng, 2005), cell-cycle regulation (W. Korver et al., 1997; R. H. Medema et al., 2000) and metabolism (D. Q. Shih et al., 1999; A. Cederberg et al., 2001; A. Barthel et al., 2005). Mutations in forkhead genes have also been linked to human disease (S. Hannenhalli and K. H. Kaestner, 2009).

Foxf2

Human FOXF2 was identified in 1994 (S. Pierrou et al., 1994). FOXF2 was first demonstrated to have a relatively restricted expression located to the lung and placenta but later studies revealed a more widespread expression including mesenchyme adjacent to the epithelium in alimentary, respiratory, and urinary tracts (M. Hellqvist et al., 1996). Foxf2 mRNA has also been observed in the central nervous system, eye, ear, and limb buds (M. Aitola et al., 2000). Foxf2 -/- mice die at birth, most probably due to a cleft in the secondary palate, which causes air filling of the gastrointestinal tract interfering with the newborns ability to breathe and suckle (T. Wang et al., 2003). They display defects and malformations of the intestine including megacolon, colorectal muscle hypoplasia and agangliosis (M. Ormestad et al., 2006). Foxf2 +/- mice appear normal and are fertile (T. Wang et al., 2003). They do, however, have multilayered epithelium in the small intestine and are prone to develop intestine adenomas (Dr. Peter Carlsson, Personal communication). Human FOXF2 has been shown to be expressed in prostate and has been implicated to be involved in the development of prostate cancer (L. van der Heul-Nieuwenhuijsen et al., 2009)

According to data from recent studies, Foxf2 also plays a role in metabolism, as it was shown to regulate the expression of at least 9 genes involved in glycolysis and beta-

oxidation (P. Philip-Couderc et al., 2008). In addition, Gerin and colleagues have shown that Foxf2 is regulated during adipogenesis and that forced expression of Foxf2 mildly stimulates adipocyte differentiation, which suggests its involvement in adipocyte metabolism (I. Gerin et al., 2009).

In Paper I, we demonstrate mice overexpressing FOXF2 in an adipose tissue-restricted fashion to have significantly lower adipocyte insulin-mediated glucose uptake and to have impaired systemic glucose tolerance.

Foxc2

The mouse gene Foxc2 was first detected in the non-notochordial mesoderm and in mesoderm of the trunk, head and limbs (K. H. Kaestner et al., 1993; N. Miura et al., 1993). Later studies have revealed a more widespread expression including paraxial mesoderm, where Foxc2 is proposed to be involved in the regulation of differentiation and proliferation of cells giving rise to skeleton and skull (G. E. Winnier et al., 1997). During embryonic development, Foxc2 has been implicated to be involved in vascular development and cardiovascular disease (K. N. Papanicolaou et al., 2008), which is discussed in more detail below. Foxc2 -/- mice die pre-, or perinatally with multiple skeletal and cardiovascular defects, including abnormal vascular remodeling and nonfunctioning blood vessels (G. E. Winnier et al., 1997). The Foxc2 +/- phenotype is indistinguishable from wild-type and appears healthy, although more recent studies reveal a Foxc2 +/- eye phenotype, including abnormalities in ocular drainage structures and iris hypoplasia (R. S. Smith et al., 2000).

Heterozygous mutations in FOXC2 in humans cause lymphedema distichiasis syndrome, a dominantly inherited primary lymphedema (LD, OMIM #153400) (J. Fang et al., 2000; R. Bell et al., 2001). Lymphedema distichiasis syndrome is characterized by lymphedema, double rows of eyelashes (distichiasis), cardiac defects, cleft palate, renal disease and diabetes (S. M. Johnson et al., 1999; C. Yildirim-Toruner et al., 2004).

FOXC2 is also expressed in adipose tissue and has been demonstrated to be a powerful regulator of adipocyte metabolism (A. Cederberg et al., 2001). Transgenic overexpression of FOXC2 in white (WAT) and brown adipose tissue (BAT) has pleiotropic effects on gene expression in mice, leading to protection against diet-

induced insulin resistance. FOXC2 transgenic mice also experience decreased total body lipid content, lower levels of serum triglycerides and reduced plasma levels of free fatty acids (FFAs) (A. Cederberg et al., 2001). A possible role of FOXC2 as regulator of metabolic efficiency is suggested as Foxc2 expression is triggered by high-fat diet.

In paper II, we demonstrate that FOXC2 transgenic mice are protected from diet-induced insulin resistance in skeletal muscle and liver. We furthermore show this to be accompanied with decreased intracellular levels of FFA metabolites.

Obesity

The emergence of an obesity epidemic

Obesity can be defined as a disease in which excess body fat has accumulated to such an extent that health may be negatively affected. The prevalence of obesity increases rapidly in many countries and is now reaching endemic proportions in some areas. The WHO estimates that more than one billion people are overweight, and of these over 300 million are considered obese (J. C. Seidell, 2000). Obesity is not only a problem of western societies as is also seen in many developing countries. Genetic susceptibility, increased availability of high-energy food and insufficient physical activity leads to alarming rates of obesity. As a consequence it has been estimated that in year 2030, 366 million people will suffer from type 2 diabetes (T2D) (S. Wild et al., 2004).

A term often used when discussing the risks connected with obesity is the metabolic syndrome; also known as insulin resistance syndrome, dysmetabolic syndrome or syndrome X. The metabolic syndrome is characterized by visceral obesity, insulin resistance, hypertension, chronic inflammation and thrombotic disorders (M. Korbonits and S. Karger (Firm), 2008). The concept of the metabolic syndrome was introduced as a diagnostic tool to identify individuals at risk of developing T2D and cardiovascular disease and is now often used in predictions of the obesity epidemic (P. Bjorntorp, 1992) (G. M. Reaven, 1988) (G. M. Reaven, 2005). Since increased adiposity is associated with many of the key components of the metabolic syndrome, obesity has been described as the central factor in the development of the metabolic syndrome.

Type 2 diabetes and insulin signaling

The development of type 2 diabetes

T2D is caused by a combination of genetic and environmental factors and are characterized by insufficient pancreatic insulin secretion and impaired peripheral insulin sensitivity. Although development of T2D is a highly complex process a general multi-step model has been described (Figure 1). The first step of this proposed model includes impaired insulin-mediated glucose uptake and failure of insulin to suppress hepatic glucose production. Prolonged high levels of plasma glucose lead to the development of insulin resistance in peripheral tissues, including liver, skeletal muscle and adipose tissue, as a consequence β-cells in the pancreas increase insulin secretion. The β-cells finally fails to produce enough insulin to overcome the insulin resistance which leads to hyperglycemia and in the end overt T2D (M. F. Saad et al., 1991).

