From Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

MOLECULAR STUDIES ON SEX DIFFERENCES IN HEPATIC GENE EXPRESSION AND FUEL METABOLISM

Louisa Cheung
張彥真

Karolinska Institutet

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SUPERVISORS:

Petra Tollet-Egnell, Associate Professor
Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

Gunnar Norstedt, Professor
Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

Per Eriksson, Professor
Department of Medicine, Solna
Karolinska Institutet, Stockholm, Sweden

FACULTY OPPONENT:

Jan Oscarsson, Professor
Department of Bioscience, AstraZeneca R&D, Mölndal, Sweden and
Department of Physiology/Endocrinology, the Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

EXAMINATION BOARD:

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Department of Biosciences and Nutrition
Karolinska Institutet, Stockholm, Sweden

Marie Öhman, Associate Professor
Department of Molecular Biology and Functional Genomics
Stockholm University, Stockholm, Sweden

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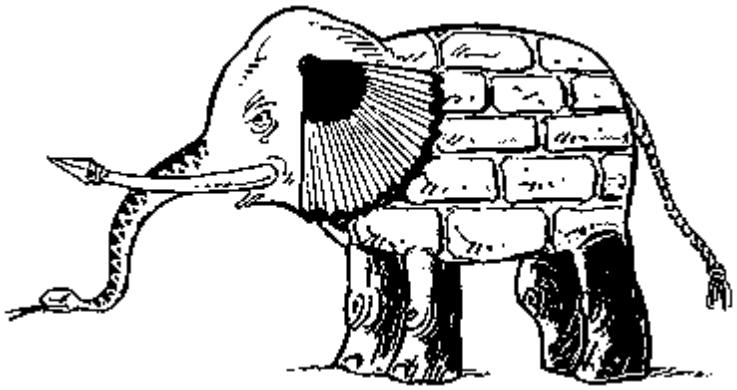
To Daniel



It was six men of Hindustan
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind)
That each by observation
Might satisfy the mind.

The first approached the Elephant
And happening to fall
Against his broad and sturdy side
At once began to bawl:
"Bless me, it seems the Elephant
Is very like a wall".

The second, feeling of his tusk,
Cried, "Ho! What have we here
So very round and smooth and sharp?
To me 'tis mighty clear
This wonder of an Elephant
Is very like a spear".

The third approached the animal,
And happening to take
The squirming trunk within his hands,
Then boldly up and spake:
"I see," quoth he, "the Elephant
Is very like a snake."

The Fourth reached out an eager hand,
And felt about the knee.
"What most this wondrous beast is like
Is mighty plain," quoth he;
"'Tis clear enough the Elephant
Is very like a tree!"

The Fifth, who chanced to touch the ear,
Said: "E'en the blindest man
Can tell what this resembles most;
Deny the fact who can,
This marvel of an Elephant
Is very like a fan!"

The Sixth no sooner had begun
About the beast to grope,
Than, seizing on the swinging tail
That fell within his scope,
"I see," quoth he, "the Elephant
Is very like a rope!"

And so these men of Hindustan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and strong,
Though each was partly in the right
And all were in the wrong.

So oft in theologic wars,
The disputants, I ween,
Rail on in utter ignorance
Of what each other mean,
And prate about an Elephant
Not one of them has seen!

The Blindmen and the Elephant
by John Godfrey Saxe (1816-1887)

ABSTRACT

In nature, sex difference is a multifactorial phenomenon. Although having a genotype of XX or XY determines the sex of an individual, the phenotypic sex differences are orchestrated by multiple factors such as developmental stages, nutritional condition, endocrine status and environmental factors. Most of the sex-dimorphic features are related to reproduction. However, an organism's reproductive system is supported, or in some cases stimulated, by core metabolic events, in particular fuel metabolism. Dysregulation in fuel metabolism is one of the causes for diseases such as obesity, type II diabetes and atherosclerosis.

The liver is a key metabolic organ coordinating various physiological processes. Males and females have slightly different metabolic needs. Therefore, this key metabolic organ is adapted to its sexual makeup and responds to physiological stimuli differently. For over 20 years, it has been known that the sex-dimorphic pattern of growth hormone (GH) secretion causes the liver to be a sex-dimorphic organ.

The aim of this thesis was to extend the knowledge of sex differences in hepatic fuel metabolism at the molecular level using high-throughput technologies. Norwegian rats (*Rattus norvegius*) were chosen to be the animal model for these studies due to their renowned sex difference in growth and liver metabolism.

