Forkhead genes in adipocytes and podocytes

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Cover image: False-colored scanning electron micrographs of a mouse adipocyte partly covered with connective tissue (left) and podocytes covering the outer surface of a mouse glomerulus (right).

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ABSTRACT

Forkhead genes are a family of transcription factors with important functions in development and metabolism. This thesis addresses tissue-specific functions of the two forkhead genes, *FOXC2* and *FOXF2*, using transgenic mouse models. Overexpression of either *FOXC2* or *FOXF2* in adipocytes resulted in opposing phenotypes in terms of insulin sensitivity. Induction of *FOXC2* increased insulin sensitivity and protected the mice against diet-induced insulin resistance based on results from hyperinsulinemic-euglycemic clamp. In addition, *FOXC2* induced the expression of ANGPT2, an angiogenic factor which in turn increased the vascular density in the adipose tissue and supported the adipocyte with increased capacity for energy supply and waste disposal. *FOXF2*, on the other hand, appeared to block insulin signaling in adipocytes by decreasing the expression of IRS1, an important component in the transduction of insulin signaling. Consistently, these mice displayed decreased insulin sensitivity in glucose and insulin tolerance tests. Finally, we generated mice with conditional deletion of *Foxc2* in podocytes and found that such deletion lead to severe proteinuria and kidney failure shortly after birth. Ultrastructural analyses revealed that the podocytes had lost their unique architecture of interdigitated foot processes, and instead, had developed microvilli structures that projected into the urinary space. In conclusion, these studies demonstrate important roles of *FOXC2* and *FOXF2* in insulin sensitivity and kidney function, roles that might also be relevant to human disease conditions.

Keywords: FOXC2, FOXF2, forkhead, transgenic animal, adipocyte, insulin signaling, insulin resistance, lipotoxicity, angiogenesis, ANGPT2, podocyte, proteinuria

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SAMMANFATTNING PÅ SVENSKA

Forkheadgener tillhör en familj transkriptionsfaktorer som visat sig spela en essentiell roll under såväl embryonalutveckling som i metabolism. Denna avhandling fokuserar på vävnadsspecifika funktioner för två forkheadgener, FOXC2 och FOXF2, genom studier av transgena musmodeller. I två av modellerna, där antingen FOXC2 eller FOXF2 överuttrycks i fettceller, visade det sig att dessa gener påverkar insulinkänslighet i varsin riktning. Genom att öka mängden FOXC2 i fettcellen så ökar också fettförbränningen. Detta är inte bara positivt för fettcellen, utan det visade sig också att övriga vävnader uppvisade en högre insulinkänslighet när detta studerades med så kallad glukos-klamp. Framförallt skyddade den ökade halten av FOXC2 i fettcellen mot den insulinresistens som möss annars utvecklar efter att ha ätit fettrikt foder, som till sammansättningen är ganska lik vanlig västerländsk mat. Den ökade energiomsättningen i fettceller med mycket FOXC2 ökar också behovet av energitillförsel till, och bortförsel av slaggprodukter från, fettväven. Blodkärlen bistår cellerna med detta och det visade sig att FOXC2 kan öka blodtillförseln genom att öka utsöndringen av det kärlstimulerande proteinet angiopoietin 2 från fettcellen. Ökad nivå av FOXF2 verkar istället blockera insulinsignaleringen i fettcellen. Detta fick till följd att mössen även utvecklade insulinresistens i andra vävnader. Utöver dessa geners roll i fettcellen har vi även studerat vilken roll FOXC2 spelar i podocyten, en väldigt specialiserad celltyp i njuren där cellen har en avgörande betydelse för njurens förmåga att hindra blodproteiner från att läcka ut i urinen. En musmodell i vilken FOXC2 tagits bort specifikt i podocyterna togs fram, och avsaknaden av FOXC2 i podocyterna resulterade i höga nivåer av protein i urinen och njursvikt strax efter födseln. När podocyterna i dessa möss studerades i hög förstoring med hjälp av elektronmikroskopi kunde man se att cellerna helt ändrat utseende. Istället för de väldigt strukturerade och sammanflätade utskott som cellerna har i friska njurar, verkar dessa utskott istället sträcka sig planlöst ut från cellkroppen när cellen saknar FOXC2. Utan podocyternas sammanflätade filter läcker protein ut i urinen och orsakar till slut njursvikt.

Sammantaget visar resultaten från de använda djurmodellerna hur viktiga de båda genreglerande faktorerna FOXC2 och FOXF2 är, både med avseende på insulinkänslighet och njurfunktion, och de båda faktorerna kan potentiellt spela betydande roller i humana sjukdomar kopplade till dessa processer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals (I-IV).

I. Kim JK, Kim HJ, Park SY, Cederberg A, Westergren R, **Nilsson D**, Higashimori T, Cho YR, Liu ZX, Dong J, Cline GW, Enerback S, and Shulman GI. Adipocyte-specific overexpression of FOXC2 prevents diet-induced increases in intramuscular fatty acyl CoA and insulin resistance. *Diabetes* 2005;54:1657-1663.

II. Xue Y, Cao R, **Nilsson D**, Chen S, Westergren R, Hedlund EM, Martijn C, Rondahl L, Krauli P, Walum E, Enerbäck S, and Cao Y. FOXC2 controls Ang-2 expression and modulates angiogenesis, vascular patterning, remodeling, and functions in adipose tissue. *Proc Natl Acad Sci U S A* 2008;105:10167-10172.

III. Westergren R, **Nilsson D**, Heglind M, Arani Z, Grände M, Cederberg A, Ahrén B, and Enerbäck 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;298:E548-554.

IV. **Nilsson D**, Heglind M, Arani Z, and Enerbäck S. Foxc2 is essential for proper podocyte function. *Manuscript***.**

PAPERS NOT INCLUDED IN THIS THESIS

Lidell ME, Betz MJ, Dahlqvist Leinhard O, Heglind M, Elander L, Slawik M, Mussack T, **Nilsson D**, Romu T, Nuutila P, Virtanen KA, Beuschlein F, Persson A, Borga M, and Enerbäck S. Evidence for two types of brown adipose tissue in humans. *Nat Med* 2013;19:631-634.

Betz MJ, Slawik M, Lidell ME, Osswald A, Heglind M, **Nilsson D**, Lichtenauer UD, Mauracher B, Mussack T, Beuschlein F, and Enerbäck S. Presence of brown adipocytes in retroperitoneal fat from patients with benign adrenal tumors: relationship with outdoor temperature. *J Clin Endocrinol Metab* 2013;98:4097-4104.

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CONTENT

ABBREVIATIONS

ACTN4	α-actinin 4
AKT2	AKT serine/threonine kinase 2
ANGPT	Angiopoietin
aP2	Adipocyte protein 2 (Fatty acid binding protein 4)
α-SMA	alpha smooth muscle actin
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
cAMP	Cyclic adenosine mono-phosphate
CD2AP	CD2 associated protein
CD31	Cluster of differentiation 31
cDNA	Complementary deoxyribonucleic acid
CG	Collapsing glomerulopathy
COL4A	Collagen IV alpha
Cre	Causes recombination
CYR61	Cysteine rich angiogenic inducer 61
DAG	Diacylglycerol
DMS	Diffuse mesangial sclerosis
DTA	Diphtheria toxin A-fragment
FAT1	Protocadherin fat 1
FFA	Free fatty acid
fl	floxed (surrounded by loxP sites)

FOX	Forkhead box
FSGS	Focal segmental glomerulosclerosis
GLUT4	Glucose transporter 4
HRP	Horse radish peroxidase
HSL	Hormone sensitive lipase
ILK	Integrin linked kinase
INSR	Insulin Receptor
IRS	Insulin Receptor Substrate
ITGB1	Integrin subunit beta 1
lacZ	Gene encoding beta-galactosidase
LMX1B	LIM homeobox transcription factor 1 beta
loxP	locus of X(cross)-over P1
MAGI	Membrane associated guanylate kinase inverted
MCN	Minimal change nephropathy
MEF	Mouse embryonic fibroblast
mRNA	Messenger ribonucleic acid
NPHS1	Nephrin
NPHS2	Podocin
NRP1	Neuropilin 1
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C

Pod	Podocin
PODXL	Podocalyxin
PPAR	Peroxisome proliferator activated receptor
qPCR	Quantitative polymerase chain reaction
RHPN1	Rhophilin Rho GTPase binding protein 1
TEK	TEK tyrosine receptor kinase
TJP1	Tight junction protein ZO-1
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WHO	World Health Organization
WT1	Wilms tumor 1

DEFINITIONS IN SHORT

Obesity	defined by the World Health Organization (WHO) as having a body mass index (BMI) (weight/(length*length)) above 30 kg/m^2, whereas individuals with BMI between 25-30 kg/m^2 are considered overweight (1).
Insulin resistance	a condition where insulin responsive tissues, primarily skeletal muscle, liver, and adipose tissue, do not respond to insulin properly and need higher insulin serum levels to absorb glucose from the blood. As a consequence, beta-cells in pancreas increase the secretion of insulin to maintain the blood glucose level.
Type 2 diabetes	a metabolic disease characterized by a fasting plasma glucose level ≥126 mg/dl (7.0 mmol/l) or a casual plasma glucose ≥200 mg/dl (11.1 mmol/l) which occurs when beta-cells loses the ability to compensate for the increased requirement of insulin seen in insulin resistance (2).
Angiogenesis	the formation of new blood vessels from pre-existing vessels, while vasculogenesis is the term used for the formation of completely new vessels, especially during formation of vasculature in the embryo (3).

INTRODUCTION

FORKHEAD TRANSCRIPTION FACTORS

Gene transcription is a complex chain of events in which the coding DNA sequence is translated into RNA, and regulation of this process is critical for a cell to function correctly. The motor in this process is the RNA polymerase, an enzyme which has the ability to bind to promoter regions of genes and synthesize RNA based on the complementary code of the DNA (4,5). Since RNA polymerase itself does not possess specificity for different promoters, specificity is provided by interaction with transcription factors. Such factors can for instance bind to specific DNA sequences close to the site of transcription initiation in order to either activate or block transcription (5). Besides providing binding site for RNA polymerase on cell-specific promoters, transcription factors have also been shown to promote transcription by actively opening up the condensed chromatin (5). One family of transcription factors are the forkhead transcription factors that share a highly conserved 100 amino acid DNA binding domain, the so-called fork head domain (6). The name comes from the Drosophila mutant that, due to mutation of the gene fork head (*fkh)*, had a forked, or split, head (7,8).

The forkhead family consists of more than 40 members in mammals, and null mutant mice for many of the genes result in early developmental defects and embryonic lethality (9). Forkhead genes have also been reported to play important roles in the adult in diverse areas, including metabolism, immunology, and disease (9-12). In response to external stimuli, the activity of forkhead transcription factors can be regulated by post-translational modifications, such as phosphorylation and acetylation (13-15). These modifications may control cellular localization of the factor as well as its ability to bind DNA. The forkhead transcription factors have in some literature been termed “winged helix” proteins, based on their structure and ability to embrace DNA (16,17). Still, several, phylogenetically unrelated, proteins display a winged helix structure (18). Therefore the nomenclature was revised and the forkhead genes are now referred to as FOX (Forkhead box) genes and divided into 19 phylogenetic subclasses denoted by the letters A-S (19,20).

FOXC2

In the early 1990s, *Foxc2* was identified as a mesenchymal forkhead gene, due to its expression in the developing mesenchyme of the mouse embryo. Hence, the gene was initially named mesenchyme fork head 1(*Mfh-1*) (21). During development, expression of this single-exon gene is first detected in non-notochordial mesoderm and subsequently in developing cartilaginous tissues, dorsal aortas, heart and the metanephros (21,22). The importance of *Foxc2* in these developing structures was highlighted in global *Foxc2* knockout mice which suffered from embryonic or perinatal lethality due to skeletal and urogenital defects as well as an interrupted aortic arch (23-25).

Human and mouse FOXC2 share more than 85 % homology in amino acid sequence, hence they are well conserved and likely to share function among species. This would explain why no homozygous mutations of human *FOXC2,* causing disruption of the gene function, have been identified so far. Nevertheless, heterozygous mutations in *FOXC2* have been shown to cause lymphedema distichiasis syndrome, a disease accompanied by lymphedema of the limbs and double rows of eyelashes (distichiasis) (26,27). Interestingly, although heterozygous *Foxc2* knockout mice appear healthy, they have been reported to display both lymphatic defects and distichiasis (28,29), confirming the conserved function between species. Some lymphedema distichiasis patients have also been reported to develop renal disease and diabetes mellitus. Genetic analysis revealed that these patients were heterozygous for a lymphedema distichiasis-causing mutation in *FOXC2* and homozygous for another allelic variation, which might affect the expression level of the functional protein (30). Mutations and expression levels of human *FOXC2* are also associated with obesity, hyperlipidemia, insulin sensitivity, and type 2 diabetes (31-34).

When *Foxc2* was identified as an adipocyte-expressed gene, this encouraged the generation of transgenic mice with adipocyte-specific overexpression of *FOXC2* (35). Induction of *FOXC2* in adipocytes increased the metabolic rate in the adipose tissue and as a consequence these mice were protected against diet-induced insulin resistance and also displayed reduced serum levels of triglycerides and free fatty acids (FFA) (35). In Paper I and II we further characterize the systemic effects in mice with increased *FOXC2* expression in adipocytes.

In the mouse kidney, *Foxc2* displays a glomerular expression mainly confined to podocytes (21,25,36,37). Yet, the *in vivo* requirement for *Foxc2* expression in the kidney has only been studied using a *Foxc2* global

knockout model. Given that *Foxc2*-deficient mice die *in uterus*, and the kidney is a complex organ with contribution from different tissue and cell types, we decided to investigate a potential role of *Foxc2* in podocytes, using a newly generated conditional knockout mouse model (Paper IV).

FOXF2

Nearly at the same time as *Foxc2* was identified in the mouse, seven human FOX genes, including *FOXF2,* were identified (38). Analyses of the *FOXF2* expression pattern initially showed that the gene is abundantly expressed in lung and placenta (38,39). In addition, *Foxf2* expression in mouse is widespread during embryogenesis in alimentary, respiratory, and urinary tracts as well as the central nervous system, eye, ear, and limb buds (40). Mice that lack *Foxf2* expression die shortly after birth due to cleft palate that renders the mice unable to suckle, causing air-filled gastrointestinal tract (41). In addition, *Foxf2* global knockout mice suffer from gut abnormalities, including ganglionic megacolon and colorectal muscle hypoplasia, perhaps due to defective paracrine signaling (42). *Foxf2* was also shown to be important for the development and maintenance of the blood brain barrier in mice (43). Recently, a mutation in human *FOXF2* has been identified in patients with cleft palate (44) suggesting a functional conservation of the gene between species, similarly to FOXC2. Decreased levels of *FOXF2* mRNA have also been associated with poor prognosis of various cancers (45-47).

Besides developmental roles, *Foxf2* expression was found to be regulated during adipogenesis where it promotes adipocyte differentiation (48). Interestingly, *Foxf2* also appears to inhibit the expression of glycolytic genes in a mouse atrial cell line (49). In Paper III we generated a transgenic mouse, which overexpresses *FOXF2* specifically in adipocytes, and characterized the effects on glucose metabolism.