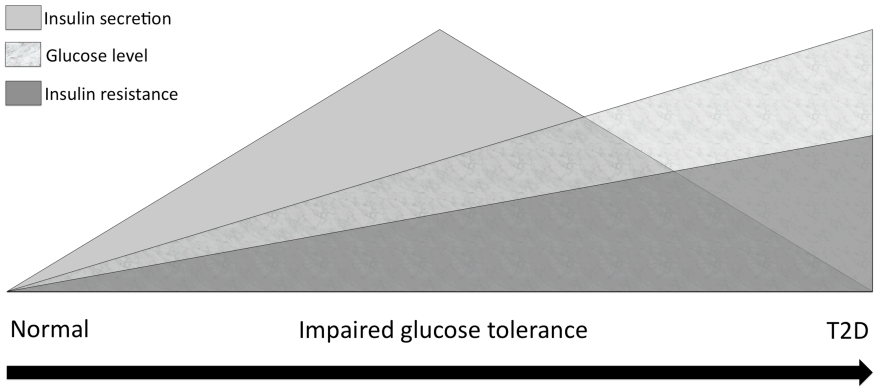

Figure 1. Progression of Type 2 diabetes.
Development of type 2 diabetes (T2D) is preceded by progressive insulin resistance in peripheral tissues, followed by an enhanced insulin secretion as well as reduced insulin clearance in the liver. Finally, the pancreatic β-cells fails to compensate for the insulin resistance, leading to impaired glucose tolerance and eventually development of overt T2D.

As described in this pathophysiologic model, insulin resistance is a key event in the development of T2D. Several factors secreted from adipose tissue have been proposed to mediate a link between obesity and insulin resistance, for example tumor necrosis factor-α (TNFα) (K. T. Uysal et al., 1997), resistin (C. M. Steppan et al., 2001), leptin (V. A. Barr et al., 1997), adiponectin (E. Hu et al., 1996) and FFAs (G. I. Shulman, 2000).

Insulin-mediated intracellular signaling

Insulin is a key anabolic hormone. Upon binding to specific surface receptors it triggers a complex intracellular signaling cascade, mainly leading to a cellular redistribution of glucose transporter 4 (Glut4). This subsequently triggers the transport of glucose from the blood into tissues (D. E. James et al., 1988). Insulin signaling is controlled by negative feedback, whereby downstream components inhibit upstream events (P. Gual et al., 2003). Alternatively, signals from other pathways can inhibit or enhance insulin signaling (Y. Zick, 2005). The insulin receptor (IR) belongs to the family of tyrosine kinase receptors that become autophosphorylated at multiple tyrosine residues upon ligand binding. This leads to the activation of an intrinsic tyrosine kinase that, in turn, can phosphorylate members of the insulin receptor substrate family (M. F. White, 1997). The most studied member of the family of insulin receptor substrates is insulin receptor substrate 1 (Irs1). The activated IR phosphorylates Irs1 on multiple tyrosine residues, which then serves as docking sites for proteins containing Src-homology-2 domains; the most important being the regulatory p85 subunit of phosphatidylinositol 3 kinase (PI3K). The binding of the PI3K p85 subunit to Irs1 activates the catalytic PI3K p110 subunit, which promotes the recruitment of PI3K to the plasma membrane. There it catalyses the phosphorylation of specific phospholipids, phosphoinositides, on the 3-position to produce phosphatidylinositol-3-phosphates (PIP3). The local insulin-induced increase in PIP3 results in the recruitment of phosphoinositide-dependent kinase (PDK1) and protein kinase B (PKB, also called Akt), the latter being one of the most important signaling mediators in metabolic insulin signaling. PIP3 dependent activation of PDK1 mediates the phosphorylation of PKB/Akt Thr308, one of the sites critical for activation of PKB/Akt (D. R. Alessi et al., 1996). PIP3 is also required for

phosphorylation of Ser473 on PKB/Akt by mTOR (D. D. Sarbassov et al., 2005). Activated PKB/Akt directly regulates a number of intracellular substrates important for glucose, protein and fat metabolism (Figure 2) (E. L. Whiteman et al., 2002; C. M. Taniguchi et al., 2006).

Insulin receptor substrate 1

The importance of Irs1 in insulin signaling is demonstrated by the phenotype of the Irs1 gene knockout mice, which exhibit impaired glucose tolerance, increased serum triglycerides and hypertension (H. Tamemoto et al., 1994; H. Abe et al., 1998). Irs1 +/- mice also present impaired glucose tolerance when given a high-fat diet (A. Shirakami et al., 2002). Impaired tyrosine phosphorylation of Irs1 has been noted in animal models of insulin resistance (M. J. Saad et al., 1992). Sequence analysis of the IRS1 gene in humans has revealed polymorphisms with higher frequency in T2D. For example the transition Gly-Arg at position 972 has shown to associate with T2D in some ethnic groups (G. A. Hitman et al., 1995). Functional studies of these patients have shown decreased activity of PKB/Akt due to defective IRS1 PI3 kinase association (M. L. Hribal et al., 2000). Furthermore, in cultured adipocytes, addition of known insulin resistance promoting proteins TNFα and interleukin-6 (IL-6) lead to decreased expression of Irs1 (V. Rotter et al., 2003)

The phosphorylation pattern of Irs1 is crucial for its function. Irs1 contains 21 putative tyrosine phosphorylation sites and more than 30 potential serine/threonine phosphorylation sites (M. F. White, 1997). Ser/Thr phosphorylation of Irs1 has shown to affect the binding of Irs1 to plasma membrane, induction of Irs1 protein degradation and inhibition of docking and binding of downstream effectors (S. Boura-Halfon and Y. Zick, 2009). Several known inducers of insulin resistance such as TNFα, FFAs and cellular stress, have all shown to promote Ser/Thr Irs1 phosphorylation (G. S. Hotamisligil et al., 1996) (E. W. Kraegen et al., 2001) (K. E. Wellen and G. S. Hotamisligil, 2005).

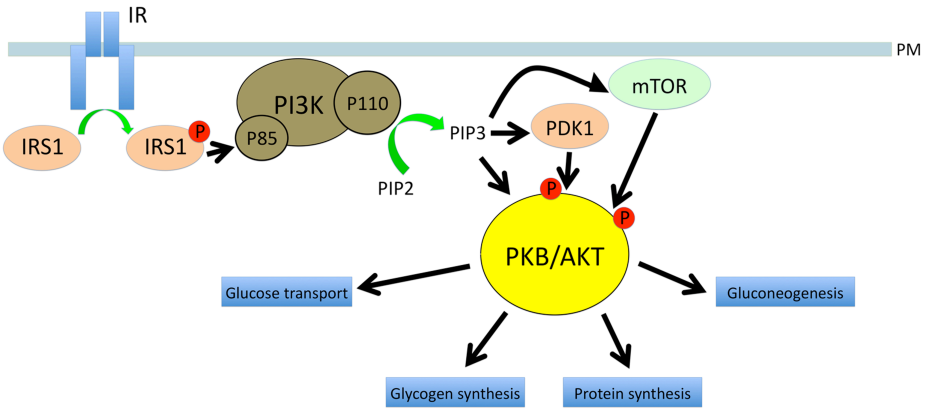

Figure 2. The Irs-dependent insulin signaling pathway.
Insulin binding to the insulin receptor (IR) results in phosphorylation of tyrosine residues on the receptor, which promotes phosphorylation of IR substrates such as insulin receptor substrate 1 (IRS1). Docking of the regulatory subunit (p85) of PI3 kinase (PI3K) to phosphotyrosine residues of IRS1 activates its serine/threonine kinase activity and the phosphorylation cascade involving phosphoinositide-dependent kinase 1 (PDK1), mammalian target of rapamycin (mTOR) and protein kinase B (PKB/Akt). For more detailed description see text.