We demonstrated that male and female rats differ metabolically at the molecular level. In serum, they displayed differences in their circulating metabolic profiles. Some of these differences might be contributed by the liver since metabolic and transcript profiles derived from hepatic samples also differed between male and female rats. Under a control basal condition, male rats displayed a higher expression of hepatic genes encoding important proteins for glucose oxidation, glycogen production, lipid synthesis, fatty acid oxidation, and amino acid turnover (paper I). After mild starvation, the male livers had lower expression of lipogenic genes and higher expression of lipolytic genes than the females. Moreover, a larger number of hepatic genes were regulated by insulin-induced hypoglycemia in females than in males (paper III). In order to gain a deeper understanding about of the regulatory mechanisms behind the sex-differentiated gene expression, a newly described sex-differentiated hepatic gene, fatty acid translocase (*FAT/CD36*), was analyzed in detail (paper II). We revealed that *FAT/CD36* has two alternative splice variants with two different first exons and corresponding promoters. Continuous infusion of GH, a female secretory pattern, induced the expression of *FAT/CD36* preferentially through the promoter of exon 1a, whereas episodic administration of GH, a male secretory pattern, repressed the expression through both alternative promoters. We also identified two sex-differentiated microRNAs, miR-29b and miR-122a (paper IV). The function of microRNA is to inhibit the protein translation of its targets. Continuous infusion of GH suppressed the level of miR-29b in parallel with an increase in protein level of its potential target, INSIG1.

In conclusion, male and female rats differ in hepatic fuel metabolism at the molecular level. The integration of biological data obtained from high-throughput screening technologies at different molecular levels and at different metabolic status might elucidate putative sex differences during the development of metabolic complications.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numbers in the text:

I. Ståhlberg N, Rico-Bautista E, Fisher RM, Wu X, **Cheung L**, Flores-Morales A, Tybring G, Norstedt G, Tollet-Egnell P. (2004) Female-predominant expression of fatty acid translocase/CD36 in rat and human liver. *Endocrinology.* Apr 145(4):1972-9.

II. **Cheung L**, Andersen M, Gustavsson C, Odeberg J, Fernández-Pérez L, Norstedt G, Tollet-Egnell P. (2007) Hormonal and nutritional regulation of alternative CD36 transcripts in rat liver--a role for growth hormone in alternative exon usage. *BMC Mol Biol.* Jul 17;8:60.

III. Gustavsson C, **Cheung L**, Yassin K, Lewitt M, Merino R, Tahir F, Brismar K, Östenson C-G, Norstedt G and Tollet-Egnell P. (2007) Sex-differences in the control of gluconeogenesis and hepatic glucose output in rats *Submitted.*

IV. **Cheung L**, Gustavsson C, Norstedt G and Tollet-Egnell P. (2007) Sex-different and growth hormone-regulated expression of microRNA in rat liver *Submitted.*

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LIST OF ABBREVIATIONS

-/-	Homozygous gene-deficiency
AR	Androgen receptor
Arg1	Arginase 1
C/EBP	CAAT/enchancer binding protein
cDNA	Complementary DNA
CIS	Cytokine-inducible SH2 protein
CoA	Coenzyme A
CPT	Carnitine palmitoyltransferase
Cutl2	Cut-like 2
CYP	Cytochrome P450
DMG	N,N'-dimethylglycine
ER	Estrogen receptor
FASN	Fatty acid synthase
FAT/CD36 or Cd36	Fatty acid translocase/CD36
G6Pase	Glucose-6-phosphatase
GFI1	Growth factor independent 1
GH	Growth hormone
GHc	Continuous infusion of growth hormone
GHi	Episodic administration of growth hormone
GHR	Growth hormone receptor
GHRHR	Growth hormone releasing hormone receptor
GR	Glucocorticoid receptor
HDL	High density lipoprotein
HGO	Hepatic glucose output
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HNF	Hepatocyte nuclear factor
IGF	Insulin growth factor
Insig1	Insulin-induced gene 1
JAK	Janus kinase
LCFA	Long-chain fatty acids
LDLR	Low density lipoprotein receptor
m-HPPA	m-Hydroxyphenylpropionic acid
miRNA	microRNA
mRNA	Messenger RNA
MUP	Major urinary protein
MS	Mass spectrometry
NAC	N-acetylglycoprotein
NEFA	Non-esterified fatty acids
Oat	Ornithine aminotransferase
PCR	Polymerase chain reaction
PEPCK	Pyruvate carboxykinase
PLS-DA	Partial least squares with discriminating data
PPAR	Peroxisomal proliferator-activated receptor
Rsl	Regulator of sex limited protein
SH2	Src-homology-2