OBESITY

Being able to store excess energy for times with low access to food has been a prerequisite for human survival during evolution. However, as a consequence of the current urbanization occurring around the globe, accompanied by high-energy food intake and a more sedentary life style, obesity is increasing at an alarming rate worldwide. The World Health Organization (WHO) has labeled obesity as an epidemic and estimated that, in 2014, 1.9 billion adults were overweight, and 600 million of them were considered obese (1). A consequence of obesity is an increased risk of

premature death due to cardiovascular diseases, fatty liver, and type 2 diabetes (50,51). The International Diabetes Foundation (IDF) estimates that the number of diabetic patients will rise from 382 million in 2013 to more than 592 million by 2035 with obesity being the major contributor to the rapid increase (52).

Despite being a risk factor for many diseases, obesity does not always cause disease. In fact, almost 25% of obese individuals are insulin sensitive (53). The mechanism behind this has not been fully understood, but could relate to the anatomical location of adiposity (54) as well as the capacity of the adipose tissue to either store energy (55,56) or dissipate energy (35,57). Nevertheless, finding means to fight obesity, or at least improve insulin sensitivity in obese individuals, is critical to ease the burden on patients as well as health care (58).

The adipocytes

The adipose tissue is a very heterogeneous tissue in terms of location, function and morphology. Initially, adipose tissue was thought to be an inert tissue, but it has now become evident that the fat cells, the adipocytes, are able to do much more than just store triglycerides for periods with low access to food, like fasting and starvation. Moreover, the adipocytes are not the same throughout the body, as at least three distinct types of adipocytes have been identified (59).

The classical adipocyte, the white adipocyte, is the most abundant. After a meal, the anabolic hormone insulin induces the white adipocyte to take up and store excess energy in a single, large lipid droplet (60). During starvation the stored energy can be released as FFA in a process called lipolysis, which is mainly induced by the catecholamines epinephrine and norepinephrine (61). The adipocyte has also been shown to be able to signal its metabolic status through secreted factors, like leptin and adiponectin (62,63), in both a paracrine and endocrine fashion. Several other factors might be secreted from adipose tissue, including cytokines, growth factors and angiogenic factors (64) but many of them are secreted from non-adipocyte cells in the stromal-vascular compartment, like immune cells and vascular cells (65).

Another type of adipocyte, the brown adipocyte, is found in the brown adipose tissue (BAT) in rodents, hibernating animals, and newborn humans. The brown adipocyte stores lipids in several small lipid droplets, but more importantly, brown adipocytes have the capability of burning fat to generate heat in response to adrenergic stimuli (66). This process is executed by

uncoupling protein 1 (UCP1), which uncouple the proton gradient that is being generated over the inner mitochondrial membrane during oxidative phosphorylation. Without this gradient, the mitochondria cannot produce ATP and energy is dissipated as heat at the expense of fatty acids (67,68). For mammals, this non-shivering thermogenesis has probably been an evolutionary advantage to maintain body temperature during neonatal periods and residence in cold environments (69). BAT has until recently been considered to be more or less absent in adult humans.

However, metabolically active and UCP1 positive adipose tissue was lately identified in supra-clavicular regions of healthy adults (70-74). Despite UCP1 expression, this newly discovered tissue was shown to be different from the classical BAT found in newborns and rodents (75,76). The adipocytes in these depots were denoted beige adipocytes, a cell type distinct from the other adipocytes (59). Beige adipocytes seem to share features of both white and brown adipocytes. They can store lipids in unilocular droplets as white adipocytes do, but upon adrenergic stimulation, expression and activity of UCP1 is induced and lipids are combusted (77,78), similarly to what happens in brown adipocytes. The exact origin of the beige adipocytes has yet to be clarified. Genetic lineage studies in mice have revealed that UCP1-positive, beige adipocytes in inguinal or perigonadal white adipose tissue (WAT) are not derived from the same lineage as brown adipocytes (79-81). Even so, recent findings indicate the existence of two types of beige adipocytes, since beige adipocytes, resident in the retroperitoneal WAT, were shown to originate from the brown adipocyte lineage (81). The possibility to induce "beiging" of WAT, i.e. increase the activity of beige adipocytes and hence the energy dissipation capacity, has attracted much attention as a mean to fight obesity (80,82-86).

LIPOTOXICITY

Lipotoxicity is a pathological condition that arises when the adipose tissue loses the ability to store excess lipids. Instead serum levels of triglycerides and FFA increases and lipids are being stored ectopically in non-adipose tissues like skeletal muscle, liver, kidney, and pancreas. Accumulation of lipid metabolites impairs the insulin signaling in these tissues, which ultimately leads to insulin resistance (87-90). This means that the tissues do not respond to insulin as expected in healthy individuals, i.e. more insulin is required to get the same metabolic response. The insulin signaling and its impairment by lipids are described in detail below.

Insulin signaling

In response to increased blood glucose levels after a meal, beta-cells in the pancreas secrete the anabolic hormone insulin (91). Insulin then acts on tissues to maximize the extraction of energy from the diet, especially by stimulating glucose uptake in skeletal muscle and adipose tissue (92,93), while suppressing glucose production in liver (94,95), and inhibiting lipolysis in adipose tissue (96,97). In this way, insulin keeps a tight control of the plasma glucose levels in healthy subjects.

The insulin signaling pathway is illustrated in Figure 1. In detail, insulin signals via its cell-surface receptor, the insulin receptor (INSR) (98,99) that, upon binding by insulin, undergo autophosphorylation via tyrosine kinase activity of the receptor subunits (100,101). Mutations in human INSR, which cause a decrease in the receptor tyrosine kinase activity, have been associated with insulin resistance and diabetes (102), emphasizing its importance in signal transduction. The activated tyrosine kinase then phosphorylates several substrates, particularly members of the insulin receptor substrate (IRS) family (103).

Based on mutational analysis, IRS1 and IRS2 seem to be the most important members of this family in the development of insulin resistance. Allelic variants of both *IRS1* and *IRS2* have been associated with insulin resistance and diabetes in humans (104-107) In addition, homozygous deletion of *Irs1* in mice causes insulin resistance and growth retardation, but, by increasing the beta-cell mass in pancreas and therefore the insulin secretion, these mice do not develop diabetes (108,109). On the other hand, mice lacking *Irs2* suffer from overt type 2 diabetes, mainly due to impaired insulin-like growth factor 1 (IGF1) signaling in pancreas, which results in decreased insulin secretion due to loss of beta-cell mass in pancreas (110,111). Insulin resistance in the liver of *Irs2* knockout mice could potentially contribute to the phenotype (112,113), whereas the insulin resistance observed in skeletal muscle is considered to be a consequence of the resulting hyperglycemia in these mice (110). The different roles of IRS1 and IRS2 in insulin signaling are further manifested by the fact that IRS1, but not IRS2, can increase the phosphorylation, and hence the activity, of INSR by inhibiting protein-tyrosine phosphatases that dephosphorylate INSR (114). In line with this, it was reported that only IRS1 was required for proper glucose uptake in muscle cells in vitro (115). Additionally, IRS1 appears to be more important than IRS2 in adipogenesis since adipocyte differentiation was only inhibited in pre-adipocytes from *Irs1* knockout mice (116).

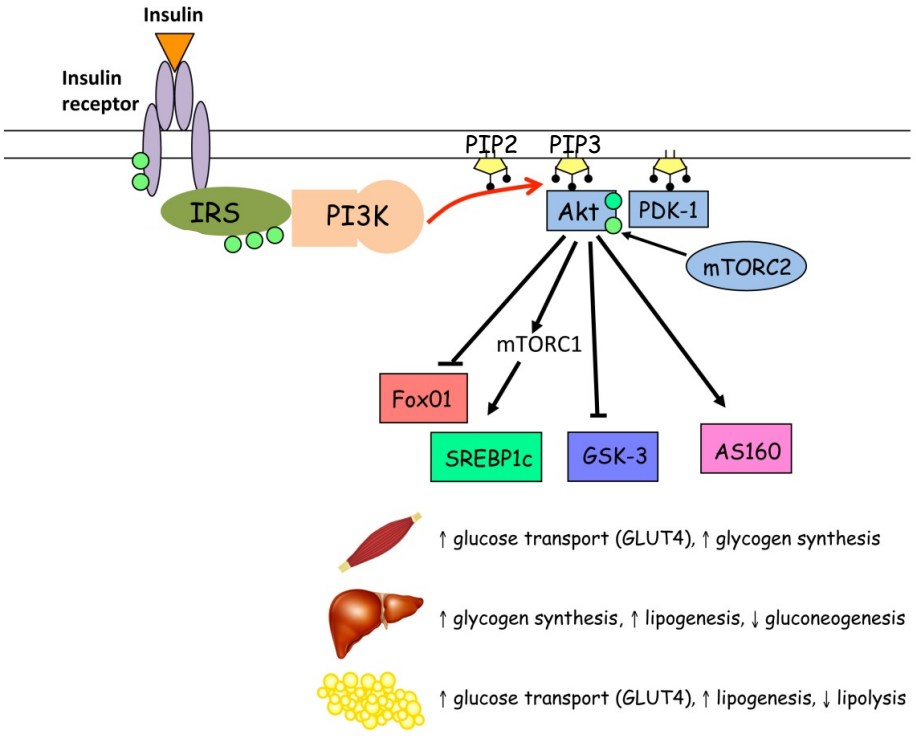

Figure 1. Insulin signaling pathway. Insulin binds to its membrane-bound receptor, initiating tyrosine phosphorylation of IRS and subsequent activation of PI3K. Activated PI3K eventually leads to phosphorylation and activation of AKT that initiate different downstream events depending on cell type. In skeletal muscle and adipose tissue, insulin induces translocation of GLUT4 to cell membrane which increases glucose uptake. Glycogen synthesis is stimulated by insulin in skeletal muscle and liver whereas gluconeogenesis is inhibited in liver. Insulin also suppresses lipolysis in adipose tissue. IRS, insulin receptor substrate; PI3K phosphatidylinositol-4,5-bisphosphate 3-kinase;PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositide-dependent protein kinase 1; mTORC mammalian target of rapamycin complex; FoxO1 Forkhead box protein O1; SREBP1c sterol regulatory element binding protein 1c; GSK-3, glycogen synthase kinase 3; AS160, 160 kDa Akt substrate. Green circles represent phosphorylations. Adapted from Nigel Turner (2013). Mitochondrial Metabolism and Insulin Action, Type 2 Diabetes, Prof. Kazuko Masuo (Ed.), InTech, DOI: 10.5772/56449. Available from: http://www.intechopen.com/books/type-2-diabetes/mitochondrial-metabolism-and-insulin-action

Taken together, these data suggest that IRS1 might contribute more to insulin signaling, particularly in the skeletal muscle and adipocytes, than IRS2 does. Deletion of either *Irs3* or *Irs4* in mice cause no or minor effects on glucose metabolism (117,118), suggesting them to have negligible roles in the development of insulin resistance.

Signal transduction via tyrosine phosphorylation of IRS1 involves interaction with, and subsequent activation of, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) (119,120), an important step in insulin stimulated glucose uptake. This interaction/activation has for example been shown to be inhibited by IKBKB (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta)-mediated serine/threonine phosphorylation of IRS1 (121), which in turn contributes to insulin resistance (122). Activation of PI3K then initiates a signaling cascade that eventually phosphorylates and activates AKT serine/threonine kinase 2 (AKT2) (123). Activated AKT2 then acts on different targets depending on cell type to mediate insulin signaling. In hepatocytes, AKT2 phosphorylates FOXO1 and consequently inhibits FOXO1-dependent expression of gluconeogenic genes (13,124). AKT2 also inactivates glycogen synthase kinase in both liver and skeletal muscle, which leads to sustained activity of glycogen synthase, the enzyme that catalyzes glycogen formation (125,126). In skeletal muscle and adipocytes, AKT2 additionally promotes translocation of glucose transporter 4 (GLUT4 or SLC2A4) to the cell membrane to increase glucose uptake into the cell (127,128). However, adipose tissue might not contribute substantially to glucose homeostasis, since insulin-stimulated glucose uptake in adipose tissue only accounts for a small part of whole-body glucose uptake (129). Nevertheless, glucose is important for the synthesis of triglycerides in adipocytes since glycerol-3-phosphate is synthesized from dihydroxyacetone-phosphate, an intermediate produced during glycolysis (130). Taken together, AKT2-mediated increase in glucose uptake by skeletal muscle, liver, and adipose tissue, as well as decreased glucose production in liver, all contribute to the normalization of postprandial increase in blood glucose levels (92-94).

Insulin also inhibits lipolysis in adipocytes by inactivating lipases, such as hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), but recent data indicate that this mechanism might be independent of AKT2 (131). The activation of both lipases is dependent on active protein kinase A (PKA), which directly phosphorylates HSL and indirectly promotes ATGL activity by perilipin phosphorylation (132,133). Perilipin, a protein that covers the surface of lipid droplets, appears to block lipolysis in the basal state and promote lipolysis upon phosphorylation by PKA (133,134). PKA activity is in turn regulated by cyclic adenosine mono-phosphate (cAMP)

levels, and insulin-stimulated inhibition of lipolysis involves degradation of cAMP by phosphodiesterase E (PDE), causing inactivation of PKA (135).

Lipid-induced insulin resistance

Insulin resistance is a feature of many metabolic disorders; in particular, type 2 diabetes (136) and the metabolic syndrome (also known as syndrome X or insulin resistance syndrome), a syndrome which involves the characterized co-occurrence of four pathogenic states: central obesity, insulin resistance, dyslipidemia, and hypertension (137). Although mutations and allelic variations in many of the genes involved in insulin signaling, including *INS* (insulin), *INSR*, *IRS1*, *PI3KR1* (PI3K regulatory subunit 1), and *AKT2*, could indeed contribute to insulin resistance and diabetes (105,138-142), the rapid increase of these conditions today is most likely due to obesity caused by high caloric diet and sedentary lifestyle (139,143).

Insulin resistance is often preceded by overweight. Many reports indicate that ectopic lipid accumulation, especially in skeletal muscle, liver, and pancreas, is a major contributor to development of systemic insulin resistance, but it might actually be initiated by insulin resistance in adipocytes as the adipose tissue reaches the limit of its expandability, i.e. its storage capacity (144). This hypothesis is supported by findings in human and mice with lipodystrophy, which suffer from severe insulin resistance and diabetes (89,145). Lipodystrophy is a condition with significant reduction or loss of adipose tissue, the designated tissue for storage of lipids. Interestingly, the insulin resistance in lipodystrophic mice could be rescued by transplantation of adipose tissue (145). The beneficial effect of antidiabetic thiazolidinediones, agonists of the adipogenic gene peroxisome proliferator activated receptor gamma (*PPARG)* (146), on insulin sensitivity also supports the adipose tissue expandability hypothesis since the major effect of this drug probably is related to the increased storage capacity of adipose tissue and subsequent decrease in the ectopic accumulation of lipids (147).

The toxic effects of ectopic lipids are further established in mice with skeletal muscle-specific deletions of either lipoprotein lipase (*Lpl*) (148) or fatty acid transport protein 1 (*Fatp1*) (149). In these mice, lipid uptake in skeletal muscle is blocked, an event which protects the mice from developing insulin resistance in skeletal muscle. Correspondingly, tissue-specific overexpression of *Lpl* causes tissue-specific insulin resistance in mice (150). Insulin resistance is also strongly correlated with high levels of FFA in serum (151,152) and ectopic fat accumulation in skeletal muscle, so-called intramyocellular lipids (153,154).