Insulin resistance in skeletal muscle and adipose tissue

Skeletal muscle is the major site for insulin-mediated glucose uptake. *In vivo* studies have shown that skeletal muscle is responsible for as much as 80-90 % of the total insulin-mediated glucose uptake (D. Thiebaud et al., 1982). Skeletal muscle insulin resistance has been shown to be tightly linked to T2D. The concepts of the development of insulin resistance in skeletal muscle are related to alterations in the insulin signaling transduction pathway, including for example decreased Irs1 protein content, effects on Irs1 phosphorylation, reduced PI3K activity or altered protein expression of the regulatory subunit of PI3K (L. Pirola et al., 2004) (A. R. Saltiel and C. R. Kahn, 2001). Alterations in the expression and/or translocation of Glut4 to the plasma membrane have also been implicated to contribute to impaired skeletal muscle insulin response (A. E. Stenbit et al., 1997).

Several studies in both human and animal models have shown a strong inverse relationship between intracellular myocyte fat content and insulin sensitivity (G. Boden et al., 2001) (M. Krssak et al., 1999; J. K. Kim et al., 2001). In addition, mice deficient in specific fatty acid transporters (e.g. FAT/CD36 and FATP1) show decreased fatty acid uptake and exhibit improved insulin sensitivity (T. Hajri et al., 2002; J. K. Kim et al., 2004). Several theories on the effect of elevated levels of FFAs in the development of insulin resistance in skeletal muscle have been proposed. Previously, FFAs was suggested to compete with glucose metabolism in skeletal muscle, leading to insulin resistance and reduced glycogen synthesis when present at high levels (the so called Randle cycle) (P. J. Randle et al., 1963).

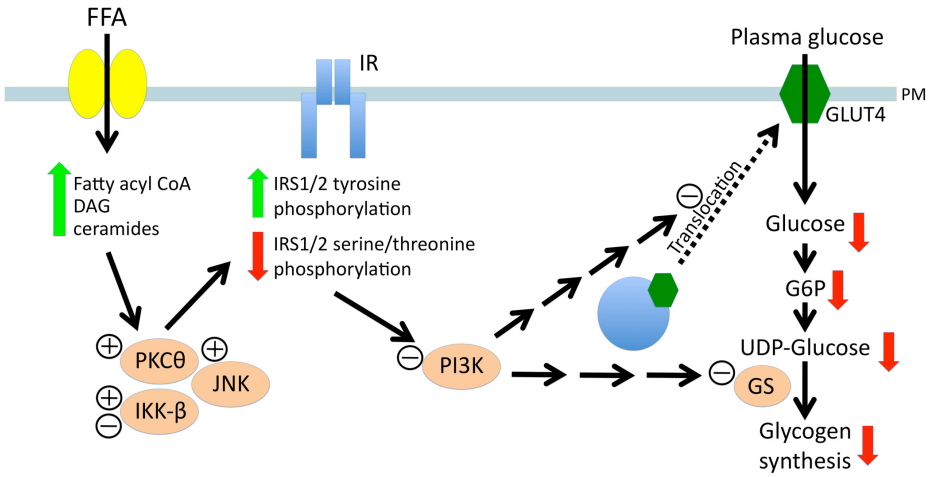

Figure 3. Mechanism of FFA-induced insulin resistance in skeletal muscle.
An increase in delivery of free fatty acids (FFAs) to muscle or a decrease in intramuscular FFA metabolism leads to an increase in intramuscular FFA metabolites such as diacylglycerol (DAG), fatty acyl CoA and ceramides. Increased intracellular levels of these metabolites activates protein kinase Cθ (PKCθ), c-Jun amino-terminal kinase (JNK) and/or inhibiting κB kinase-β (IKK-β) promoting phosphorylation of serine/threonine sites on insulin receptor substrates 1 and 2 (IRS1/2). This in turn reduces the ability of IRS1/2 to activate phosphatidylinositol 3-kinase (PI3K). This subsequently decreases glucose transport by inhibiting translocation of glucose transporter 4 (GLUT4) to the plasma membrane and inhibiting activity of glycogen synthase (GS). Glucose-6-phosphatase (G6P), Uridine diphosphate glucose (UDP-Glucose). *Adapted from (G. I. Shulman, 2000)*

However, more recent studies have gained deeper insight into the induction of insulin resistance by increased availability of FFAs (G. I. Shulman, 2000). FFA-induced insulin resistance in skeletal muscle is associated with activation of protein kinase Cθ (PKCθ) (C. Yu et al., 2002), interfering with the insulin signaling cascade with reduced insulin-mediated Irs1 tyrosine phosphorylation and reduced Irs1 associated PI3 kinase activity (M. E. Griffin et al., 1999). This correlates well with the observation that obese insulin resistant human subjects exhibit increased intramuscular activity of PKCθ (S. I. Itani et al., 2000). Several other mediators, such as, κB kinase-β (IKK-β) and c-Jun amino-terminal kinase (JNK), have also been suggested to mediate FFA-induced insulin resistance (M. Yuan et al., 2001; J. Hirosumi et al., 2002) (Figure 3).

Historically, adipose tissue has been considered to be a relatively passive energy-storing organ. However, the adipose tissue is now also recognized as a major endocrine organ, secreting factors that play an important role in appetite regulation, energy homeostasis and development of insulin resistance (B. M. Spiegelman and J. S. Flier, 1996). There are several possible links between adipose tissue function and insulin resistance in other organs, such as, resistin (C. M. Steppan et al., 2001), adiponectin (T. Yamauchi et al., 2001), leptin (K. F. Petersen et al., 2002) and TNFα (G. S. Hotamisligil et al., 1993). Perhaps the most obvious link is the regulation of FFA delivery from the adipose tissue to the rest of the body. Simplistically, an expanding adipose tissue mass delivers more FFA into the bloodstream to be utilized by, for example, skeletal muscle as discussed above. The increased FFA release from adipose tissue also promotes insulin secretion from pancreatic β-cells, contributing to the hyperinsulinaemia seen in subjects with insulin resistance (Y. Itoh et al., 2003).

In papers I and II, we show that adipocyte overexpression of two forkhead transcription factors, Foxf2 and Foxc2, regulates peripheral insulin signaling.