Slp	Sex limited protein
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SREBP	Sterol regulatory element binding protein
SRY	Sex determining region Y
STAT	Signal transducer and activator of transcription
T2D	Type II diabetes mellitus
TAG	Triacylglyerides
TF	Transcription factor
TFBS	Transcription factor binding site
TMAO	Trimethylamine-N-oxide
TOF-MS	Time of flight
Tox	Thymus high-mobility group box protein
Trim24	Tripartite motif-containing 24
VLDL	Very low density lipoprotein
ZDF	Zucker diabetic fatty rats

Abbreviations used only once are described where they appear in the text.

PREFACE

Reproduction is important for life to carry on one generation after another. Complex organisms like most plants and most animals reproduce sexually. Sexual reproduction occurs when gametes of each sex fuse and form a new individual. Male refers to the sex that produces small mobile gametes, spermatozoa. Female is defined as the sex possessing the larger gametes, ova.

The sex in most mammals is determined genetically by the XY-determination system. There are also animals in other classes or phyla that are sex-determined by environmental factors. For instance, some reptilian species are made male or female depending on the temperature when the egg is incubated. More fascinating is that some animals can perform sex changes. Clownfish are by default male and the dominant individual in a group becomes female. In addition, many invertebrate species (including worms and snails) are hermaphrodites, which possess both male and female reproductive organs. This thesis will focus on mammalian sex differences using rat as the model animal.

The front cover of this book shows two sketches with five distinct differences. Please take some time to find them before going to the solutions in the appendix. Males and females differ genetically, biologically, physiologically and psychologically. Some sex differences are already obvious during childhood, while many of them become noticeable during puberty and adulthood. Interestingly, a number of sex differences diminish in the aged years.

This thesis will describe sex differences in the hepatic fuel metabolism in the liver. Some of these differences might underlie the different risk between men and women in the development of metabolic diseases. As individualized medicine will be the future of medical diagnosis and treatment, it is imperative to understand that medical research should include the whole human population, ethnicity as well as sex. So, is it necessary to re-do all the research where a sex-perspective was missing? I would leave this question for you to think about while you read this thesis.

INTRODUCTION

In nature, sex difference is a multifactorial phenomenon. Although having a genotype of XX or XY determines the sex of an individual, the phenotypic sex differences are orchestrated by multiple factors depending on developmental stages, nutritional condition, endocrine status and environmental factors (Figure 1). Most of the sex-dimorphic features are related to reproduction. However, an organism's reproductive system is supported, or in some cases stimulated, by core metabolic events, in particular fuel metabolism.

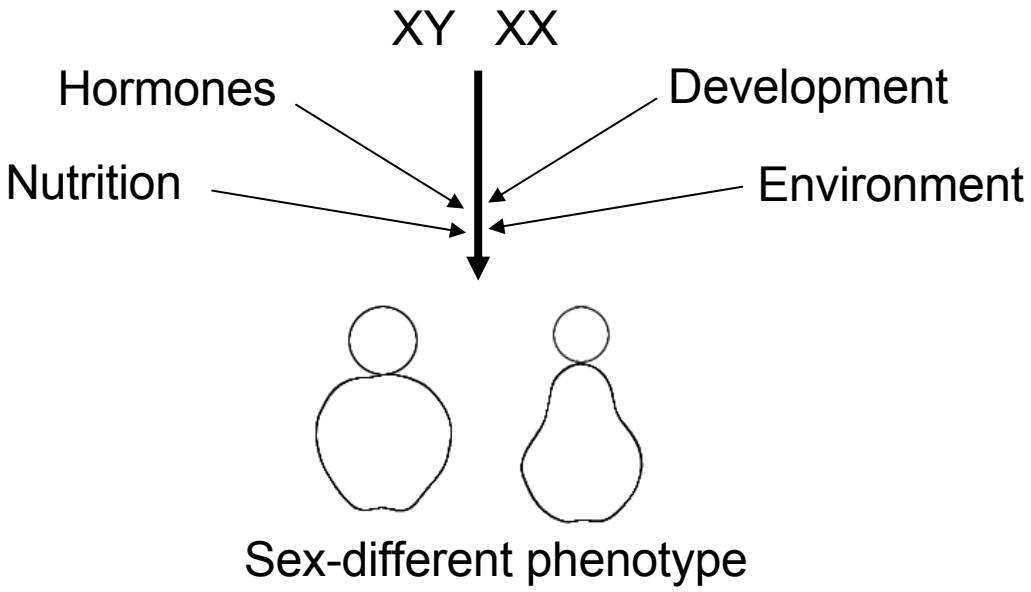

Figure 1. Factors contributing to the sex-different phenotypes.