There are several mechanisms that could explain the lipotoxic effects on insulin signaling in the cell. Increased levels of fatty acyl CoA in skeletal muscle, for example, are associated with insulin resistance, possibly by activating a serine kinase cascade that eventually phosphorylates serine residues of IRS1, hence blocking the PI3K activation (155). In muscle and liver, diacylglycerol (DAG) accumulation, due to inadequate esterification of FFA and DAG into triglycerides, also appears to be of importance (155,156). Increased level of DAG is associated with induced activity of protein kinase C (PKC), which in turn blocks insulin signaling by inhibiting INSR tyrosine kinase activity (157) and IRS1-associated PI3K activity (155). Conversely, inactivation of PKC protects against lipid- or diet-induced insulin resistance in liver (157). In addition, transgenic mice with skeletal muscle overexpression of acyl coenzyme A:diacylglycerol acyltransferase 1 (DGAT1), the enzyme catalyzing the production of triglycerides from FFA and DAG (158), were protected from fat-induced insulin resistance, probably due to the decreased DAG content in skeletal muscle (159). This model also suggests that the inert triglycerides *per se* are not harmful for the cell. Ceramide is another lipid that might be involved in the development of insulin resistance. This intermediate in lipid metabolism prevents the phosphorylation and activation of AKT which in turn inhibits GLUT4 translocation (160). Accordingly, increased ceramide levels in skeletal muscle are associated with insulin resistance in humans (161). Consistently, overexpression of sphingosine kinase 1 (SPHK1), the enzyme involved in the catabolism of ceramides, prevented ceramide accumulation and protected mice from diet-induced insulin resistance (162).

Exactly how lipids accumulate in tissues like skeletal muscle and liver is not fully understood, but plasma FFA could contribute to the development of insulin resistance (151,152). Lipolysis in the adipose tissue is the major source of plasma FFA and defective insulin-signaling in adipocytes, resulting in increased lipolysis, might be the initiating step in the development of insulin resistance (144,163). For example, decreased levels of IRS1 in adipose tissue, resulting in impaired insulin signaling, could predict the development of type 2 diabetes (164), at least in part due to the loss of the anti-lipolytic effect of insulin. Besides reduced anti-lipolytic activity of insulin, lipolysis can be induced by several secreted factors. Leptin, the adipocyte-secreted hormone that signals the storage-status of the adipocyte, can directly induce lipolysis in adipocytes, but this lipolysis is only accompanied by release of glycerol and not FFA from the cells (165). On the other hand, leptin might also induce lipolysis and release of FFA indirectly by induction of catecholamine secretion from the adrenal gland (166). Release of cytokines, preferentially from infiltrating macrophages, has also

been reported to induce lipolysis and release of FFA to serum (167,168). However, inflammatory responses might be secondary to adipose tissue reaching its storage limit since adipocyte death precedes macrophage infiltration (169).

In conclusion, there is substantial support for the hypothesis that, as long as the adipocyte can maintain its monopoly in storing lipids, insulin sensitivity in other tissues is preserved. However, when adipose tissue reaches its storage capacity limit, insulin resistant adipocytes start to release lipids which in turn are being ectopically stored in tissues like muscle and liver. Overfilled adipocytes undergo apoptosis which recruits macrophages and even more lipids are released. In line with this hypothesis, insulin sensitivity can be improved by either increasing energy expenditure or by increasing storage capacity. In Paper I and III we demonstrate the opposing roles of *FOXC2* and *FOXF2* in adipocytes during the development of insulin resistance.

Type 2 diabetes

The blunted effect of insulin, which is observed in insulin resistance, forces the pancreatic beta-cells to compensate with increased production and secretion of insulin to the blood, causing hyperinsulinemia. Eventually the beta-cell function declines, both by a decrease in the ability to sense glucose levels as well as a decrease in beta-cell mass, resulting in reduced insulin production and development of type 2 diabetes (170,171). Without enough insulin to remove glucose from the blood and suppress glucose production in liver, the blood glucose levels raise to cause hyperglycemia (92,93). Recent reports challenges the compensatory model of insulin secretion. Mice lacking three out of four insulin alleles (*Ins1*$^{+/-}$; *Ins2*$^{-/-}$) were protected from obesity and hence it was concluded that hyperinsulinemia might precede obesity (172,173). Still, these mice do not display improved insulin sensitivity. On the contrary, trying to genetically induce obesity in these mice, by co-deletion of leptin, caused increased serum FFA and triglyceride levels as well as diabetes (173). Additionally it has been shown that a substantial part of non-diabetic obese subjects have normal glucose levels and are insulin sensitive (53).

Prolonged hyperglycemia cause injury to endothelial cells, rendering increased vasoconstriction, stiffer vessel walls, and damage of underlying smooth muscle cells, which might contribute to hypertension (174,175). As a consequence, diabetic patients have increased risk of developing micro- and macrovascular diseases such as diabetic nephropathy (176), stroke (177), myocardial infarction (177), diabetic retinopathy (178), and circulation

problems causing amputation in lower extremities (179). Especially the renal and cardiovascular diseases are major causes of the increased mortality associated with type 2 diabetics (180-182).

ANGIOGENESIS IN ADIPOSE TISSUE

The adipose tissue is unique in its ability to expand and regress depending on nutritional status. With this plasticity comes a requirement to continuously remodel its vasculature (183,184). Interestingly, adipose tissue has been reported to have pronounced angiogenic activity (185,186) and has even been used to promote vessel regeneration after myocardial infarction (187). Several factors expressed by adipocytes are involved in the vascular remodeling in adipose tissue. Leptin and resistin promote angiogenesis (188,189), whereas adiponectin has been demonstrated to possess antiangiogenic properties (190). In addition, traditional angiogenic factors, like vascular endothelial growth factors (VEGFs), placental growth factor, angiopoietins, and hepatocyte growth factor, could influence the vascular remodeling in adipose tissue (191-195).

Angiopoietins and vascular remodeling

Angiopoeitin 1 and 2 (ANGPT1, also known as ANG-1, and ANGPT2, also known as ANG-2) are two important angiogenic factors that work together with VEGFs to orchestrate vessel pattering, maturation, and stabilization (196,197). Both of these factors bind to the same endothelial cell-specific receptor, the TEK receptor tyrosine kinase (TEK, also known as TIE2), but with opposing effect on angiogenesis. Binding of ANGPT1 to TEK activates the receptor (198) leading to vessel stabilization (199,200), whereas the action of ANGPT2 seems to be more dependent on the context of the expression. In the adult, *Angpt2* is expressed in tissues with active vessel remodeling, perhaps providing the initiating step by destabilizing the vessel (201-203). Mice lacking *Angpt2* revealed the necessity of the protein for postnatal angiogenic remodeling and lymphatic pattering (203). Co-expression with VEGF at sites of active vascular remodeling, such as ovary and tumors, generated the hypothesis that ANGPT2 promotes sprouting in the presence of VEGF whereas vessel regress in the absence of VEGF (196,201,204). In addition, *Angpt2* mRNA levels are elevated in adipose tissue of obesity mouse models (194) suggesting a role in adipose tissue expansion.

FOXC2 and the vasculature

As discussed above, FOXC2 has diverse and important roles during development. Disrupted aortic branch development in the *Foxc2* global knockout mouse suggests an important role for *Foxc2* in cardiovascular development (23,205). *FOXC2* is also important in the development of lymphatic vasculature, particularly necessary for the generation and maintenance of lymphatic valves, and mutations in FOXC2 could result in lymphedema distichiasis in both human and mice (26,27,29,36,206). Additionally, mutations in *FOXC2* have been linked to defective venous valve formation (207).

Recently FOXC2 has attracted attention for its putative involvement in angiogenesis, especially tumor angiogenesis. *Foxc2* can induce the formation of microvessels (208) and mediate migration of endothelial cells (208,209). Work on heterozygous *Foxc2* knockout mice also showed that tumor growth was inhibited in these mice, which was suggested to depend on decreased angiogenic capacity (210).

In Paper II we characterize the vascular phenotype in adipose tissue from mice with adipocyte-specific overexpression of *FOXC2* and identify ANGPT2 as the mediator of the phenotype.

DIABETIC NEPHROPATHY

In diabetic subjects, diabetic nephropathy, together with cardiovascular disease, is the major cause of premature death (180-182). Diabetic nephropathy is also the leading cause of chronic kidney disease in most Western societies and, besides overt diabetes, it is clinically characterized by persistent albuminuria, decline in glomerular filtration rate, and hypertension (211). Albuminuria, or presence of protein in urine, is one of the earliest signs of kidney damage and progression of nephropathy (212). Notably, there seems to be a substantial genetic predisposal for diabetic nephropathy since it is more prevalent in African Americans, Asians, and Native Americans than Caucasians (213,214). The major contributor to the disease is thought to be the high blood glucose levels and the high blood pressure associated with diabetes, and hence these areas are main targets in preventing and treating diabetic nephropathy (211,212).

The kidney and the nephron

During metabolism, several waste products are being produced that need to be dealt with by the body. While carbon is mainly disposed of via the lungs as CO_2 in the exhaled air, nitrogen is excreted as urea via the kidney. The main function of the kidney is to filter the blood so that waste products are disposed of via urine, but it is also a crucial regulator of fluid and electrolyte balance in the body by secreting hormones to control blood pressure and red blood cell production (215,216).

The human kidney consists of about one million filtration units, so-called nephrons (Figure 2). These units control waste disposal, fluid and electrolyte balance as well as reabsorption of vital nutrients like glucose, amino acids, and electrolytes (217). The filtration process starts by blood entering the nephron via an afferent arteriole. Within the renal corpuscle, the arteriole forms a tuft called the glomerulus, which is the filtration site in the nephron. Blood cells and proteins are retained in the blood while fluid and small molecules pass freely through the glomerular filter to produce primary urine, also referred to as ultrafiltrate, which is collected by the Bowman´s capsule. Purified blood exits the glomerulus via an efferent arteriole whereas the primary urine is drained through the tubules. The primary urine contains vital nutrients, including glucose, electrolytes, and amino acids, which are reabsorbed by cells in the proximal and distal tubules. To avoid dehydration, water is also reabsorbed in this process.

The podocyte and its role in the glomerulus

The glomerulus (Figure 3A) is the filtration site in the nephron. It mainly consists of three cell types; mesangial cells, endothelial cells, and podocytes (Figure 3B). The filtration barrier between the blood and urinary space is made up by fenestrated endothelial cells, the adjacent glomerular basement membrane, and the surrounding podocytes (Figure 3C). The importance of each of these three layers is highlighted by the fact that damage to either of them is sufficient to cause kidney injury (218). Fenestrated endothelial cells line the glomerular capillaries and form a mesh with pore size of about 100 nm (219). These pores allow fluid and macromolecules to pass, but retain blood cells in the capillary. The basement membrane is built by components produced by endothelial cells and podocytes, and provides support for these cells as well as a size barrier for larger proteins, like transferrins (220). The last layer of the filtration barrier consists of podocytes that sit on the urinary side of the glomerulus, attached to the basement membrane by so-called foot processes.

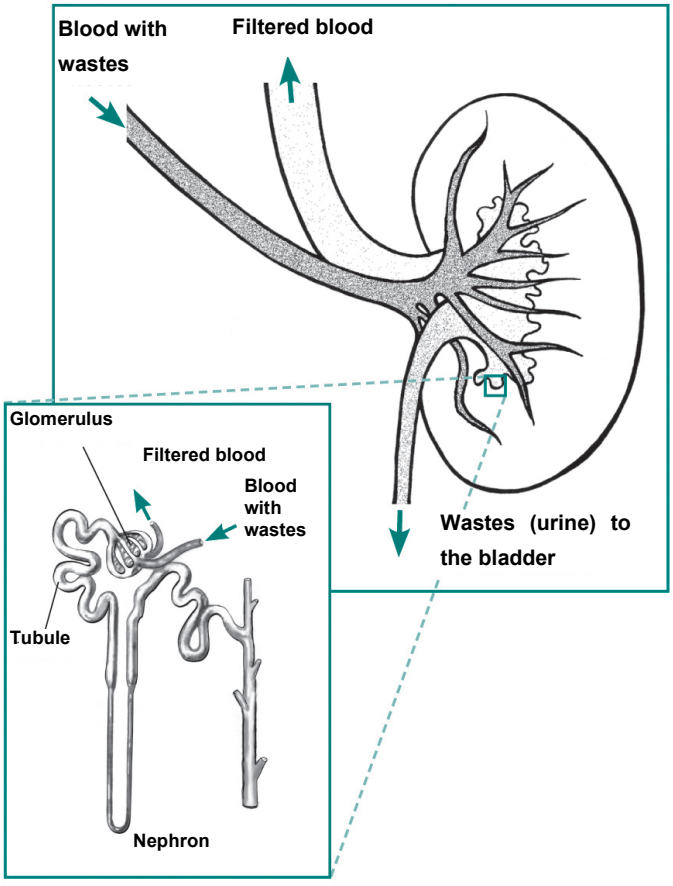

Figure 2. The kidney and the nephron (inset), the filtration unit of the kidney. Arterial blood with wastes is filtered through the glomerulus, producing an ultrafiltrate and vital nutrients (such as glucose, electrolytes and amino acids) and water are reabsorbed by the tubules whereas urine is disposed of via the ureter to the bladder. Adapted from National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.

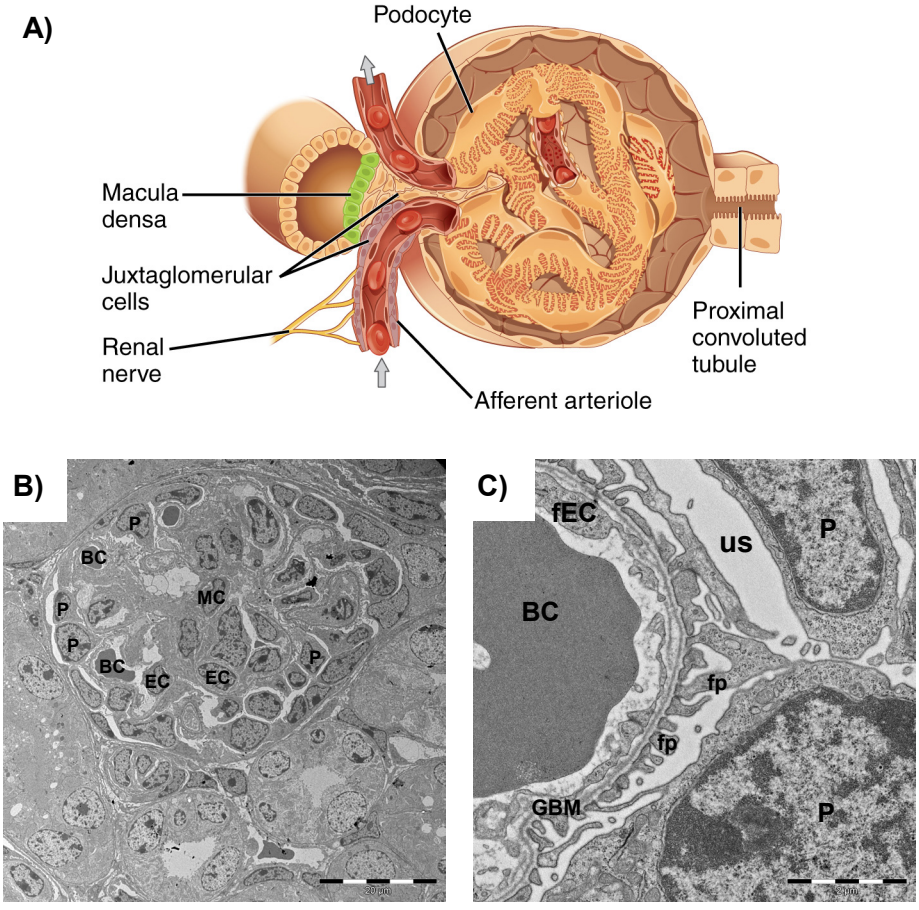

Figure 3. The glomerulus and the glomerular filtration barrier. A) Illustration of a glomerulus showing podocytes covering the surface of the arteriole in Bowman´s capsule. B) Transmission electron micrograph of a cross section of a mouse glomerulus with some epithelial podocytes (P), capillary endothelial cells (EC), and mesangial cells (MC) marked. Red blood cells (BC) also visible. C) Increased magnification of the glomerular filtration barrier. Podocytes (P) are "floating" in the urinary space (us) of the Bowman´s capsule, attached to the glomerular basement membrane (GBM) by its foot processes (fp). Lining the capillary side of the GBM, the fenestrated endothelial cells (fEC) is visible with an adjacent red blood cell (BC). Bars 20µm in B) and 2 µm in C). Illustration in A) from OpenStax, Anatomy & Physiology, 2013.