Adipose tissue vasculature

Vasculogenesis and angiogenesis

The formation of the vascular network is vital for embryonic development as well as in postnatal life. This process can be divided into two separate events. The formation of new vessels is called vasculogenesis, while angiogenesis refers to the formation of new vessels from already existing ones (S. F. Gilbert et al., 2006). Briefly, vasculogenesis can be described as undifferentiated endothelial cells, referred to as angioblasts, upon the influence of external stimuli differentiate into mature endothelial cells in order to form the primitive capillary plexus (S. W. Jin and C. Patterson, 2009). It was generally thought that vasculogenesis only occurred in the developing embryo, but recent data suggests that new vessels are also formed in the adult organism. Circulating endothelial progenitor cells is found to be able to contribute, to varying degrees, to the formation of new blood vessels, for example during events such as tumor growth or to the revascularization process following trauma (D. Ribatti et al., 2001) (W. Tang et al., 2008). The main triggering events of postnatal vasculogenesis are thought to be increased demand for delivery of oxygen and pro- or anti-angiogenetic agents.

The process of angiogenesis can be divided into two types. Sprouting angiogenesis refers the process where endothelial cells escape from the vessel wall and migrate into the surrounding tissue towards a source of angiogenic stimuli (P. H. Burri et al., 2004). The second type of angiogenesis is called intussusceptive angiogenesis or splitting angiogenesis, where endothelial cells from the vessel wall extend into the lumen, in order to split a single vessel in two (A. N. Makanya et al., 2009).

Angiogenesis in adipose tissue

Unlike most tissues, adipose tissue constantly undergoes expansion and regression. This requires a continuous remodeling of the tissue vasculature in order to adapt to the new tissue size and demands (G. J. Hausman and R. L. Richardson, 2004). In expanding adipose tissue the formation of new blood vessels contributes both to adipocyte hyperplasia (the generation of more adipocytes) and adipocyte hypertrophy (increasing the size of already existing adipocytes). The vessels supply the newly formed tissue with nutrients and oxygen as well as growth factors and cytokines. Furthermore the

vessel network also supplies the expanding tissue with circulating stem cells, derived mainly from the bone marrow. These stem cells have the capacity to differentiate into preadipocytes, adipocytes and vascular cells (Y. Cao, 2007). BAT is one of the most highly vascularized tissues in the body. The ample perfusion of BAT provides brown adipocytes with high amounts of oxygen and substrates needed for thermogenesis. Furthermore the dense vasculature provides a means to dissipate the heat generated to the rest of the body. A tissue specific feature of BAT is the ability to activate angiogenesis in response to cold exposure (Y. Xue et al., 2009).

Key Players in adipose tissue angiogenesis

Angiogenesis is a highly complex process and several regulatory systems are involved in the balance of promoting and inhibiting vessel formation. The main players in adipose tissue angiogenesis are vascular endothelial growth factors (VEGF) and its receptors (VEGFR) (G. Neufeld et al., 1999) (M. Papetti and I. M. Herman, 2002). VEGF have been shown to be a highly potent stimulator of angiogenesis when binding to the VEGFR. VEGF binding gives rise to a complex intracellular signaling cascade which leads to stimulation of vessel permeability, proliferation and migration of immature endothelial cells (G. Neufeld et al., 1999). VEGF expression is influenced by a number of factors, including hypoxia, insulin and several cytokines (S. Liekens et al., 2001). Many negatively acting regulators of angiogenesis have also been described, such as proliferin-related protein (D. Jackson et al., 1994), angiostatin (M. S. O'Reilly et al., 1994) and endostatin (M. S. O'Reilly et al., 1997).

Angipoietins

Some 15 years ago a second family of growth factors, termed angiopoietins, was described. To date, four members of this family have been identified (Angiopoietin 1-4)(S. Davis et al., 1996; P. C. Maisonpierre et al., 1997; D. M. Valenzuela et al., 1999)). They all act by binding to two tyrosine kinase receptors (Tie-1 and Tie-2), which are expressed by vascular and lymphatic endothelial cells. Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are specific ligands of the Tie-2 receptor. Tie-2 activation promotes vessel assembly and maturation which involves the interactions between endothelial cells and vascular smooth muscle cells (vSMC) / pericytes, which, amongst

other things, are important for developing and maintaining a mature vessel structure (N. Jones et al., 2001) Ang-1 promotes maturation and stabilization of vessels (D. Hanahan, 1997; G. D. Yancopoulos et al., 2000), while Ang-2 serves as an Ang-1 antagonist and is expressed at sites of vascular remodeling (G. Thurston, 2003). Ang-2 plays a complex role in the regulation of vascular remodeling and its function depends on expression of other angiogenic stimuli. In the presence of VEGF, Ang-2 promotes angiogenic sprouting, while in the absence of VEGF, Ang-2 contributes to vascular regression (J. Holash et al., 1999). Increased expression of Ang-2 is observed during adipose tissue growth, supporting the notion that adipose tissue development requires continuous remodeling, maturation and pattering of the vasculature (J. V. Silha et al., 2005). Increased levels of Ang-2 have also been observed during hypoxia (H. Oh et al., 1999; P. Pichiule et al., 2004) as well as in pathological conditions, for example in the endothelium of different types of tumors (L. Zhang et al., 2003; J. Oliner et al., 2004). Mice with transgenic overexpression of Ang-2 display severe vascular defects including disruption of vessel integrity (P. C. Maisonpierre et al., 1997), highly reminiscent of the phenotype of Ang-1 deficient mice (C. Suri et al., 1996). Further strengthening the hypothesis that Ang-2 exerts antagonistic function on Ang-1/Tie-2 signaling are observations that endothelial specific overexpression of Ang-2 in adult mice leads to a complete suppression of Ang-1 mediated Tie-2 signaling (Y. Reiss et al., 2007).

Foxc2 as regulator of angiogenesis

It has previously been shown that Foxc2 is involved in the regulation of embryonic development, where it is required for remodeling of the branchial arch arteries in order to form the aortic arch (K. Iida et al., 1997; G. E. Winnier et al., 1999). Foxc2 also has been shown to have important functions in lymphatic vasculature and mutations in the human FOXC2 gene are responsible for lymhedema distichiasis as previously discussed. Furthermore, the phenotype seen in Foxc2 +/- mice mimics several of the features seen in humans with lymphedema-distichiasis syndrome (B. M. Kriederman et al., 2003). Apart from this, recent research has pointed out Foxc2 as an important regulator of angiogenesis in adipose tissue, where it directly affects several proteins in remodeling, maturation and function of blood and lymph vessels (B. M. Kriederman et al., 2003; S. L. Dagenais et al., 2004). New data has revealed two cell surface molecules

present in vascular endothelial cells to serve as direct Foxc2 downstream targets. Promoters of both chemokine receptor 4 (CXCR4) and integrin β3 are activated upon Foxc2 binding, supplying strong evidence that Foxc2 is directly involved in angiogenesis (H. Hayashi and T. Kume, 2008; H. Hayashi et al., 2008).

In Paper III we describe the angiogenetic phenotype of mice overexpressing FOXC2 in adipose tissue. We furthermore provide mechanistic insight showing FOXC2 to directly control Ang-2 expression.