SEX DIFFERENCE

Genetic

At the chromosome level, the most obvious sex difference is the presence of a Y chromosome in male and the double copies of X chromosome in female. The Y chromosome carries a very important gene in male sex determination, sex determining region Y (*SRY*). SRY is essential for the initiation of testis development. A mutation of this gene can lead to XY female development. A group of scientists under the lead of Arthur P. Arnold translocated this gene from the Y chromosome to an autosome (non-sex chromosome), so that female mice would produce this protein. Interestingly, the transgenic female mice showed some male behaviour, whereas male mice lacking SRY protein behaved more like female mice (De Vries et al. 2002). Besides, this gene can also modulate gene expression that leads to other sex differences. However, it is not certain that the same would happen in humans.

Autosomes present in double copies, one from the mother and one from the father. More than 99% of the genes are expressed from both maternal and paternal chromosomes. However, there are a few genes which are expressed from either a maternal or a paternal chromosome. In that case, the gene expression of one of the

chromosomes will be silenced by a process called imprinting. The most important example is the X-inactivation, which happens solely in females. Females carry two copies of the X chromosome and one of them has to be silenced to maintain a normal development (Tycko and Morison 2002).

Body composition

The bodies of men and women look differently. These differences exist in the foetus and during childhood but become more obvious during puberty and adulthood. Some major differences are located in fat, muscle and bone. Interestingly, some of these sex differences diminish in the aged years (Wells 2007).

In general, muscle mass is higher in male than female. The fiber type and size in males and females are different as well (Staron 1997; Fox et al. 2003). Males have more lean mass than females at birth, and this becomes more obvious after puberty when females start to gain fat (Wells 2007). Moreover, the fat storage differs between sexes. When there is excess fat, men prefer to store them in the abdominal region (visceral fat) whereas women's excess fat is all over the body with higher concentration in the hip and thigh region (gluteal-femoral fat). This results in an apple-shaped body in overweight men and a pear-shaped body in overweight women.

Metabolism

Sex differences in intermediary metabolism most likely contribute to the sex-different body composition. After a meal, men use 56% of the fat through fatty acid oxidation, 7% is stored in the lower body, 14% in the upper body and 23% in other part of the body. For women, 41% of fat intake is oxidized, 22% is stored in the lower body, 25% in the upper body subcutaneous and 12% in other parts of the body (Votruba and Jensen 2007).

The locations of fat storage also affect the speed in energy release. Upper body subcutaneous fat has higher lipolytic activity than fat in the lower body region. Therefore, visceral fat is more readily available in acute need, whereas gluteal-femoral fat is more long-lasting. The gluteal-femoral fat might be evolved as a mechanism to survive through famine for energy balance and heat production (Hoyenga and Hoyenga 1982), which is more important for women during pregnancy and child-caring periods.

Prediabetes development

A high concentration of non-esterified fatty acids (NEFA, in other words free fatty acids) in the circulation can cause a metabolic imbalance in glucose metabolism. It impairs the peripheral and hepatic insulin sensitivity and can also impair pancreatic β cell function (Carpentier 2008). NEFA released from the visceral fat will first be delivered to the liver. If the liver takes up more fat than it can degrade or release back to the circulation, fatty liver will develop. Several studies have shown that liver fat is one of the main risk factors for the metabolic syndrome (Marchesini et al. 2001; Kotronen et al. 2008). The male type of visceral fat distribution, if maintained

as obesity develops, is associated with adverse health consequences such as Type II diabetes mellitus (T2D) and cardiovascular complications.

Women, before menopause, are thought to have a lower risk to develop metabolic complications. There is increasing evidence for antidiabetic actions of estrogen in both humans and rodents (Louet et al. 2004). In a population study on subjects participating in the Stockholm Diabetes Prevention Programme, the prevalence of early abnormalities of glucose metabolism, with an increased risk of T2D, was shown to be two to three times higher in men compared to women at middle-age (Kuhl et al. 2005). Two different studies analyzing the diabetes prevalence in Chinese, Japanese and 13 European populations showed that young and middle-aged men have up to two times higher risk in terms of impaired fasting glycemia, whereas women (up to age 50 in Asia and 70 in Europe) have approximately 1.5 times higher risk in terms of impaired glucose tolerance (DECODE-Study-Group 2003; Qiao et al. 2003). It is clear that the pathological status of diabetes is different between men and women, but it also depends on age and ethnicity.