The podocyte is a highly specialized epithelial cell. It embraces the capillaries with its extended primary foot processes from which secondary foot processes extend (Figure 4). The primary foot processes are arranged so that their secondary foot processes can interdigitate with corresponding secondary foot processes on adjacent podocytes. These foot processes cover the entire outer surface of the vessel. Since the interdigitated secondary foot processes are separated by approximately 50 nm, the podocytes were initially not considered to contribute to filtration (219). The importance of podocytes in glomerular filtration was first realized when it was shown by transmission electron microscopy that adjacent foot processes are connected by a zipper-like structure, the so-called slit diaphragm (221). The rod-shaped structures that form these slit diaphragms act as a sieve with a pore size almost identical to the size of albumin (70 Å) (221). This finding revealed that the slit diaphragm of the podocyte foot processes is the main contributor to keep proteins in the blood from leaking into urine.

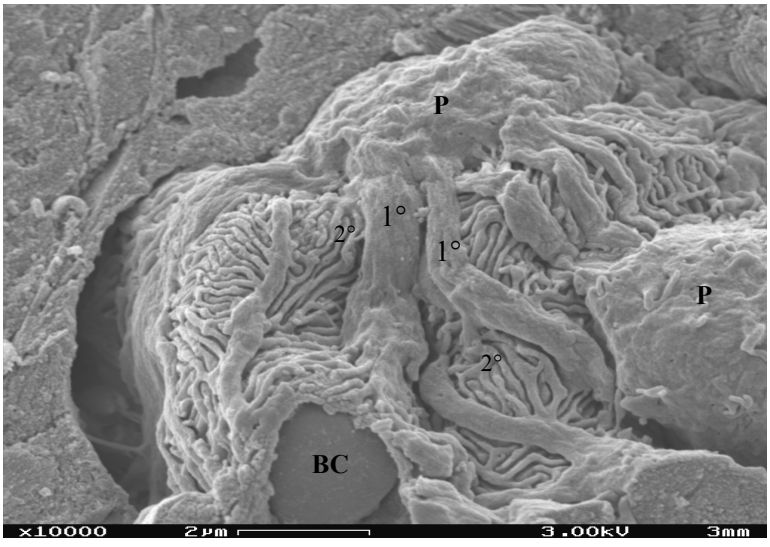

Figure 4. Scanning electron micrograph of mouse glomerulus. Podocyte cell body (P) with primary (1°) and secondary (2°) foot processes embracing the capillary, constituting the outer layer in the glomerular filtration barrier. Red blood cell (BC) in the capillary lumen has been exposed during sample preparation. Bar 2 µm.

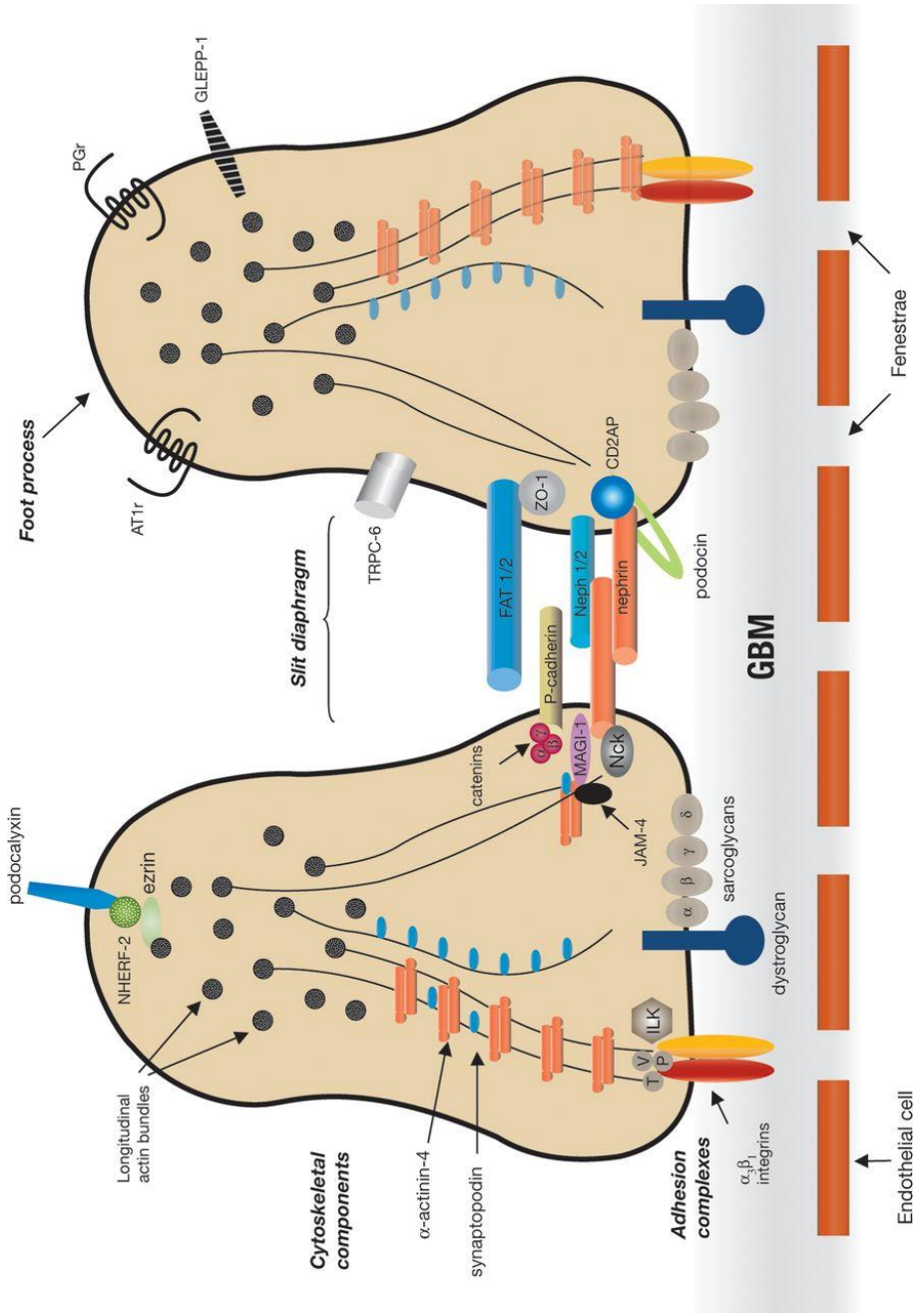
Foot process
PGr
GLEPP-1
AT1r
Slit diaphragm
TRPC-6
FAT 1/2
ZO-1
CD2AP
Neph 1/2
nephrin
podocin
P-cadherin
catenins
MAGI-1
Nck
JAM-4
podocalyxin
NHERF-2
ezrin
Longitudinal actin bundles
Cytoskeletal components
α-actinin-4
synaptopodin
ILK
T
V
P
α
β
γ
δ
sarcoglycans
dystroglycan
Adhesion complexes
α3β1 integrins
GBM
Fenestrae
Endothelial cell

Figure 5. Molecular overview of the slit diaphragm, cytoskeletal components, and podocyte cell–matrix interactions in two interdigitated podocyte foot processes. At the slit diaphragm, nephrin (NPHS1), kirrel (NEPH1), P-cadherin (CDH3), and protocadherin fat 1 and 2(FAT1/2), builds up the fine sieve that keeps protein from leaking into urine, with the Ca^{2+} channel TRPC6 in close proximity. The slit diaphragm is attached to the membrane and cytoskeleton via podocin (NPHS2), and adaptor proteins like CD2 associated protein (CD2AP),tight junction protein 1 (TJP1/ZO-1),membrane-associated guanylate kinase inverted 1(MAGI1) and non-catalytic region of tyrosine kinase adaptor protein (NCK). α-actinin 4 (ACTN4) links the cytoskeleton to both the slit diaphragm and the adhesion complexes, where integrins bind to laminins in the glomerular basement membrane. Other adaptor proteins for integrin are integrin linked kinase (ILK), talin (T), paxilin (P) and vinculin (V). At the apical membrane, podocalyxin (PODXL), solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator (NHRF or NHERF), and ezrin (EZR) build up an important cytoskeleton remodeling complex. From Michaud, 2007 (222).

The molecular structure of the podocyte foot processes (Figure 5) has been thoroughly investigated since the 1990s. Nephrin (NPHS1), responsible for congenital nephrotic syndrome of the finnish type (223), was identified in the rod-like structures of the slit diaphragm by electron microscopy (224). To date, several other components of the slit diaphragm have been identified, including kirrel/neph1 (NEPH1) (225), protocadherin fat 1 (FAT1) (226), and P-cadherin (CDH3) (227). The slit diaphragm is anchored to the plasma membrane by interaction with the transmembrane protein podocin (NPHS2) (228). Inside the foot process numerous proteins have been identified as critical for podocyte function by linking the slit diaphragm to the cytoskeleton. These include CD2 associated protein (CD2AP) (228), tight junction protein ZO-1 (TJP1/ZO-1) (229), alpha-actinin 4 (ACTN4) (230), membrane-associated guanylate kinase inverted 1 and 2 (MAGI1/2) (231,232), and non-catalytic region of tyrosine kinase adaptor protein 1 and 2 (NCK1/2) (233,234). In addition, transient receptor potential cation channel subfamily C member 6 (TRPC6), a calcium-permeable cation channel important for increased intracellular Ca^{2+} concentration (235), has been linked to the slit diaphragm, possibly providing a mechanosensing ability (236).

Attachment of the foot processes to the glomerular basement membrane is also important. Especially the interaction between integrins in the podocyte plasma membrane and laminins in the matrix appears to be critical (237-240). Intracellular integrin signaling and integrin binding to actin via integrin linked kinase (ILK) (241), vinculin (VCL) (242), and talin 1 (TLN) (243), have been suggested to be necessary for functional foot processes. Collagen IV subunit a3 and a4 (COL4A3 and COL4A4), matrix components of the

glomerular basement membrane, are additional factors demonstrated to be necessary for correct podocyte attachment (244,245).

Apart from the slit diaphragm and attachment to the basement membrane, structural proteins of importance for functional foot processes include podocalyxin (PODXL), a glycoprotein, that provides the podocyte with a negative apical cell surface (246) and is connected to the cytoskeleton via adaptors like solute carrier family 9 (sodium/hydrogen exchanger) member 3 regulator 1 and 2 (NHRF1/2 also known as SLC9A3R1 and SLC9A3R2) (247,248) and ezrin (EZR) (249). Recently schwannomin interacting protein 1 (SCHIP1) (250) and rhophilin Rho GTPase binding protein 1 (RHPN1) were identified as being important for the cytoskeletal structure in the podocyte (251).

Several transcription factors have also been identified as important for the podocyte. Wilms tumor 1 (WT1) (252) is a zinc-finger transcription factor that regulates the expression of important podocyte genes like *Podxl* (253), *Nphs1* (254,255), and perhaps also *Nphs2*, *Cd2ap*, and *Vegfa* (256). Reduced levels of WT1 are consequently associated with impaired kidney development and glomerular injury (255-257). LIM homeobox transcription factor 1 beta (LMX1B) is another podocyte transcription factor required for normal podocyte development (258). Several studies of the *Lmx1b* global knockout mouse have proposed that COL4A3, COL4A4, NPHS2, and CD2AP are LMX1B targets in the podocyte (259-261). Still, deletion of *Lmx1b* specifically in podocytes of mice could not confirm decreased expression of any of these proteins (262). Thus, the defective podocytes observed upon global knockout of *Lmx1b* might be explained by developmental arrest of podocytes, or other secondary effects, rather than loss of LMX1B-mediated transcriptional regulation of these genes.

A third transcription factor with documented expression in mouse podocytes is FOXC2. *Foxc2* is one of the earliest podocyte markers during mouse glomerular development, with sustained expression in the adult podocyte (21,25,36,37). Studies of the global *Foxc2* knockout mouse model revealed that, like *Lmx1b*, *Foxc2* is required for proper podocyte development (25), and COL4A3, COL4A4, and NPHS2 were identified as putative transcriptional targets (25). In addition, reduced levels of *Rhpn1* mRNA and PODXL protein were detected in mice lacking *Foxc2* (25). Lack of any of these targets could, in principle, contribute to the kidney phenotype seen in the global *Foxc2* knockout, including abnormal glomerular shape, dilated and blood-filled capillary loops, and failure to produce proper fenestrae of the endothelial cells. Nonetheless, the specific role of FOXC2 in differentiated

podocytes was not clarified in this model. Particularly, learning from the LMX1B story, gene regulations observed in global knockout mice might be indirect, resulting from developmental defects rather than direct transcriptional regulation. In Paper IV we generated a conditional *Foxc2* deletion to be able to investigate the postnatal role of *Foxc2* specifically in podocytes.

Podocyte injury

The importance of podocytes in maintaining the glomerular filtration barrier is emphasized by the fact that podocyte damage frequently causes proteinuria. Podocytes often responds to injury by so-called foot process effacement, a state in which foot processes are flattened and the interdigitating pattern is lost (263). During effacement, the slit diaphragms, and hence the fine sieve that keeps proteins from leaking into urine, are disrupted. Consequently, foot process effacement is strongly linked to proteinuria (264-266), although it should be noted that not all proteinuric cases are caused by podocyte damage. Another possible morphological alteration of a damaged podocyte is the so-called podocyte microvillus transformation. This characteristic involves development of thin protrusions, microvilli, from the podocyte cell body into the urinary space of the glomerulus. Podocyte microvillus transformation has been observed in experimental animal models and in various human kidney diseases with confirmed proteinuria (241,267-270).