Results and discussion

Paper I – Adipocyte overexpression of FOXF2 regulates insulin signaling

Numerous forkhead proteins have been shown to be expressed in adipose tissue and regulating glucose metabolism e.g. FOXC2, FOXO1 and FOXA2 (A. Cederberg et al., 2001; C. Wolfrum et al., 2003; J. Nakae et al., 2008).

The FOXF2 transgenic mice model

In the current study we have, by screening of a human adipocyte cDNA library, shown that forkhead transcription factor F2 (FOXF2) is expressed is adipocytes. To investigate the role of FOXF2 *in vivo* we decided to create transgenic mice overexpressing FOXF2 in adipose tissue. We made a DNA construct in which the adipose-specific aP2 (N. R. Coe et al., 1999) enhancer/promoter, known to promote transcription in WAT and BAT *in vivo* (S. R. Ross et al., 1990), was fused to a cDNA encoding FOXF2. This construct was injected into male pronucleus of (C57BL/6J x CBA) F_1 zygotes, cultured overnight and transferred to pseudopregnant females, producing mice overexpressing FOXF2 in adipose tissue (Figure 2A, Paper I). The FOXF2 transgenic mice appeared to develop normally and to be normal regarding general behaviour and reproduction. No significant difference in terms of weight curves, adiposity, adipocyte size or food intake could be observed (Unpublished data).

Native Foxf2 expression

Using quantitative real time PCR we detected the native expression of Foxf2 in various fat depots and other tissues known to be involved in glucose metabolism. In all examined WAT depots (abdominal, inguinal and perirenal) and BAT, significant amounts of Foxf2 expression could be detected (Figure 1A, Paper I). However, no substantial Foxf2 expression was observed in pancreas, liver or skeletal muscle. To eliminate the possibility that the Foxf2 expression seen in adipose tissue originating from other cell types than the adipocyte, we measured the relative expression levels of

Foxf2 in adipocyte and stromal-vascular fraction. Foxf2 mRNA was significantly more abundant in adipocytes compared with adipose tissue stromal-vascular cells (Figure 1C, Paper I). Since the expression of several other forkhead genes have shown to be regulated during adipogenesis (I. Gerin et al., 2009), we decided to study Foxf2 expression during a prolonged differentiation of 3T3-L1 cells. Foxf2 expression displays a biphasic profile peaking at 4 and 18 days after differentiation start (Figure 1B, Paper I).

FOXF2 regulates systemic glucose tolerance

Overexpression of other forkhead genes in an adipocyte-restricted manner has been shown affect whole body glucose metabolism (A. Cederberg et al., 2001), therefore we set out to study whether a similar phenotype was present in the FOXF2 transgenic mice.

Using an intravenous glucose tolerance test, we investigated the response to an intravenously administrated glucose load. No significant difference in plasma glucose levels could be observed when comparing FOXF2 transgenic mice with wild-type littermates (Figure 2B, Paper I). However, FOXF2 transgenic mice secreted significantly more insulin in response to a given glucose load (Figure 2C, Paper I). They were also less efficient in eliminating blood glucose when given a fixed dose of insulin, as determined by glucose plasma levels (Figure 2D, Paper I). These observations suggest that FOXF2 transgenic mice have impaired insulin signaling. To study if FOXF2 affects adipocyte glucose uptake by direct regulation of glucose transport, expression of glucose transporters 1 and 4 (Glut1, Glut4) was measured in WAT and differentiated mouse embryonic fibroblasts (MEFs) from wild-type and FOXF2 transgenic mice (unpublished data). No significant difference in expression of glucose transporters could be detected in FOXF2 transgenic mice when compared with wild-type littermates, which indicates the impaired insulin-mediated glucose uptake of the FOXF2 transgenic mice to involve other mechanisms than lower expression of glucose transporters.

Based on the observation that other forkhead genes have been shown to be regulated by a high-fat diet and to be involved in the development of diet-induced insulin resistance (A. Cederberg et al., 2001) (J. K. Kim et al., 2005), we measured Foxf2 steady state mRNA levels in WAT from mice after 8 weeks of high-fat diet (35.9

% fat). A significant increase in Foxf2 expression could be detected in WAT from high-fat fed mice as compared with mice fed standard chow (4 % fat) (Figure 7, Paper I). This indicates that diet-induced alterations in Foxf2 expression could contribute to the development of insulin resistance in adipose tissue, and possibly also systemically.

FOXF2 regulates genes involved in insulin resistance

To further study the role of FOXF2 as a metabolic regulator in adipose tissue we quantified mRNA levels of genes known to regulate adipocyte metabolism (Figure 3, Paper I). Several genes known to be involved in the pathogenesis of insulin resistance, including Irs1, perilipin (J. T. Tansey et al., 2001; P. K. Saha et al., 2004), Acrp30 (adiponectin) (A. H. Berg et al., 2001; T. Yamauchi et al., 2001; C. Menzaghi et al., 2002) and carnitine palmitoyltransferase 2 (Cpt2) (J. P. Bonnefont et al., 2004) were all significantly downregulated in WAT from FOXF2 transgenic mice. The expression of sterol regulatory element binding protein 1 (Srebp1) (T. F. Osborne, 2000) however was significantly upregulated. Since the insulin secretion phenotype of FOXF2 transgenic mice appeared to be isolated to the early phase of insulin secretion, we decided to study the regulation of Irs1 further.

FOXF2 negatively regulates expression of Irs1

There is a firm body of evidence that regulation of Irs1 is involved in the development of insulin resistance and T2D. Low levels of mRNA and protein levels of IRS1 in human adipocytes have been proposed to be predictive of insulin resistance and T2D (E. Carvalho et al., 2001). Some human IRS1 mutations have also been shown to be associated with impaired insulin signaling (J. O. Clausen et al., 1995).

Apart from Irs1 mRNA levels being significantly reduced in WAT from FOXF2 transgenic mice (Figure 3A, Paper I) we could also, as judged by western blots, detect a downregulation of IRS1 protein levels in response to enhanced levels of FOXF2, in both WAT (Figure 4A, Paper I) and MEF adipocytes (Figure 4B, Paper I).

Phosphorylation of the IRS1 protein has been described as a key step in insulin signaling under both physiological and pathological conditions (J. F. Tanti et al., 1994; Y. Zick, 2005) (B. Draznin, 2006). The fact that serine/threonine phosphorylation of IRS1 impairs its ability to associate with the insulin receptor, subsequently inhibiting

insulin-mediated stimulated glucose uptake (K. Paz et al., 1997), made us study the phosphorylation pattern of IRS1 in WAT from FOXF2 transgenic mice and wild-type littermates. Previous studies have shown that increased phosphorylation of both Ser^{307} (L. Rui et al., 2001) and Ser^{612} (K. De Fea and R. A. Roth, 1997) inhibiting IRS1 mediated insulin signaling. Using western blot we could detect lower levels of both phosphorylated IRS1 Ser^{307} (Figure 5B, Paper I) and IRS1 Ser^{612} (Figure 5C, Paper I) as could be expected due to lower levels of IRS1 protein (Figures 4A and 5A, Paper I). The same level of downregulation of IRS1 tyrosine phosphorylation was also observed (Figure 5D, Paper I). Since the decrease of IRS1 phosphorylation seen in transgenic mice is fully explained by the reduced levels of IRS1 protein, we suggest that any effect on insulin signaling seen in transgenic mice is due to lower total levels of IRS1 protein rather than an altered pattern of IRS1 phosphorylation.