Gale and Gillespie reviewed the relationship between sex and diabetes prevalence and suggested that fathers with Type I diabetes are more likely to transmit the condition to their offspring whereas in T2D, children to a diseased mother are more likely to "inherit" the condition (Gale and Gillespie 2001). Although these speculations remain controversial, it is evident that sex and sex-related differences are important factors in understanding the development of metabolic imbalance.

T2D poses different stress on diseased men and women. Dr. Marianne Legato (a advocate in sex-specific healthcare in the United States) and associates analyzed the consequences of T2D in men and women by surveying available population studies published between 1995 and 2005 that includes diabetic patients (Legato et al. 2006). Unfortunately, some studies were restricted to men while others included both sexes but did not assess the effect of sex. They concluded that diabetic women suffered more severe lipid abnormalities than diabetic men. Women with diabetes also had a higher increase in the risk of developing and a higher risk of death from cardiovascular disease.

Animal models

T2D results from a subnormal response of tissues to insulin and a failure of the insulin-secreting pancreatic β cells to compensate for this insensitivity. The importance of insulin signalling in the liver in regulating glucose homeostasis was demonstrated by Michael and co-workers (Michael et al. 2000), using liver-specific insulin receptor knockout (LIRKO) mice. These animals exhibit dramatic insulin resistance, severe glucose intolerance, and a failure of insulin to suppress hepatic glucose production.

A "protective effect" of estrogen against diabetes was demonstrated using estrogen receptor (ER) α knockout (ERKO) mice. This animal model show characteristics which resemble those in humans lacking ERα (Smith et al. 1994) or aromatase (Morishima et al. 1995). Both male and female ERKO mice develop insulin resistance

and impaired glucose tolerance (Heine et al. 2000) and insulin resistance is largely localized to the liver (Bryzgalova et al. 2006).

Impaired hepatic insulin action has been shown to precede the development of peripheral insulin resistance in rats fed high-carbohydrate (Pagliassotti et al. 1996) or high-fat (Samuel et al. 2004) diets. Female rats, with the same age and on the same diet as the males, did not develop these metabolic changes (Horton et al. 1997), indicating a sex-different sensitivity towards high-carbohydrate diets. Similar findings have been reported in animal models of high-fat diet-induced diabetes. The obese Zucker diabetic fatty (ZDF) male rat (Stern et al. 1972) has become a widely used animal model of T2D. In contrast, the obese ZDF females rarely develop T2D. Interestingly, ZDF males develop T2D even on low fat-content diets, whereas only the highest fat-content diet is able to induce insulin resistance in ZDF females (Corsetti et al. 2000).

LIVER – A KEY METABOLIC AND SEX-DIMORPHIC ORGAN

The liver is a key metabolic organ coordinating various physiological processes. One of the most important functions of the liver is to remove harmful substances from the circulation. It is also the main organ in maintaining carbohydrate and lipid homeostasis. After a meal, carbohydrates are stored in the liver as glycogen and chylomicron remnants are cleared from the bloodstream. Between meals or during fasting or exercise, glucose and fat are released from the liver to meet the energy demand from other organs. About 80% of cholesterol production in the body is from this visceral organ. It also produces bile acid to facilitate absorption of dietary lipids. Moreover, the liver takes part in amino acid turnover by making certain amino acids and excreting unwanted amino acids in form of urea. Other important functions include, plasma protein production, degradation of blood cells, storage of vitamins and minerals and immune response.

Males and females have slightly different metabolic needs. Therefore, this key metabolic organ is adapted to its sex and responds to physiological stimuli differently. Sex dimorphic gene expression patterns have been reported in metabolic organs including liver, muscle and adipose tissue (Yang et al. 2006). Studies in the 1960s discovered several sex-specific proteins in the liver: Bond's protein, α-2u-globulin and senescence marker proteins 1 and -2 in rat, major urinary proteins (MUPs) in mouse and vitellogenin (the precursor of the egg yolk proteins) in chicken (reviewed in (Roy and Chatterjee 1983)). The sex hormones estradiol and testosterone were found to regulate the synthesis of these proteins.