The exact mechanism behind the morphological changes seen in the injured podocyte, such as foot process effacement and microvillus transformation, has not been established, but can be caused by several factors. Many proteins of the foot process architecture, either of the slit diaphragm or the podocyte cytoskeleton, have been confirmed as critical, since mutations in either of them are associated with podocyte dysfunction. Thus, defects in many of the proteins included in Figure 5 have been linked to proteinuria or podocyte dysfunction, either in human disease or animal models (271). Their importance for glomerular filtration is almost like a house of cards: if you take one card out, the whole house collapses, resulting in proteinuria. In addition, mutations in the podocyte transcription factors *WT1*, *LMX1B*, and *FOXC2* in humans have all been linked to renal disease (30,272-275), indicating that they are essential for transcription of genes important for kidney function, also in humans.

Kidney diseases that are thought to be caused by podocyte damage or dysfunction are referred to as podocytopathies (276). Although the

pathological presentation of the podocytopathies might be very similar, the response to treatments varies, suggesting that the underlying causes may be different. Podocytopathies are generally divided into four categories based on histological manifestations: minimal change nephropathy (MCN), focal segmental glomerulosclerosis (FSGS), diffuse mesangial sclerosis (DMS), and collapsing glomerulopathy (CG) (277). MCN displays normal histology until examined at the ultrastructural level, where podocyte foot process effacement and microvillus transformation is evident. Conversely, FSGS, DMS, and CG are presented by histological changes in respect of solidification of the glomerular capillary tuft, i.e. deposits of extracellular matrix, or mesangial expansion, within the capillary network. Unlike the other podocytopathies, there is no change in the number of podocytes in MCN glomeruli. MCN may be caused by adverse immune responses, and as such they can be treated with steroids (278). Still, about 20% of MCN are steroid-resistant, and MCN has also been associated with mutations in NPHS2 (279), dysferlin (DYSF) (280), and NPHS1 (281,282). Lack or redistribution of the important slit diaphragm proteins NPHS1 and NPHS2 have also been demonstrated in MCN (277,283). FSGS has been associated with mutations in several of the proteins critical for podocyte function, and the pathology of FSGS involves apoptosis and/or detachment of podocytes (277). DMS and CG, on the other hand, involves de-differentiation and proliferation of podocytes. In DMS this leads to mesangial expansion while it is probably caused by viral infections in CG (277). Relatively few mutations have been identified as causing DMS and CG, indicating that the podocytes in these conditions are injured by external factors.

Besides being dependent on internal structure, podocytes can also be damaged by physiological conditions, one of them being diabetes. High concentrations of glucose, for example, cause apoptosis in cultured podocytes (284). This effect of glucose might explain the reduced number of podocytes seen in diabetic patients (285). In addition, the high blood pressure associated with diabetes could potentially cause damage to podocytes (286). Prolonged hyperglycemia, e.g. in diabetes, increases the amount of advanced glycation end products, so-called AGEs, in serum (287). AGEs have been implicated in the pathogenesis of diabetic nephropathy, possibly by affecting podocyte migration (288). Podocytes can also be damaged, directly or indirectly, by infections, like HIV (269) and Hepatitis C (289), or autoimmune diseases, like lupus nephritis and membranous nephropathy (290).

Podocytes that were deprived of *Foxc2 in vivo* were characterized in Paper IV in terms of viability, morphology, and expression of critical components.

AIM

The general aim of this thesis was to investigate the roles of the related forkhead genes *FOXC2* and *FOXF2* in adipocytes and, for *Foxc2*, also in podocytes.

In Paper I, the specific aim was to investigate insulin sensitivity and whole-body glucose metabolism in *aP2-FOXC2* mice, which have adipocyte-specific overexpression of *FOXC2*, and try to understand why these mice are protected against diet-induced insulin resistance.

The aim in Paper II was to characterize the vascular phenotype of *aP2-FOXC2* mice and to identify factors involved in this phenotype.

In Paper III, the aim was to generate a transgenic mouse with overexpression of *FOXF2* specifically in adipocytes and assess the effect of such overexpression on glucose homeostasis.

The specific aim in Paper IV was to generate a mouse with conditional deletion of *Foxc2* specifically in podocytes, explore the phenotype of this mouse model, and try to identify the mechanism underlying the observed phenotype.

METHODS

A selection of the methods used in this thesis will here be described in general terms, explaining the purpose and considerations made.

ANIMALS

The foundation of this thesis is based on work on genetically modified mice. Where applicable we have utilized cell cultures or other *in vitro* studies to address our questions. But to be able to study complex biological functions, it is often essential to study them in their natural context, with, for example proper vascularization, innervation and other cell-to-cell interactions present. This is especially important when studying conditions involving multiple organs and cell types, such as lipid and glucose metabolism. The strategies for generating the genetically modified mice are presented below. Detailed information about the generation of each mouse strain can be found in the denoted paper.

aP2-FOXC2 transgenic mice

Our lab has previously generated transgenic mice where human *FOXC2* is overexpressed in adipose tissues (35). In paper I and II, we further characterize the systemic effects resulting from the adipose-specific induction of *FOXC2* in mice. This mouse carries a transgenic construct where expression of human *FOXC2* coding sequence is controlled by the enhancer/promoter of *aP2* (official gene name: fatty acid binding protein 4, *Fabp4*) (Figure 6A). The *aP2* enhancer/promoter has been shown to be active in differentiated adipocytes (291) and was hence chosen to drive the expression of *FOXC2* in adipose tissue of transgenic mice.

aP2-FOXF2 transgenic mice

Foxf2 is another forkhead gene that was found to be expressed in adipocytes (48). The dramatic effect on gene expression and adipocyte metabolism in *aP2-FOXC2* mice (35), in addition to reports of other forkhead genes possessing central roles in the adipocyte (48,292,293), encouraged us to investigate the role of *FOXF2* in the adipocyte. For this purpose, we generated a construct where human *FOXF2* cDNA was cloned under the control of the same *aP2* enhancer/promoter that was successfully used before (Cederberg et al 2001) (Figure 6A). The construct was injected into the pronucleus of a male zygote (fertilized egg) and transgenic founders were

screened by quantitative PCR (qPCR) for induced expression of *FOXF2* in adipose tissue.

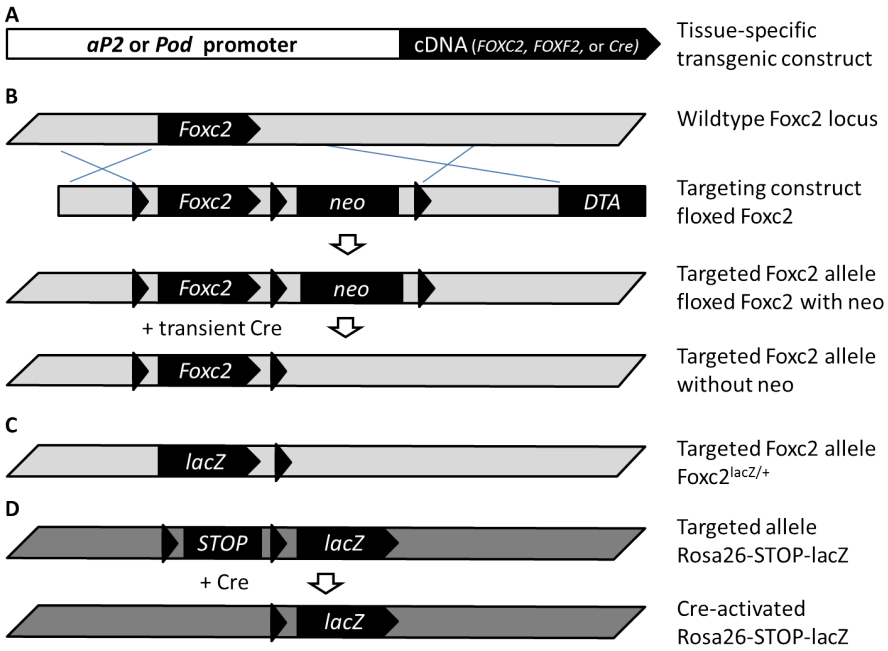

Figure 6. Design strategies for transgenic animals used in this thesis. A) General design for transgenic overexpression of cDNA (FOXC2, FOXF2, or Cre) under the control of tissue-specific promoter (aP2 or Pod). B) Homologous recombination into the Foxc2 locus (light grey) using a targeting construct with three loxP sites (black arrow-head) flanking the single exon of Foxc2 as well as the neo-cassette (positive selection). For negative selection a DTA-cassette was included. Subsequent transient transfection of correctly targeted ES cells with Cre generated clones, with floxed Foxc2 remaining but with neo-cassette deleted, that was used for generation of floxed Foxc2 mice. C) Targeted Foxc2 allele in the Foxc2lacZ knockin model, where homologous recombination was performed with a similar vector as in (B) except that the Foxc2 gene was replaced by the β-galactosidase gene lacZ. D) Rosa26 locus (dark grey) in Rosa26-STOP-lacZ transgenic mice. When subjected to Cre recombinase, for example in podocytes of Pod-Cre transgenic mice, the floxed STOP is removed and transcription of lacZ is initiated. Black pentagon = coding sequence, black rectangle = cassette with selection marker or STOP sequence, black arrow-head = loxP sequence, white = promoter in transgenic construct, light grey = genomic DNA in Foxc2 locus, dark grey = genomic DNA in Rosa26 locus.

Floxed *Foxc2* mice

Apart from overexpression of a gene, where a gain-of-function is supposed to shed light on gene function, deleting the gene could also give information on its specific role(s). *Foxc2* has been knocked out in mice by several groups (23,24) resulting in severe developmental effects, including premature death and cardiovascular defects. To evade these developmental effects, especially the embryonic or early postnatal lethality, we decided to generate a mouse where *Foxc2* is surrounded by loxP (locus of X-over P1) sites, a so-called floxed *Foxc2* mouse, to study cell or tissue-specific roles of the gene (Paper IV). These loxP sites can be recognized by the enzyme Cre recombinase that recombines, i.e. breaks and rejoins, these sequences (294). Depending on the orientation of the loxP sequences there will be different outcomes. DNA between two loxP sites oriented in the same direction will be excised, whereas DNA will be inverted if the loxP sequences have opposite orientation (294).

A targeting construct, containing genomic DNA from the mouse *Foxc2* locus, was assembled and loxP sites were inserted on either side of the single *Foxc2* exon (Figure 6B). Since our purpose was to make a conditional deletion of *Foxc2*, the loxP sites were oriented in the same direction. As a positive selection marker, the gene *neo* was included in the construct. This gene confers resistance to geneticin (G418), an antibiotic that inhibits protein biosynthesis. As a result, only cells that carry the construct survive when growing the cells in culture media containing the antibiotic. In addition, the gene for diphtheria toxin A-fragment (*DTA*) was included as a negative selection marker (295). The final construct was electroporated into RW4 embryonic stem cells to enable homologous recombination into the genome. During homologous recombination, any construct DNA outside of the homologous arms (light grey in Figure 6), including the *DTA* gene, should be discarded. If not, the expression of the *DTA* gene generates a lethal toxin that kills the host cell. Hence, the number of false positive clones should decrease. Homologous recombination was confirmed by southern blot. To identify clones with correct insertion of the construct, the probes were designed to reside outside of the genomic DNA region used to build the construct.

Introducing extrachromosomal DNA into the genome always possess a potential risk for undesired side effects. This is especially true when such DNA involves the expression of a transgene like *neo,* which have been shown to alter gene expression and metabolism (296). To avoid any potential adverse effects of *neo* expression, the *neo* gene was flanked by loxP sites. Hence, it was possible to remove the gene from the genome by transient

transfection of the positive ES clones with a Cre expression plasmid. Correctly targeted clones were used to generate chimeric founder mice that were subsequently screened for germline transmission of the floxed *Foxc2* allele, hereafter denoted *Foxc2*^{fl}.

Other mouse strains

In Paper IV we made use of the *Foxc2*$^{lacZ/+}$ mice (35), where the bacterial gene *lacZ* has been used as a marker gene for *Foxc2* expression (Figure 6C). The gene *lacZ* encodes the enzyme beta-galactosidase that bacteria use to cleave lactose into glucose and galactose. The enzyme can also cleave a lactose analog called X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) resulting in a blue precipitate that is easily detected. Since expression of *lacZ* is under regulation of the endogenous *Foxc2* promoter, detection of beta-galactosidase activity would indicate *Foxc2* expression. Considering that this is an enzymatic reaction, it also provides a more sensitive detection method as compared to immunohistochemistry or *in situ* hybridization. In other words, low levels of *lacZ* (and hence *Foxc2* promoter activity) is possible to detect by increased incubation times that will accumulate blue precipitate. However, it will not provide hard evidence for presence of either mRNA or protein of the gene of interest.

With the generation of the floxed *Foxc2* allele, it was possible to delete the gene in specific cells using appropriate Cre drivers. In Paper IV we used *Pod-Cre* transgenic mice to specifically delete *Foxc2* in podocytes. *Pod-Cre* mice are transgenic mice that express Cre recombinase under the control of the human podocin (*NPHS2*) promoter (297). In parallel to using *Pod-Cre* transgenic mice to delete *Foxc2*, we crossed it with a *Rosa26* Cre reporter strain (298) to confirm that the expression of Cre was specific to podocytes. Cre activity in these mice removes a floxed 4 x polyadenylation signal that blocks the transcription of *lacZ* (Figure 6D). To be able to investigate the role of *Nrp1* in podocytes, we also crossed *Pod-Cre* mice with mice carrying the floxed *Nrp1* allele (299) to generate mice with *Nrp1* null alleles in podocytes.

ASSESSMENT OF GLUCOSE METABOLISM

Typical signs of insulin resistance involve dysfunctional glucose uptake from blood. Fasting glucose and insulin levels in plasma is the initial measurement to make, but unless the subject is diabetic or severely insulin resistant, differences in glucose metabolism might not be detected under these circumstances due to the tight regulation of glucose levels in plasma (300). Therefore methods to measure these variables under challenged conditions

have been developed. There are several available methods to assess this and the methods used in Paper I and III are described here.

Hyperinsulinemic-euglycemic clamp

The hyperinsulinemic-euglycemic clamp is widely considered the golden standard for measuring insulin sensitivity and was used in Paper I to assess the insulin sensitivity of *aP2-FOXC2* transgenic mice. The basis of this method includes a constant infusion of insulin, rendering hyperinsulinemia. To compensate for the insulin-stimulated glucose uptake, glucose is simultaneously infused at variable rates to clamp the glucose levels, meaning that glucose is kept at a constant level in plasma. The infusion rates that are required to maintain euglycemia, i.e. normal plasma glucose levels, are indicative of how efficient insulin stimulates glucose uptake. In evidence, insulin sensitive subjects have higher infusion rates than insulin resistant during the clamp (301).

By including and measuring the activity of ^{3}H-labelled glucose, during basal as well as clamp period, it is also possible to perform more accurate estimations on whole body glucose turnover and hepatic glucose production (302). During glucose metabolism in cells, the tritium atom from [3-^{3}H] glucose then mainly ends up in either H_2O molecules during glycolysis or glycogen during glycogen synthesis (303). Hence it is also possible to use the $^{3}H_2O$ levels in plasma as a measure for glycolysis and measure the activity of ^{3}H in glycogen to determine glycogen synthesis.