FOXF2 reduces insulin-mediated glucose uptake

Is the impaired insulin mediated glucose uptake seen in FOXF2 transgenic mice a consequence of effects on adipocyte insulin signaling? To address this question, we studied insulin-mediated glucose uptake in differentiated MEFs derived from FOXF2 transgenic and wild-type mice. In the range of physiological insulin levels, a significant reduction (~25 %) in glucose uptake was detected for FOXF2 transgenic adipocytes as compared to wild-type adipocytes (Figure 6, Paper I). An observation that could be compared to the ~50 % reduction in insulin-mediated glucose uptake seen in adipocytes from Irs1 deficient mice (Y. Kaburagi et al., 1997).

Paper II – Adipocyte overexpression of FOXC2 prevents diet-induced insulin resistance

Many techniques have been deployed in order to study insulin signaling in peripheral insulin responding tissues. However hyperinsulinemic-euglycemic clamp technique is considered to be the golden standard for measuring insulin action *in vivo* (R. A. DeFronzo et al., 1979). With this technique, a constant rate of insulin is infused intravenously in order to increase the uptake of circulating blood glucose and inhibiting endogenous glucose production by the liver. The decline in plasma glucose is prevented by a variable rate of intravenous glucose infusion. The amount of exogenous glucose required to maintain plasma glucose at its initial level is quantified by the glucose infusion rate. Since as much as 80–90 % of the infused glucose is taken up by skeletal muscle, insulin sensitivity measured with this technique primarily reflects skeletal muscle metabolism (D. Thiebaud et al., 1982). Clamp experiments can be significantly enhanced by the use of glucose tracers. Glucose can be radioactively labeled and the concentration of glucose metabolites can be detected in individual organs after finished clamp experiments. Mice overexpressing FOXC2 have previously been shown to be protected from diet-induced obesity and glucose intolerance (A. Cederberg et al., 2001). In order to study the effect of FOXC2 overexpression on tissue-specific glucose metabolism and insulin signaling *in vivo*, we subjected mice to a hyperinsulinemic-euglycemic clamp experiment. Studies were performed on FOXC2 transgenic and wild-type mice, before and after 3 weeks of high fat feeding.

FOXC2 regulates lean and fat mass distribution

As a first step, we set out to explore whole-body lean and fat mass distribution in FOXC2 transgenic compared with wild-type mice. We have previously shown, by comparing the mass of dissected fat depots, that FOXC2 transgenic mice have altered adipose tissue distribution and morphology (A. Cederberg et al., 2001). They have enlarged interscapular BAT depots whereas the intraabdominal WAT depot is clearly reduced in size and displays a more "brownish" color. These features are most probably due to a FOXC2 dependent regulation of adipocyte gene expression. WAT from FOXC2 transgenic mice displays a more BAT like phenotype with upregulated

expression of genes associated with mitochondrial function and biogenesis, e.g. cythochrome oxidase subunit II (*coxII*) and PPARγ coactivator 1 (*PGC-1*). Significant expression of the BAT specific gene uncoupling protein 1 (Ucp1) is also seen in WAT from FOXC2 transgenic mice (A. Cederberg et al., 2001). Using electron microscopy it was shown that WAT adipocytes from FOXC2 transgenic mice contained more and larger mitochondria as compared with wild-type adipocytes (A. Cederberg et al., 2001).

In the current study, we could by using ^{1}H magnetic resonance spectroscopy estimate FOXC2 transgenic mice to have significantly lower whole-body fat mass and higher whole-body lean mass than weight matched wild-type littermates (Figure 1AB, Paper II). After 3 weeks on high-fat diet, transgenic mice had gained significantly less whole-body fat mass but more whole-body lean mass than wild-type mice (Figure 1AB, Paper II), a result that can be explained by the elevated adipocyte metabolism seen in FOXC2 transgenic mice.

FOXC2 regulates glucose metabolism

The failure of insulin to inhibit hepatic glucose production (HGP) is a basic feature of insulin resistance in the liver and a major factor contributing to the hyperglycemia seen in T2D.

On standard chow (4 % fat), HGP rates did not differ between the FOXC2 transgenic and wild-type mice. However, following high-fat diet (55 % fat), typical signs of liver insulin resistance with significantly blunted insulin-mediated suppression of HGP could be observed in wild-type mice, while FOXC2 transgenic mice appear, at least to some extend, to be protected from diet-induced insulin resistance in the liver (Figure 1D, Paper II). Whole-body glucose turnover gives an indication of the rate at which glucose is eliminated from the blood stream and is defined as the ratio of the glucose infusion rate and plasma glucose levels at the end of the clamp experiment. As could be expected, insulin-mediated whole-body glucose turnover was significantly decreased in wild-type mice on high-fat diet, indicating impaired insulin-stimulated glucose uptake (Figure 2A, Paper II). In contrast, FOXC2 transgenic mice seem to be completely protected from diet-induced decrease in whole-body glucose uptake, indicating a less affected insulin signaling pathway (Figure 2A, Paper II).

Based on the notion that the skeletal muscle takes up most of the infused glucose, levels of glucose transferred to the skeletal muscle and stored in the form of glycogen were detected using ion-exchange chromatography (as described in (H. J. Kim et al., 2004)). No significant difference in skeletal muscle glucose uptake could be observed when comparing FOXC2 transgenic and wild-type mice fed standard chow. FOXC2 transgenic mice however seem to have a moderately affected skeletal muscle glucose uptake following high-fat diet as could be compared with the ~40 % decrease in glucose uptake seen in wild-type mice. This indicates that FOXC2 transgenic mice are to some extent protected from diet-induced insulin resistance in skeletal muscle.

FOXC2 regulates skeletal muscle insulin signaling

Obesity and T2D are usually associated with elevated levels of circulating free fatty acids, leading to accumulation of fatty acid metabolites in skeletal muscle. This subsequently leads to fat-induced skeletal muscle insulin resistance for which several possible mechanisms have been proposed (L. A. Consitt et al., 2009). Recent studies have shown fat-induced insulin resistance in skeletal muscle to involve elevated levels of intramuscular fatty acyl CoA leading to activation of PKC-θ (C. Yu et al., 2002). Activation of PKC-θ promotes Irs1 serine phosphorylation, which eventually interferes with its ability to activate PI3 kinase leading to impaired insulin signaling and insulin resistance.