During the clamp it is also possible to calculate insulin-stimulated tissue-specific glucose uptake by adding a bolus dose of ^{14}C-labelled deoxy-glucose, a glucose analog that cannot undergo either glycolysis or glycogen synthesis. Although this glucose analog cannot be metabolized, it can be phosphorylated by hexokinase into deoxy-glucose-6-phosphate, a metabolite that is trapped intracellularly (304). Therefore, the amount of deoxy-glucose-6-phosphate in a tissue is a direct measurement of its glucose uptake.

Glucose and insulin tolerance tests

Another commonly used method to assay insulin sensitivity is the glucose tolerance test. It provides an easier and quicker method than the clamp, and gives an estimate of the systemic capacity to handle a given glucose load. The glucose can be delivered orally, intraperitoneally, or intravenously, and the plasma glucose concentration is then monitored until it reaches normal levels. In Paper III, glucose was administered intravenously to avoid gastrointestinal effects accompanying oral administration (305), and

uncertainties of correct dosage that comes with intraperitoneal injections. Upon intraperitoneal injection, the injected solution might end up in for example urinary bladder or abdominal adipose depots.

The glucose levels plotted over time gives a curve of how well glucose is being taken up by the body. In insulin resistant subjects, the initial rise in blood glucose is higher than normal and the removal rate is also delayed. However, insulin resistant subjects might display normal glucose removal curves if they are able to secrete more insulin. Therefore, in addition to knowledge of the glucose removal rate, measuring the insulin levels during a glucose tolerance test can provide additional information of the insulin resistant state.

However, the glucose tolerance test might not be efficient enough to distinguish between insulin resistance and problems related to insulin secretion. Insulin sensitivity can be more directly evaluated by an insulin tolerance test, as in Paper III, where insulin is injected and plasma glucose levels are monitored after the administration. Insulin resistant subjects display lower capacity to remove glucose from the blood and hence the drop in glucose levels is not as prominent as in healthy subjects.

GENE EXPRESSION ANALYSES

To understand mechanisms behind biological processes and diseases, studying the expression of genes can provide useful information. A wide range of methods have been developed to study gene expression, either direct measurements of transcripts from genes, i.e. the RNA molecules, or the translated proteins. But gene expression can also be analyzed by assaying the promoter activity of the gene of interest.

qPCR

The most commonly used method for analyzing individual transcripts is the qPCR, a sensitive method that can identify even low-abundant mRNA (306). This technique, which was employed in Paper II, III, and IV, utilizes the amplification of targeted DNA by PCR, which, if efficiency is 100%, will double the amount of template DNA in each cycle. Since RNA cannot be amplified by the *Taq* DNA polymerase used in PCR, the RNA molecules first need to be transcribed into complementary DNA (cDNA) by reverse transcriptase. This is a critical step in the analysis since RNA quality will affect the cDNA amplification efficiency (307).

Detection of the amplified target is accomplished by excitation of fluorophores in real-time PCR systems. A very common and convenient fluorophore is SYBR® Green, which binds unspecifically to double-stranded DNA and, when bound, absorbs blue light (excitation maximum at 498 nm) and emits green light (emission maximum at 522 nm) (308,309). The amount of bound SYBR® Green is relative to the amount of double-stranded DNA in the sample. Yet, since SYBR® Green binds DNA unspecifically, it will detect all double-stranded DNA, including primer-dimers and unspecific PCR products. Specificity for the target gene is accomplished by careful primer design using available software, either web-based such as Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or stand-alone applications like PerlPrimer (http://perlprimer.sourceforge.net/).

To detect small differences in expression levels by qPCR an appropriate normalization method is necessary to be able to compensate for sample-to-sample and run-to-run variations due to differences in RNA quality, cDNA synthesis and pipetting errors. Expression levels of so called house-keeping or reference genes are often utilized as an internal measurement of the amount of starting material. Several genes, including 18S ribosomal RNA (*RNA18S5)*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)*, actin beta (*ACTB)*, and ribosomal protein lateral stalk subunit P0 (*RPLP0,* also known as *36B4*), have been suggested as reference genes, but it has become evident that there are no such thing as a universal reference gene (310). Instead, one or several genes should be validated for each new experimental condition (310) and expression level of the gene of interest is then presented as expression relative to the reference gene(s).

Promoter-reporter constructs

An indirect measurement of the expression of a particular gene is the analysis of the promoter activity for that gene. The promoter is the genomic sequence located upstream (5´) of the transcription initiation site of a gene and it contains the regulatory units, such as transcription factor binding sites, needed for correct transcriptional control. By cloning the promoter region into a reporter construct, where luciferase was the reporter gene of choice in Paper II, it is then possible to transfer it to a cell and analyze its activity under various circumstances. For example, as was done in Paper II, the effect of a transcription factor like FOXC2 on the promoter activity can be analyzed by using co-transfections in cultured cells, i.e. transferring the promoter construct and an expression vector for the transcription factor into the same cell. If the transcription factor is a regulator of the promoter activity, the expression of the reporter gene will then be either up- or downregulated

depending on whether the transcription factor acts as an activator or repressor.

When regulation of the promoter activity has been established, it is possible to map the regulation site(s), either by site-directed mutagenesis (311), which was utilized in Paper II, or by making truncated versions of the promoter (312). When the active binding site is deleted, the ability for the transcription factor to regulate promoter activity is lost.

Western blot

Although RNA levels could be very informative, the translated protein often provides the function of a gene. Western blot, or protein immunoblot, is a widely used analytical method that involves size separation of proteins by gel electrophoresis and subsequent electrophoretic blotting of the proteins onto a membrane (313). This method was also used to study protein expression in Paper III and IV. Making use of the epitope specificity of antibodies (314), individual proteins, and even post-translational modifications like phosphorylations (315), can be identified by probing the membrane with antigen-specific antibody (313). An antibody can be either polyclonal; recognizing several epitopes of a target molecule, or monoclonal; recognizing a single epitope. The western blot technique can be combined with an initial step of immunoprecipitation if, for example, the target protein is low-abundant or if interaction partners are being investigated (316).

To detect the primary antibody, some kind of label is needed. This label can be directly conjugated, i.e. bound, to the primary antibody. Alternatively, the label is attached to a secondary antibody, which possesses affinity for the immunoglobulin isotype of the primary antibody. This labeling method is referred to as indirect labeling. In western blot, horse radish peroxidase (HRP) is often used as reporter conjugate, but labeling with radioisotopes and fluorescent dye is also possible. The activity of HRP can be detected either colorimetrically, as it produces a colored precipitate from certain substrates (317), or chemiluminescently, if provided a substrate that becomes luminescent upon peroxidase activity (318). The intensity of the signal gives an indication of the amount of the target in that sample.

Immunohistochemistry

In Paper II and IV the use of antibodies was further extended to immunohistochemistry, where microscopic techniques are utilized to identify proteins and visualize their location *in situ* in fixed cells or tissue sections. The principal is the same as for Western blot, i.e. immunoblotting on a

membrane, in the sense that primary antibody raised against the target of interest is hybridized to a sample. The primary antibody could be direct-conjugated with a reporter or detected by an appropriate secondary antibody. Also in this application, HRP have been extensively used, particularly with colorimetric detection (319). The invention of the confocal scanning microscope by Marvin Minsky in the 1950s (320), in combination with laser technology (321), has increased the use of fluorescent labels in the detection of proteins *in situ*. Combining different fluorescent labels for different targets also makes it possible to analyze the expression of several proteins or structures in the same sample (322).

RESULTS AND DISCUSSION

PAPER I – OVEREXPRESSION OF *FOXC2* IN ADIPOCYTES PROTECTS AGAINST DIET-INDUCED INSULIN RESISTANCE

Previous work on *aP2-FOXC2* mice has shown that the metabolic rate in adipocytes was increased in these mice, protecting them from diet-induced obesity (35). Expression of *FOXC2* made the WAT resemble BAT, both in terms of morphology and gene expression. BAT is unique in its ability to burn fat to generate heat (323). While ectopic fat, at least in part, might explain development of insulin resistance in non-adipose tissues (87), increased oxidation of fat in adipocytes could have beneficial effects on whole-body homeostasis (35). In Paper I we investigated the systemic effects of the increased metabolic capacity of the adipose tissue in *aP2-FOXC2* mice. To assess this we studied *aP2-FOXC2* mice fed either standard chow diet (4% fat content by weight) or a high-fat diet (~30% fat content by weight) that resembles ordinary human diet in the western world and is well-known to induce obesity and insulin resistance in mice (324,325).

Despite increased oxidation of fat, overexpression of *FOXC2* in adipocytes did not affect body weight as compared to wild-type littermates, either on standard chow or high-fat diet (Table 1, Paper I). Nevertheless, consistent with previous findings (35) there was a significant decrease in the fat mass of *aP2-FOXC2* mice (Figure 1A, Paper I). The lack of difference in body weight between transgenic and wild-type mice was explained by an increase in whole-body lean mass of these mice (Figure 1B, Paper I). In evidence, *aP2-FOXC2* mice gained as much weight as wild-type mice, but instead of storing excess energy in adipose tissue they seemed to provide substrates to other tissues. Or rather, by removing lipids, with potential toxic effects, from the system through increased oxidation in adipocytes, tissues remain more insulin sensitive and hence respond better to the anabolic effects of insulin.

Insulin resistance is an early indicator of type 2 diabetes and is characterized by the need to increase insulin levels to maintain normal glucose concentrations in plasma (136). High-fat diet induced a prominent insulin resistance in wild-type mice as demonstrated by an increase in fasting plasma glucose levels and a dramatic increase in fasting plasma insulin levels (Table 1, Paper I). Insulin resistance in wild-type mice after high-fat feeding was further established using the hyperinsulinemic-euglycemic clamp which

showed a marked reduction in glucose infusion as compared to mice fed standard chow diet (Figure 1C, Paper I), indicating that insulin-stimulated disposal of glucose from the blood was hampered. In parallel, the basal hepatic glucose production was not suppressed by insulin during the clamp on wild-type mice after high-fat feeding (Figure 1D, Paper I), a sign of insulin resistance specifically in the liver (94).

The majority of insulin-stimulated glucose uptake is taking place in skeletal muscle (326). Hence, changes in insulin-stimulated whole-body glucose turnover could be attributed mainly to skeletal muscle. In wild-type mice high-fat feeding caused a ~40% decrease in whole-body glucose turnover (Figure 2A, Paper I), as expected in mice with insulin resistance. This was accompanied by a 40% decrease in skeletal muscle glucose uptake (Figure 2B, Paper I), confirming that this tissue is the major source of insulin-stimulated glucose uptake. In line with development of insulin resistance and decreased glucose uptake, the wild-type mice also displayed decreased insulin-stimulated glycolysis as well as glycogen and lipid synthesis following high-fat feeding (Figure 2C and 2D, Paper I).

In contrast to the wild-type mice, mice with adipocyte-specific overexpression of FOXC2 seemed protected against diet-induced insulin resistance. The high-fat fed *aP2-FOXC2* mice were just as efficient in removing plasma glucose during the clamp as mice fed standard chow diet (Figure 1C, Paper I). They also maintained the ability of insulin-mediated suppression of basal glucose production in the liver (Figure 1D, Paper I). In addition, whole-body glucose turnover, as well as glucose uptake in skeletal muscle, was normal in *aP2-FOXC2* mice, even after high-fat diet (Figure 2A and 2B, Paper I). Consequently, whole-body glycolysis and glycogen plus lipid synthesis was comparable to that of mice fed a standard chow (Figure 2C and 2D, Paper I). This indicates that increased fat oxidation in the adipose tissue protects against insulin resistance in other tissues.

Interestingly, the *aP2-FOXC2* mice were protected from all of the effects that normally accompany diet-induced insulin resistance as it was indifferent from mice fed standard chow in virtually all aspects of the clamp experiment. Fat, both in terms of serum levels of FFA and intracellular levels of fatty acyl CoA, has been shown to positively correlate with the development of insulin resistance (151,153). To investigate whether the intramuscular fat levels where associated with the observed diet-induced changes in insulin sensitivity, we measured the levels of fatty acyl CoA by LC/MS/MS, a mass-spectrometry based method for quantitative measurements. Indeed, high-fat diet increased the accumulation of fatty acyl CoA in skeletal muscle of wild-

type mice (Figure 4B, Paper I) whereas the levels in *aP2-FOXC2* mice remained normal. Intramuscular fatty acyl CoA hampers insulin signaling, probably through activation of a serine kinase cascade leading to serine phosphorylation of IRS1 (155). Serine phosphorylation of IRS1 prevents tyrosine phosphorylation of IRS1 which in turn decreases the activation of PI-3 kinase (155,327,328), causing insulin resistance. To investigate the insulin signaling pathway in skeletal muscle, we measured the activity of the IRS1 associated PI3K. Wild-type mice fed a high-fat diet had significantly decreased activity of PI3K, indicating improper insulin signaling. On the other hand, the activity in skeletal muscle from high-fat fed *aP2-FOXC2* mice were equivalent to what was observed in mice, wild type and transgenic, fed a standard chow (Figure 3B, Paper I). This further establishes that *aP2-FOXC2* mice, which possess an increased capacity for oxidation of fat in adipocytes, are protected from diet-induced insulin resistance.

PAPER II – *FOXC2* STIMULATES ANGIOGENESIS IN ADIPOSE TISSUE

In previous work, it was established that adipocyte-specific overexpression of *FOXC2* lead to decreased fat mass associated with increased expression of genes involved in the oxidation of fat, such as *Ucp1* and *Ppara* (35). The WAT from *aP2-FOXC2* mice also contained more mitochondria and exhibited a higher oxygen consumption rate as compared to wild-type mice. An increase in energy consumption would also increase the need for energy supply as well as waste disposal via the blood stream. The metabolically highly active BAT is consequently more vascularized than the WAT (183). These findings, in combination with the reddish appearance of WAT in *aP2-FOXC2* mice (Figure 1A, Paper II), made us investigate the vascularization of the adipose tissue in these mice.

To characterize the vascular network in the adipose tissue, we utilized antibodies directed against several well-characterized cell-specific markers. CD31 was used as an endothelial cell marker (329), smooth muscle cells were detected by staining for α-SMA (alpha smooth muscle actin, ACTA2) (330) whereas pericytes were labelled using anti-NG2 antibody (331). In *aP2-FOXC2* transgenic mice, the adipose tissue showed increased vascular density based on CD31 labeling, regardless of the depot studied (Figure 1B-E). Interestingly, the increased vascular density of the epididymal WAT in *aP2-FOXC2* mice approached the pattern seen in wild-type BAT, giving further support to the browning effect of the adipose tissue described earlier (35). The vessels in *aP2-FOXC2* transgenic mice were not only increased in

number, but also formed a denser network (plexus) with more connections between each other. In addition, adipocyte-specific induction of *FOXC2* led to a redistribution of vascular smooth muscle cells and pericytes to microvessels (Figure 2, Paper II).

Since angiogenesis in adipose tissue was clearly affected by the increased expression of *FOXC2* in adipocytes, we decided to conduct a more global investigation of the gene expression. We performed microarray analyses and compared the mRNA expression profiles of WAT from *aP2-FOXC2* and wild-type mice. Strikingly, several angiogenic factors were induced by *FOXC2*. We reinvestigated a subset of these by qPCR and found that the most prominent effect was a more than five-fold induction of *Angpt2*. In addition, a significant upregulation could also be detected for placental growth factor (*Pgf/PlGF*) (Figure 3A, Paper II). We could not confirm microarray data which suggested that ephrin B2 (*Efnb2*), notch 3 (*Notch3)*, and platelet derived growth factor receptor beta (*Pdgfrb*) were differentially regulated in adipose tissue from aP2-FOXC2 mice, but at least the trend was that they were induced.

Angpt2 is involved in vascular remodeling, as well as neovascularization, by destabilizing blood vessels (201). Since ischemia, i.e. low blood supply and availability of oxygen, can induce the expression of *ANGPT2* (332), we wanted to explore if the observed effect was caused by direct regulation by FOXC2 or merely a result of the increased metabolic rate. Evidence that *Angpt2* might be transcriptionally regulated by FOXC2 was provided from experiments on mouse embryonic fibroblasts (MEFs) derived from *Foxc2* heterozygous knockout intercrosses. In MEFs with one remaining functional *Foxc2* allele, both *Foxc2* and *Angpt2* expression was reduced to approximately half of the wild-type expression (Figure 3B, Paper III). It is worth pointing out that not all *Angpt2* expression is dependent on FOXC2, since MEFs from homozygous *Foxc2* knockout embryos, i.e. completely lacking *Foxc2* expression, still express *Angpt2* at levels comparable to heterozygous MEFs.

We could provide further support for a direct FOXC2 regulation of *Angpt2* expression by studying its ability to activate the promoter of *Angpt2*. Using a co-transfection luciferase assay, we showed that FOXC2 was able to activate the *Angpt2* promoter in a dose-dependent manner (Figure 3C, Paper II). Sequence analysis of the promoter region revealed five putative forkhead-binding sites, based on the forkhead-binding consensus sequence (38). By site-directed mutagenesis, promoter constructs were generated with each and one of these sites mutated. Using these modified constructs we were able to

identify one of these sites as responsible for practically all of the FOXC2 dependent activation of the *Angpt2* promoter (Figure 3C, Paper II). Collectively these data confirm that FOXC2 is able to directly induce *Angpt2* expression.

Since ANGPT2 possesses a signal peptide for secretion (201), we wanted to investigate if ANGPT2 could contribute to the angiogenic phenotype via paracrine secretion from the adipocytes. To explore this, *aP2-FOXC2* transgenic mice and wild-type littermates were treated with subcutaneous injections of an ANGPT2-specific inhibitor. Interestingly, treatment with this inhibitor virtually reversed the angiogenic phenotype seen in *aP2-FOXC2* mice, whereas no effect was observed in wild-type animals. The reddish appearance of the subcutaneous adipose tissue in *aP2-FOXC2* mice was reduced after treatment (Figure 4A, Paper II), and accompanied by a decreased number of blood vessels, particularly microvessels, as judged by CD31-staining (Figure 4B and 4C). The vascular network in adipose tissue from transgenic mice also became more organized and contained less branching after treatment with the inhibitor (Figure 4C and 4E). In addition, α-SMA staining showed that vascular smooth muscle cells had relocated from microvessels back to large arterial vessels where they are normally found (Figure 4D, 4F and 4G). Altogether, the results from treatment with a specific ANGPT2 inhibitor strongly suggest that most, if not all, of the angiogenic phenotype observed in adipose tissue of *aP2-FOXC2* mice, comes from ANGPT2 secretion. Still, contribution of other angiogenic factors cannot be excluded.

PAPER III – OVEREXPRESSION OF *FOXF2* IN ADIPOCYTES ALTERS INSULIN SIGNALING

Members of the forkhead family of transcription factors often possess important functions and many are critical for proper development (9). Several forkhead genes have also been implicated in adipocyte metabolism (35,48,292,293) When screening an adipocyte cDNA library for forkhead genes, we identified *FOXF2* as a novel adipocyte gene and subsequently confirmed expression in adipose tissue by qPCR analysis (Figure 1, Paper III). Since *Foxf2* expression was regulated during adipocyte differentiation (Figure 1B, Paper III), and has been reported to promote adipocyte differentiation (48), we wanted to explore its role in adipocytes *in vivo*.

Due to postnatal lethality of the global *Foxf2* knockout mouse, we generated transgenic mice with adipocyte-specific overexpression of human *FOXF2*,

so-called *aP2-FOXF2* mice. In this model, mRNA levels of *FOXF2* were readily induced in adipose tissue to about 4 times that of the wild-type levels (Figure 2A, Paper III). To investigate if this increased expression affected whole-body glucose metabolism, we performed an intravenous glucose tolerance test. Although the glucose removal rate was the same between transgenic and wild-type animals (Figure 2B, Paper III), the amount of insulin needed to dispose of the glucose was significantly increased in *aP2-FOXF2* mice (Figure 2C, Paper III), suggesting that these mice were insulin resistant. Insulin resistance in these mice was further tested by an insulin tolerance test, which revealed that *aP2-FOXF2* mice were less efficient than wild-type mice in removing glucose from serum after receiving a given insulin dose (Figure 2D, Paper III), indicating a defect in insulin signaling in insulin-responsive tissues rather than hampered insulin secretion from pancreas.

Having established the metabolic dysfunction in the *aP2-FOXF2* mice, we then analyzed the gene expression in WAT, focusing on genes linked to the insulin signaling pathway or diabetes. Several genes were indeed identified as regulated by *FOXF2* overexpression in WAT. The qPCR data suggests that FOXF2 mainly functions as a repressor, at least for the subset of genes that displayed a differential expression, since expression of *Irs1*, perilipin (*Plpn*), adiponectin (*Adipoq*, synonym *Acrp30*), and carnitoyl palmitoyltransferase 2 (*Cpt2*) was decreased, whereas only expression of sterol regulatory element binding transcription factor (*Srebf1*, synonym *Srebp1*) was found to be induced (Figure 3, Paper III).

Several of these genes have been reported to be involved in the pathogenesis of insulin resistance. What attracted our attention the most was the decreased levels of *Irs1,* since it is one of the earliest mediators of insulin signaling (333). Low levels of IRS1 have been linked to insulin resistance in both humans (164) and mice (108). Since the effect on all the other putative target genes potentially could be explained by decreased insulin signaling, we decided to examine the IRS1 levels in *aP2-FOXF2* mice further.

Although mRNA is crucial for subsequent synthesis of protein, there is not always a direct correlation between mRNA and protein levels of specific proteins (334). However, in this study we could confirm a 50% reduction of IRS1 protein in adipose tissue from *aP2-FOXF2* mice as compared to the wild-type (Figure 4A, Paper III).

MEFs possess the ability to differentiate into adipocytes (335) and have the advantage of being more homogenous than adipose tissue in terms of cell

types. Hence, adipocyte-specific effects could potentially be more distinct in MEFs as compared to adipose tissue, especially in terms of gene regulation. Indeed, the *FOXF2*-dependent down-regulation of IRS1 was even more pronounced when we analyzed compared protein levels in MEFs derived from *aP2-FOXF2* and wild-type mice that were differentiated into adipocytes (Figure 4B, Paper III).

Although lower levels of IRS1 alone could explain the observed insulin resistance, several studies have shown the importance of the phosphorylation status of IRS-1. Increased serine phosphorylation, as well as decreased tyrosine phosphorylation, seems to inhibit the ability of IRS1 to bind and activate PI3K (121,327,336). Although we could detect lower levels of both serine and tyrosine phosphorylated IRS1, there appears to be no change in the ratio between the different types of phosphorylations (Figure 5, Paper III). In other words, the observed defect in insulin signaling appears to be due to lower levels of IRS-1 rather than altered phosphorylation status.

From the *in vivo* data, we could observe that the amount of insulin needed to dispose of a given glucose load was increased in mice with adipocyte-specific overexpression of *FOXF2* as compared to wild type mice (Figure 2, Paper III). Since insulin-stimulated whole-body glucose uptake is mainly taking place in skeletal muscle (326), the observed difference indicated development of insulin resistance also in skeletal muscle, despite the fact that transgenic modulation of gene expression was adipocyte-specific. To validate the hypothesis that the observed phenotype is originating from hampered insulin signaling in adipocytes, insulin-stimulated glucose uptake was assessed in MEFs that were differentiated into adipocytes. As expected from the lower IRS1 levels, the insulin-stimulated glucose uptake was significantly reduced in MEFs with *FOXF2* overexpression at 50 mM insulin (Figure 6, Paper III). Notably, at higher insulin levels, there was no significant difference between *aP2-FOXF2* and wild-type MEFs. Perhaps this effect resembles the situation in insulin resistant mice, where normal serum glucose levels are maintained by high serum insulin levels.

Taken together, our data suggest that *FOXF2* is involved in the development of insulin resistance by suppressing the expression of IRS1. Intriguingly, *FOXF2* seems to have the exact opposite effect on adipocyte metabolism as compared to *FOXC2*, which instead protects against diet-induced insulin resistance (Paper I and (35)). It has also recently been shown that *FOXF2* could directly suppress *FOXC2* expression *in vitro* (337) which further establishes their counteracting roles.

PAPER IV – PODOCYTE-SPECIFIC DELETION OF *FOXC2* CAUSES PROTEINURIA AND KIDNEY FAILURE

Studies of *Foxc2* in knock-out mouse models have contributed substantially to our knowledge of FOXC2 functions during embryonic development. That the gene is crucial for correct formation of the aortic branch and vertebral column was, for example, derived from these mice (23,24). Nevertheless, the embryonic or perinatal lethality that comes with homozygous *Foxc2* deletion have hindered functional studies in adult animals. In our efforts to unveil the functions of *Foxc2*, we had developed a knock-in model, where the *Foxc2* coding sequence was replaced by the coding sequence for the marker gene *lacZ* (338). Since expression of *lacZ* is under the control of the endogenous *Foxc2* promoter, it is possible to use the *lacZ* expression as a marker for *Foxc2* expression. By establishing a MEF cell line from this mouse, it was possible to use *lacZ* expression as a read-out to screen for effectors of *Foxc2* expression (338). Additionally, this model also turned out to be a valuable tool in gaining insight into the *in vivo* expression pattern of *Foxc2*, both during embryogenesis and in the adult.

Staining heterozygous $Foxc2^{lacZ/+}$ embryos for *lacZ* expression revealed widespread expression during embryogenesis in developing structures including bone, vertebrae, heart, and nephrons (Figure 1, Paper IV). This pattern correlates well with previous reports on *Foxc2* expression during development (21-25) thus validating the model. We could also show that, in the adult kidney of $Foxc2^{lacZ/+}$ mice, *lacZ* was expressed in glomeruli as well as tubuli and ureter (Figure 2, Paper IV), suggesting that *Foxc2* is also expressed in these structures. Although expression of *Foxc2* in kidney has been confirmed by several groups, it has only been reported in the glomeruli, more specifically, in podocytes, of normal kidney (21,25,36,37). Interestingly, induction of *Foxc2* expression has recently been reported also in injured tubuli (339). One of the reasons for using *lacZ* as a marker gene is that it is presumed to provide a more sensitive detection method as compared to immunohistochemistry and *in situ* hybridizations. Hence, the observed *lacZ* expression in tubuli of $Foxc2^{lacZ/+}$ kidney probably corresponds to low *Foxc2* expression levels that are not detectable by the methods previously used in other studies.

In diabetic patients, kidney damage, or diabetic nephropathy, is one of the most severe complications and it is accompanied by poor prognosis on survival rate (176). Mutations in *FOXC2* have been linked to dyslipidemia, type 2 diabetes, and proteinuria (30,32,340), as well as lymphedema distichiasis (26,27). Development of proteinuria is one of the major

contributing factors for diabetic complications (341) and damage to podocytes is strongly linked to proteinuria (264,265). Previous studies of *Foxc2* in kidney have focused on the global knockout model, assuming podocyte-specific expression in the kidney (25). Due to perinatal lethality such studies have been limited to developmental effects of *Foxc2,* and its potential role in the development of diabetic nephropathy is unknown. To be able to bypass the developmental roles of *Foxc2*, we generated a mouse carrying a floxed *Foxc2* allele (Figure 3, Paper IV). By crossing this $Foxc2^{fl/+}$ mouse with *Pod-Cre* transgenic mice (297) that possess podocyte-specific Cre expression (Figure 2B, Paper IV), it was possible to specifically delete *Foxc2* in podocytes.

When genotyping litters from such crossings at 4 weeks of age, we could not identify any mice with complete deletion of *Foxc2* in podocytes (Figure 4A, Paper IV). All other possible genotypes were present at the expected Mendelian ratio, which indicated that neither the presence of Cre nor homozygosity for the loxP insertions appeared to cause any harm to the mice. Pups were then monitored from late gestational stages to see if the podocyte-specific deletion of *Foxc2* could cause embryonic or early postnatal lethality. Until about 10 days of age, all expected genotypes were observed, even $Foxc2^{fl/fl}$; *Pod-Cre* (Figure 4B, Paper IV) demonstrating that the genetic modification was not prenatally lethal. At around 10 days after birth, mice with homozygous deletion of *Foxc2* in podocytes had to be euthanized due to: poor vital signs, including growth retardation, decreased motility, and dehydration.

To assess the kidney function of $Foxc2^{fl/fl}$; *Pod-Cre* mice, urine samples from newborn pups were analyzed for protein content on a Coomassie-stained polyacrylamide gel. This analysis demonstrated very high protein content in the urine of 5-day-old $Foxc2^{fl/fl}$; *Pod-Cre* pups as compared to heterozygous $Foxc2^{fl/+}$; *Pod-Cre* littermates (Figure 4C, Paper IV). Since Coomassie staining has a rather low sensitivity, the urine was also analyzed for albumin (the major plasma protein) content using an ELISA approach, to investigate the possible presence of microalbuminuria in mice with other genotypes. None of the other genotypes, not even the heterozygous $Foxc2^{fl/+}$; *Pod-Cre* mice, displayed increased levels of albumin in the urine (Figure 4D, Paper IV). Hence it seems as only one functional *Foxc2* allele is sufficient for podocyte function.

Standard histological studies revealed that kidney sections of $Foxc2^{fl/fl}$; *Pod-Cre* was undistinguishable from $Foxc2^{fl/fl}$ until end stage renal failure, when signs of kidney damage, including enlarged glomeruli and dilated tubuli with

proteinaceous content become evident (Figure 5A, Paper IV). The fact that onset of proteinuria precedes any observable histological change suggests that the condition fits with the classification of MCN (277). The pronounced proteinuria already in 5-day-old *Foxc2*$^{fl/fl}$; *Pod-Cre* mice was a strong indicator for podocyte damage. Since podocytes in MCN had also been reported to undergo structural changes (264), we decided to investigate the ultrastructure of the podocytes. Using transmission electron microscopy, it became obvious that, already at day 5, podocytes exhibit foot process effacement, the hallmark of podocyte injury (263). The effacement was even more evident at day 9 in kidneys from *Foxc2*$^{fl/fl}$; *Pod-Cre* mice (Figure 5B, Paper IV). Other important parts of the glomerular filtration barrier, such as the fenestrated endothelial cells and glomerular basement membrane, which also could contribute to the observed phenotype if damaged (240,244,342), appeared unaffected even at end stage renal failure. The observed changes seems to be, at least structurally, isolated to podocytes in *Foxc2*$^{fl/fl}$; *Pod-Cre* mice, which is a discrepancy compared to the phenotype of global *Foxc2* knockout mice that, for example, more or less lack fenestrae (25). Thus, the observed phenotype indicates that the effects were more related to podocytes, which also was one of the purposes for generating a podocyte-specific deletion of *Foxc2*.