In the current study we measured Irs1-associated PI3 kinase activity and fatty acyl CoA concentrations in skeletal muscle from both FOXC2 transgenic and wild-type mice, before and after high-fat diet. Irs1-associated PI3 kinase activity was shown to be markedly reduced in wild-type skeletal muscle as an effect of high-fat diet (Figure 3C, Paper II). In contrast, skeletal muscle from FOXC2 transgenic mice show moderately affected Irs1-associated PI3 kinase activity. Using liquid chromatography tandem mass spectrometry (LC/MS/MS), intramuscular fatty acid content in dissected quadriceps was detected by concentrations of fatty acyl CoA. Total fatty acyl CoA concentrations did not differ between wild-type and FOXC2 transgenic mice on standard chow. In contrast, following high-fat diet levels of individual long-chain fatty acyl CoA (C16:0, C16:1,

C18:0, C18:1, C18:2 and C18:3) as well as total fatty acyl CoA were significantly lower in FOXC2 transgenic mice as compared with wild-type mice (Figure 4B, Paper III).

We show that the protective phenotype from diet-induced insulin resistance in skeletal muscle, seen in the FOXC2 transgenic mice is associated with normal levels of intramuscular fatty acyl CoA following high-fat feeding. We would like to propose this to be, at least in part, due to elevated adipocyte metabolism (A. Cederberg et al., 2001) in which FFAs are mainly metabolized by the adipose tissue. This in turn prevents their accumulation in the skeletal muscle. The elevated metabolism seen in adipocytes from FOXC2 transgenic mice includes increased adipocyte expression of perisome proliferators-activated receptor-γ (PPAR-γ). Studies have shown PPAR-γ agonist rosiglitazone to improve insulin resistance in skeletal muscle in A-ZIP/F-1 lipodystrophic mice by redirecting FFAs to the liver rather then the skeletal muscle (J. K. Kim et al., 2003). The increase expression of PPAR-γ seen in FOXC2 transgenic mice most probably contributes to the redistribution of FFA from the liver and skeletal muscle towards the adipose tissue. The characteristics of the FOXC2 transgenic metabolic phenotype resemble those of mice lacking protein-tyrosine phosphatase-1B (PTP-1B), which have been shown to exhibit lower body fat stores, increased energy expenditure, altered glucose homeostasis and enhanced skeletal muscle insulin sensitivity (L. D. Klaman et al., 2000). However, the increased metabolic rate in the PTP-1B mice has been shown to be mainly due to the upregulation of mitochondrial biogenesis in skeletal muscle rather than altered adipocyte metabolism.

Paper III – FOXC2 regulates angiogenesis in adipose tissue

The growth of adipose tissue mass leading to obesity is associated with both enlarged size and increased numbers of adipocytes. Associated with the ability of adipose tissue to grow and regress is its vasculatures capacity to adapt and meet metabolic needs (Y. Cao). Forkhead proteins have previously been shown to be directly or indirectly involved in the development of the vascular system (T. Hosaka et al., 2004; M. Potente et al., 2005; S. Seo et al., 2006) (Z. Wang et al., 2007). Members of at least five different subfamilies of forkhead transcription factors are expressed in endothelial cells or their precursors (K. N. Papanicolaou et al., 2008). One example is FoxO1, where FoxO1-null embryos die embryonically as a consequence of incomplete vascular development (T. Hosaka et al., 2004). Foxc2 has previously been implicated as a regulator of vascular development (K. N. Papanicolaou et al., 2008) and during embryonic development, Foxc2 is required for proper development and remodeling of both blood vessels and lymphatic vasculature (S. Seo et al., 2006) (T. V. Petrova et al., 2004).

In the present study we describe the vascular phenotype of transgenic mice overexpressing FOXC2 in adipocytes. We also describe the role of FOXC2 in vascular development in adipose tissue and findings that FOXC2 act as a direct regulator of Ang-2 expression.

Vascular phenotype of FOXC2 transgenic mice

The phenotype of mice overexpressing FOXC2 in adipose tissue includes reduced size of the intraabdominal WAT depot in a combination with it having a more brownish color (A. Cederberg et al., 2001). Electron microscopy examination revealed an increased number and size of mitochondria in WAT from FOXC2 trangenes. Activation of the adrenergic/cAMP/protein kinase A signaling pathway in combination with the observation that FOXC2 transgenic WAT consumes significantly more oxygen (4-fold) suggests an elevated metabolism in FOXC2 transgenic mice compared with wild-type littermates (A. Cederberg et al., 2001). With increased metabolism comes increased demands regarding for example metabolite exchange, transport of adipokines and hormones. With this in mind we decided to perform immunohistochemical analysis of

epididymal and inguinal WAT as well as interscapular BAT from FOXC2 transgenic and wild-type mice.

Using an anti-CD31 antibody specific for endothelial cells, we could show a significant increase (2- to 3-fold) in vascular density in both WAT and BAT from FOXC2 transgenic mice (Figure 1, Paper III). This agrees well with the description of WAT from transgenic mice exhibit a more BAT-like phenotype, as BAT is significantly more metabolic active and more vascularized than WAT.

These observations led us to further study vascular remodeling and maturation. Using an anti-SMA antibody we detected the presence of vascular smooth muscle cells (vSMCs). As expected, vSMCs in the wild-type tissue were localized to the large arterial blood vessels, as the main function of these cells is to support the endothelial cell in the vessel wall (J. E. Hungerford and C. D. Little, 1999). However no vSMCs could be detected in micro vessels (Figure 2, Paper III). Interestingly, both WAT and BAT from FOXC2 transgenic mice contained significantly fewer SMA positive arterial vessels compared with wild-type mice (Figure 2B, Paper III). On the other hand a significant amount of SMA positive cells could be observed with the micro vessels (Figure 2A, Paper III).

Using a pericyte specific anti-NG2 antibody we could also detect a similar trend of redistribution of pericytes in microvasculature of WAT and BAT of FOXC2 transgenic mice. Pericytes are mainly found in smaller vessels serving as vessel supporting cells and in wild-type mice were mainly associated with the arterial vessels and broadly distributed in intervascular spaces. However, they were found in a relatively low degree associated with microvessels (Figure 2A, Paper III). In transgenic tissue an altered distribution of NG2 positive pericytes to the microvessels and large arterial vessels could be observed (Figure 2A, Paper III). Quantification revealed a significant increase in the number of vSMCs in FOXC2 transgenic WAT and BAT as compared with wild-type tissue. In contrast, the number of pericytes seemed to be decreased in the transgenic tissue (Figure 2B, Paper III). These findings demonstrate that overexpression of FOXC2 in adipose tissue leads to alterations of both vessel numbers and vascular structure. We would like to speculate that overexpression of FOXC2 affects vessel maturation by regulation of vSMCs and pericyte distribution, in

order to adapt to the higher metabolic rate seen in adipose tissue of FOXC2 transgenic mice.