In the transmission electron micrographs, finger-like structures, extending into urinary space, were observed in the *Foxc2*$^{fl/fl}$; *Pod-Cre* glomeruli. To examine these finger-like structures further, kidney sections were analyzed by scanning electron microscopy, which confirmed the development of microvilli projecting from the cell body of the podocytes (Figure 6, Paper IV). A small number of microvilli had developed already in podocytes from 3-day-old *Foxc2*$^{fl/fl}$; *Pod-Cre* mice, and as the microvilli increased in number over time, the interdigitated pattern of foot processes were completely lost at day 5, when proteinuria was detected. The extensive foot process effacement observed in 5-day-old *Foxc2*$^{fl/fl}$; *Pod-Cre* mice explains why the glomeruli were unable to maintain their filtration ability (263). Although the mouse glomeruli are not fully mature when Cre activity is initiated in *Pod-Cre* mice (297), the minor differences observed in 3-day-old mice suggest that removal of *Foxc2* did not disturb the kidney formation.

In summary, when podocytes are deprived from *Foxc2* expression, they undergo dramatic and, for the host, lethal structural changes. Such changes could have many causes, like disruption of the slit diaphragm (224,228), improper anchoring to the basement membrane (237,238,244,245), cytoskeletal rearrangements (230,243,247,251), or dysfunctional cell-cell signaling/interactions (343-345). To attempt to dissect the mechanism behind

the observed phenotype of *Foxc2*$^{fl/fl}$; *Pod-Cre* mice, immunohistochemical analyses were employed. No difference between *Foxc2*$^{fl/fl}$; *Pod-Cre* and *Foxc2*$^{fl/fl}$ mice was observed in terms of: podocyte number, degree of apoptosis, or proliferation, based on WT1, TUNEL-assay and KI67 staining respectively. These are all conditions that have been reported to cause proteinuria (269,285). However, staining for ACTA2 indicated an increase in mesangial cell number (Figure 7, Paper IV), which points to glomerular damage (268,285). The minor effect on these parameters fits well with the classification of the condition as MCN (277).

A number of glomerular proteins, with reported links to podocyte damage and/or FOXC2, were also analyzed. Some intriguing potential targets, like NPHS2 (228,346), TJP1 (347), COL4A3 (244), and COL4A4 (245) were inferred from work on the *Foxc2* global knockout model, where reduced expression of these candidates was observed on either protein or mRNA level. In addition, NPHS1, ACTN4, NES, and CD2AP, proteins with known function or expression in podocytes (224,230,348,349), were analyzed in kidneys from *Foxc2*$^{fl/fl}$; *Pod-Cre* mice. Loss or reduction of the expression of any of these targets could possibly explain the phenotype of the *Foxc2*$^{fl/fl}$; *Pod-Cre* mice. However, based on the immunohistochemical staining, we could not confirm any change in the expression level for any of the analyzed proteins (Figure 8, Paper IV). This was particularly surprising for the targets which expression was affected in the global knockout model of *Foxc2* (25). Nevertheless, it does resemble the situation observed for another podocyte transcription factor, namely *Lmx1b*. Global knockout of this gene caused, similarly to the *Foxc2* global knockout, loss or reduction of important glomerular proteins like NPHS2, COL4A3 and COL4A4 (259,260). These are effects that could not be confirmed when conditionally deleting *Lmx1b* in podocytes (262,350). The examples of *Foxc2* and *Lmx1b* highlight the importance of using cell-specific, rather than global, knockouts to study gene functions.

Although no effect on expression levels could be determined by immunohistochemical analyses, a change in the distribution was observed for some proteins in glomeruli of *Foxc2*$^{fl/fl}$; *Pod-Cre* mice. Interestingly, the observed redistribution was limited to proteins known to be important for the structure of either the slit diaphragm (NPHS1, NPHS2, TJP1, ACTN4, and CD2AP) or the foot processes (PODXL and NES) (Figure 8C, Paper IV). These proteins normally locate along the capillary lining, but in *Foxc2*$^{fl/fl}$; *Pod-Cre* mice these components demonstrate a cytoplasmic, sometimes granular, distribution. None of the proteins involved in anchoring to the basement membrane (ITGB1, ILK, and CYR61 (cysteine rich angiogenic

inducer 61)), or constituting the basement membrane (COL4A3 and COL4A4), display this change in distribution (Figure 8A and 8B, Paper IV), suggesting that the damage is defined to the structure of the foot processes. Redistribution of podocyte markers has previously been observed in other conditions with foot process effacement, for example in mice with podocyte-specific deletion of *Tjp1* (347), and in patients with MCN (283).

The search for candidate genes that could explain the observed phenotype was expanded by performing gene expression analyses by qPCR on glomerular mRNA isolated from 9-day-old pups. The expression level of *Foxc2* correlated very well with genotype, with only about 10% of the wild-type expression level left in glomeruli from *Foxc2*$^{fl/fl}$; *Pod-Cre* mice. The remaining glomerular expression could either be due to incomplete Cre activity in podocytes of these mice or originate from other cell types. Yet, the majority of glomerular *Foxc2* mRNA indeed originates from podocytes. Screening the glomerular mRNA for candidate targets identified three genes, semaphorin 5a (*Sema5a*), glucocorticoid induced 1 (*Glcci1*) and neuropilin 1 (*Nrp1*), as significantly downregulated after podocyte-specific *Foxc2* deletion (Figure 9A, Paper IV).

Notably, podocytes only constitute a fraction of the total number of cells in the glomeruli. Hence, the observed effects on glomerular mRNA could be related to expression levels in other cell types than podocytes. Therefore we conducted a knockdown experiment in a podocyte cell line, using siRNA directed against *Foxc2*. In this experiment, only *Nrp1* seemed to be dependent on the *Foxc2* expression (Figure 9B, Paper IV), suggesting that the observed reduction of *Sema5a* and *Glcci1* transcripts in the glomeruli from *Foxc2*$^{fl/fl}$; *Pod-Cre* mice, might originate other cell types than podocytes. Co-regulation of NRP1 and *Foxc2* was also confirmed at protein level in these cells using a Western blot approach (Figure 9C, Paper IV).

NRP1 is a transmembrane co-receptor with affinity for VEGF and semaphorins (299). This protein has previously been reported to be regulated by *Foxc2* in endothelial cells (351). To confirm the regulation in podocytes *in vivo*, kidneys from 9-day-old *Foxc2*$^{fl/fl}$; *Pod-Cre* and *Foxc2*$^{fl/fl}$ mice were stained for NRP1. Since expression of NRP1 was widespread in the glomeruli, we co-stained for NPHS2 as a marker for podocytes. In *Foxc2*$^{fl/fl}$ control mice, these two membrane-associated proteins co-localized around the capillary tuft of the glomeruli. Strikingly, this co-localization was completely lost in glomeruli from *Foxc2*$^{fl/fl}$; *Pod-Cre* mice (Figure 10, Paper IV), where the cytoplasmic, granular redistribution of NPHS2 was evident. Encouraged by the co-regulation of NRP1 and *Foxc2*, and the reported

findings that NRP1 is expressed in podocytes where it could be involved in the development of proteinuria (288,352-354), made us study this target further. Previous generation of mice carrying floxed *Nrp1* alleles (299) made it possible to make podocyte-specific *Nrp1* knockout mice.

Unlike *Foxc2*$^{fl/fl}$; *Pod-Cre* mice, podocyte-specific deletion of *Nrp1* did not seem to affect kidney function at all. The mice appeared healthy and all genotypes were born at expected ratio (Figure 11A, Paper IV). To validate the deletion of *Nrp1* in podocytes, genomic DNA was assessed by PCR. Amplification by PCR, which was only possible if Cre recombinase had excised the floxed sequence, could only be confirmed in genomic DNA from kidney from *Foxc2*$^{fl/+}$; *Pod-Cre* (Figure 11C, Paper IV). Given the podocyte-specific expression of Cre in *Pod-Cre* mice, the observed band that was amplified by PCR on kidney DNA most likely corresponds to recombination events in podocytes. Unexpectedly, deletion of the *Nrp1* gene in podocytes did not affect glomerular mRNA levels of *Nrp1* at all (Figure 11D, Paper IV). However, this could potentially be due to high expression in other glomerular cells, such as endothelial cells, concealing the podocyte-related reduction. Therefore we checked the kidney function by analyzing the albumin content in urine from young as well as old mice. In neither case there was a difference between the different genotypes (Figure 12, Paper IV). Considering the unchanged *Nrp1* mRNA levels in glomeruli from *Nrp1*$^{fl/fl}$; *Pod-Cre*, together with the unaffected kidney function as judged by urinary albumin content, *Nrp1* does not seem to have a critical role in podocytes. The data also suggest that, although regulation of NRP1 by *Foxc2* was observed *in vitro*, the reduced levels of glomerular *Nrp1* mRNA in the podocyte-specific *Foxc2* knockout could potentially be explained by reduced expression in other cell types than podocytes.

CONCLUSION

Many forkhead genes have been shown to have important, often critical functions, particularly during development. This thesis establishes the importance of *FOXC2* and *FOXF2* for adipocyte metabolism *in vivo* and the crucial impact of *Foxc2* on podocyte function *in vivo*.

In Paper I and II, we continued the characterization of the metabolic phenotype of the *aP2-FOXC2* transgenic mice. From these findings we can conclude that:

- *FOXC2* overexpression in adipocytes protects against diet-induced insulin resistance in skeletal muscle and liver. Insulin stimulated glucose uptake and glycogen synthesis, as well as insulin suppression of gluconeogenesis, is as efficient in *aP2-FOXC2* mice fed a high-fat diet as wild type mice fed a standard diet.

- The increased metabolic rate in the adipocytes of *aP2-FOXC2* mice consumes the excess energy instead of storing it. As a consequence, the accumulation of fat in skeletal muscle following a high-fat diet in wild type mice is abolished in these mice.

- Keeping the lipid levels in skeletal muscle at a low level maintains the insulin sensitivity by sustaining the ability of IRS1 to activate PI3K upon insulin stimulation. This can be achieved either by low calorie diet or by increased capacity of the adipocyte to handle the excess energy, which in *aP2-FOXC2* mice means increased combustion of fat.

- *FOXC2* induces the expression of, not only genes involved in lipid oxidation, but also angiogenic factors, in particular ANGPT2. In this way, *FOXC2* also make sure that the metabolically active adipocyte is supplied with energy and clearance of waste products via newly formed blood vessels.

In Paper III, we characterized the role of *FOXF2* in adipocytes and found that:

- *Foxf2* is expressed in adipocytes and is induced *in vitro* during differentiation.

- Increased expression of *FOXF2* in adipocytes *in vivo* causes insulin resistance, initiated by decreased levels of IRS1 in adipocytes and subsequent decreased sensitivity to insulin. Insulin resistance in adipocytes of *aP2-FOXF2* mice probably leads to increased lipolysis in adipocytes and increased levels of FFA in serum, triggering the observed systemic insulin resistance.

In Paper IV we generated mice that lack either *Foxc2* or *Nrp1* in podocytes. From these animals we can draw the following conclusions:

- *Foxc2* expression in the podocyte *in vivo* is a prerequisite for mice to uphold normal glomerular filtration. Without *Foxc2* expression, the podocytes cannot maintain its unique architecture, involving interdigitating foot processes, causing protein to leak into the urine and eventually leading to kidney failure and death.

- None of the examined podocyte proteins, known to maintain the slit diaphragm, cytoskeletal arrangement, or anchorage of the podocyte to the glomerular basement membrane, were presented with obvious changes in expression level in glomeruli of *Foxc2* conditional knockout mice. However, the distribution of structural proteins was dramatically altered, establishing the structural defect in these mice.

- Conditional knockout of *Foxc2* provides a better tool than the global knockout to study its cell-specific roles. This is highlighted by the fact that the kidney phenotypes are different in these two models. More importantly, none of the proteins that were downregulated in global *Foxc2* knockout mice, such as NPHS2, COL4A3, and COL4A4, seemed to be affected by *Foxc2* deletion in podocytes.

- Although NRP1 seemed like a promising target, it does not appear to be necessary for the podocyte to maintain its function in the filtration barrier.

FUTURE PERSPECTIVES

The benefits of having increased levels of *FOXC2* in adipocytes are obvious from our studies on the *aP2-FOXC2* transgenic mice. Even so, many of the observed effects in these mice could be secondary to the increased metabolic rate. Although induction of several adipocyte genes has been reported, the only direct target that is validated so far is ANGPT2. The newly generated mouse strain, with a floxed *Foxc2* allele, would provide a useful tool to identify direct targets and further explore the role of *Foxc2* in adipocytes, since it will be possible to generate mice with deletion of *Foxc2* specifically in adipocytes. The first characterizations of such mice would of course be of the insulin signaling and lipid metabolism. Still, with this model it would maybe also be possible to establish whether the angiogenic phenotype of *aP2-FOXC2* transgenic mice is dependent on *Foxc2* or, if it is initiated by the increased metabolic rate in the adipocytes.

Although most evidences suggest that the phenotype of *aP2-FOXC2* transgenic mice is due to increased metabolic rate of the adipocytes, there are reports that suggest that modulating the vascularization of the adipose tissue could have effect on obesity and insulin resistance. Therefore it would be interesting to see whether the metabolic phenotype of *aP2-FOXC2* mice is affected by treating the mice with the ANGPT2 inhibitor used in Paper II.

Overexpression of *FOXF2* in adipocytes causes blunted insulin signaling in adipocytes, due to decreased levels of IRS1, but also in other tissues as judged from glucose and insulin tolerance tests. It is tempting to assume that the blunted insulin signaling in the adipocyte would cause increased lipolysis and subsequent rise in serum FFA levels and intramyocellular lipids. However, this was not measured in Paper III, and therefore it would be interesting to see if that is the case or not. Also, subjecting these mice to a clamp experiment could possibly provide more detailed information about the insulin resistance in these mice. Furthermore, the direct regulation of IRS1 expression by *FOXF2* has not been reported and hence investigating the interaction between *FOXF2* and the *Irs1* promoter, using for example promoter-reporter constructs or chromatin immunoprecipitation, could shed light on this.

In terms of identifying the mechanism behind the dramatic phenotype of the podocyte-specific *Foxc2* knockout, several different ideas are already under progress. Since the effect of *Foxc2* deletion is taking place shortly after birth when using the *Pod-Cre* transgenic mice, we cannot exclude the possibility

that we still have been investigating developmental roles of FOXC2. Therefore, an inducible deletion in podocytes of adult mice would be valuable to establish the FOXC2 function in this cell type. This model might also offer a better tool to generate enough material, i.e. number of isolated glomeruli or podocytes, for subsequent global analyses of either the transcriptome or the proteome. An alternative way of increasing the sample size for such analyses is provided by cell cultures, although such systems lack the proper biological context seen *in vivo*. Precise genome editing techniques, such as CRISPR technology, could theoretically be utilized to delete *Foxc2* in a mouse podocyte cell line. Cells in such a cell line would potentially mimic the podocytes in the podocyte-specific *Foxc2* knockout mouse. In this model it might also be possible to study functional aspects of *Foxc2* deletion, like cell-cell interactions and the organization of cytoskeleton and slit diaphragms.

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