As previous studies have shown that enhanced FOXC2 expression in adipose tissue is able to affect whole body metabolism we decided to study blood vessel formation in non-adipose tissue. Using the same anti-CD31 antibody as described above we detected vessel organization in dermal and subcutaneous tissues. This revealed a similar phenotype of that of WAT and BAT, including a denser vascular network tissue in FOXC2 transgenic mice as compared with wild-type littermates (Figure 1, supplementary information, Paper III). We also observed the vasculature in tissue from FOXC2 transgenic mice to be more irregular and having significantly shorter inter-capillary distance. As a consequence of the reorganized vessel network a significantly delayed skin wound healing could be observed in FOXC2 transgenic mice compared with wild-type littermates (Figure 5, Paper III).

FOXC2 regulates angiogenesis

A growing body of evidence shows that the adipocyte is able to cross-communicate with neighboring endothelial cells (L. J. Hutley et al., 2001) (B. Cohen et al., 2001; Y. Cao, 2007). In an attempt to identify the paracrine and endocrine factors produced by FOXC2 transgenic adipocytes we performed Affymetrix microarray and quantitative real time PCR, comparing the expression profiles of abdominal WAT from FOXC2 transgenic and wild-type mice. Expression of several proteins known to affect angiogenesis seemed to be regulated. Among these a number of genes that could contribute to the vascular phenotype seen in adipose tissue of FOXC2 transgenic mice (Figure 3A, Paper III). Ang-2 turned out to be the most regulated gene (almost 6-fold induction). Since Ang-2 is known to regulate vascular pattering, remodeling and maturation (E. Geva et al., 2002; B. Hu et al., 2003) and the fact that several studies have shown Ang-2 to be upregulated in expanding adipose tissue (J. V. Silha et al., 2005; G. Voros et al., 2005) we choose to study this further. Using real time PCR we could demonstrate the expression level of Ang-2 to correlate with the expression of Foxc2 in tissue from Foxc2 +/- mice (Figure 3B, Paper III), a observation that also suggests Foxc2 to be able to regulate Ang-2 expression.

FOXC2 transcriptionally regulates Ang-2 expression

Using the forkhead consensus binding sequence, a sequence analysis of the Ang-2 promoter revealed several putative binding sites for FOXC2. An Ang-2 promoter sequence was fused with a luciferase reporter vector and transfected into 3T3-L1 adipocytes in the presence and absence of FOXC2. A FOXC2 dependent induction of Ang-2 promoter activity could be observed (Figure 3C, Paper III). To further delineate the ability of FOXC2 to be a direct regulator of Ang-2 expression, all of the putative FOXC2 promoter binding sites were individually mutated. Inactivation of a forkhead binding *cis*-element (Fkh4) almost completely abolished the FOXC2 induced activation of the Ang-2 promoter (Figure 3C, Paper III). The contribution of Fkh1-3 and Fkh5 on the other hand, appeared to be minor. These results strongly support the role of FOXC2 as being a direct regulator of Ang-2 expression.

Ang-2 mediates the vascular phenotype of FOXC2 transgenic mice

To further study the relation between FOXC2 and Ang-2 we set out to determine whether a known Ang-2-specific inhibitor could affect the angiogenic phenotype seen in FOXC2 transgenic mice. The Ang-2 inhibitor L1-10 was administrated to FOXC2 transgenic mice in a dose known to block Ang-2 function, and were shown to completely reverse the FOXC2 angiogenic phenotype, based on histology of WAT and BAT (Figure 4, Paper III). These observations suggest that the angiogenic effect of FOXC2 is mediated trough activation of Ang-2.

Conclusions and concluding remarks

In paper I, we identify FOXF2 as an adipocyte expressed forkhead gene. We also show that mice overexpressing FOXF2 in adipose tissue have decreased glucose uptake *in vivo* and display a significantly increased insulin secretion in response to an intravenous glucose load. This is, at least in part, due to an impaired adipocyte insulin signaling associated with decreased levels of Irs1. This is in agreement with the observation that mice deficient in Irs1 expression, display impaired glucose tolerance (H. Abe et al., 1998), and that low levels of IRS1 mRNA and protein in human adipocytes is linked with insulin resistance and T2D (E. Carvalho et al., 2001). Furthermore we show that FOXF2 transgenic adipocytes, similar to adipocytes from Irs1 deficient mice (Y. Kaburagi et al., 1997), exhibit a significantly impaired insulin stimulated glucose uptake as compared with wild-type adipocytes. We also show Foxf2 expression to be induced in WAT following high fat feeding, which suggests involvement in diet-induced insulin resistance.

In paper II, we show that mice overexpressing FOXC2 in adipose tissue are protected from diet-induced alterations in liver and skeletal muscle insulin signaling. We furthermore provide evidence that this is associated with decreased levels of intramuscular fatty acid metabolites. Previous studies have pointed out intramuscular accumulation of fatty acid metabolites (e.g. fatty acyl CoA) to cause the development of fat-induced insulin resistance (C. Yu et al., 2002). In the present study we show levels of intramuscular fatty acyl CoA to be significantly increased in wild-type skeletal muscle after 3 weeks on a high-fat diet. In contrast, FOXC2 transgenic mice had more modest levels of intramuscular fatty acyl CoA. We suggest this to be due to increased adipocyte lipid metabolism of the FOXC2 transgenic mice, which prevents lipid accumulation in liver and skeletal muscle. Furthermore, we confirm previous data (A. Cederberg et al., 2001) showing FOXC2 transgenic mice to have significantly reduced whole body fat mass as compared with wild-type littermates, both when fed standard and high-fat diet.

In paper III, we describe the angiogenic phenotype seen in mice overexpressing FOXC2. This includes a denser and more irregular vascularization in WAT and BAT. Immunohistochemistry examinations reveal an altered distribution of smooth muscle cells and pericytes in adipose tissue from transgenic mice, which indicates effects on vessel remodeling and maturation. We propose this to be, at least in part, due to the higher metabolic rate of transgenic adipocytes (A. Cederberg et al., 2001). In BAT it is well established that increased oxygen consumption and elevated metabolic rate is associated with increased vessel growth and remodeling in response to metabolic needs (A. Asano et al., 1997; C. Tonello et al., 1999). We could further demonstrate regulation of several potent angiogenetic factors, for example Ang-2, in WAT from FOXC2 transgenic mice as compared with wild-type littermates. Since Ang-2 has been shown to be important for regulation of vascular pattering, remodeling and maturation (E. Geva et al., 2002; B. Hu et al., 2003) we investigated this further. Using a luciferase based experimental approach we could point out the capacity of FOXC2 to act as a direct transactivator of the Ang-2 promoter. To further establish the role of FOXC2 as regulator of Ang-2 expression, we could show an almost complete reversal of the FOXC2 transgenic angiogenic phenotype when administrating a known Ang-2 inhibitor. Taken together, our observations indicate that elevated adipocyte metabolic rate seen in mice overexpressing FOXC2, is associated with altered vascularization of adipose tissue and this to be, at least in part, due to transcriptional regulation of Ang-2.

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