However, it was later shown that an intact pituitary is required for hepatic sex dimorphism to take place (Kumar et al. 1969; Denef 1974; Gustafsson and Stenberg 1976) and the existence of a *feminizing factor* in the pituitary was postulated. In the late 1970s, Edén observed that the secretory pattern of growth hormone (GH) from the pituitary was different in male and female rats (Eden 1978; Eden 1979). A few years later, Mode and colleagues discovered that the *feminizing factor* is identical to the sex-dimorphic pattern of GH secretion (Mode et al. 1981; Mode et al. 1982; Mode et al. 1983). As illustrated in figure 2, GH secretion in males is episodic, with high peaks approximately every 3 to 5 hours. In females, GH is secreted more frequently and the differences between peaks and troughs are smaller than that in male, resulting in a continuous presence of GH. It is now established that sex differences in GH secretion control sex differences in growth and also sex differences in liver functions. A high dose of testosterone is secreted from the testis at one or more critical moments during prenatal and neonatal stage in males. This re-programs the genes in hypothalamus and changes the secretion patterns of somatostatin and growth hormone releasing hormone (Jansson et al. 1985). If such androgen re-programming did not occur, when puberty is reached, the secretion pattern would remain as the feminine continuous pattern. The sex differences in GH secretion pattern are most marked in rats, which has the most evident sex differences in body size in mammals. Key features are also found in mouse (MacLeod et al. 1991) and human (Ho et al. 1987; Hindmarsh et al. 1999).

During recent years, novel screening technologies have expanded the list of sex-different liver functions. One has also gained insights on how GH regulates sex-

specific gene transcription in the liver. Independent research groups have reported that GH secretion patterns regulate the expression of many sex-dependent transcripts and proteins (Tollet-Egnell et al. 2000; Gardmo et al. 2002; Ahluwalia et al. 2004; Laz et al. 2004), suggesting that GH secretory pattern is the determinant of liver sex (reviewed in (Mode and Gustafsson 2006)).

Steroids and drug metabolism

The liver is the main organ that removes and metabolizes excess or harmful substances. These can be endogenous (waste products and steroid clearance) or exogenous (drugs, solvents, alcohol and pollutants) in nature. There are two groups of enzymes responsible, namely Phase I and Phase II enzymes. Most of the Phase I enzymes are encoded by the cytochrome P450 (CYP) genes. The P450 enzymes make the lipophilic molecules more water soluble by adding polar groups, such as hydroxyl groups. At present, there are 57 sequenced P450 genes in human and 88 in rats; the precise functions of many of them are yet unknown. Phase II enzymes attach a water soluble moiety (for instance sugar or peptide) onto the polar group, thus making the molecule less active and facilitating renal excretion.

Sex differences in hepatic steroid and drug metabolism have been known for more than 50 years (reviewed in (Gustafsson et al. 1983b; Mode and Gustafsson 2006)). The first report came in 1932 by Nicolas and Barron showing that half of the dose of amobarbital required for a male rat was enough to anesthetize a female rat. In human, there are also sex differences in pharmacokinetics for several important drugs (Fletcher et al. 1994; Franconi et al. 2007; Schwartz 2007).

Early studies showed that sex hormones control hepatic sex differences, but later it was shown that most of the effects of sex hormones are indirect and mediated by the sex-dimorphic secretory pattern of GH (Gustafsson et al. 1983a). The most studied sex-different P450 enzymes in rat liver are the male-specific CYP2C11 (encoding steroid 2α- and 16α-hydrolyase) and female-specific CYP2C12 (encoding steroid sulphate 15β-hydrolyase). Other sex-different P450 enzymes are the male-enriched CYPs 2A2, 2C13, 2D9, 2E1, 3A2, 4A2, 4A12 and 7B1 and the female-enriched CYPs 2A1, 2A4, 2B6, 2B9, 2B13, 2C7, 3A4 and 17A1 (Waxman and O'Connor 2006). It is known that men also have higher capacity for phase II metabolism (glucuronidation, conjugation, glucuronyltransferases, methyltransferases, dehydrogenases) than women. For instance, the activities of glucuronidation, thiopurine methyl transferase, dihydropyrimidine dehydrogenase and UDP-gluronosyl transferase are higher in men (Franconi et al. 2007). Sex-dependent and GH-dependent transcriptional regulation of some of the sex-differentiated CYPs will be discussed below.

Intermediary metabolism

Carbohydrates

After a meal, carbohydrates are stored in the liver as glycogen. Within 2-6 hours after a meal, liver releases glucose from the glycogen store by glycogenolysis. During prolonged fasting, glucose is produced by gluconeogenesis – de novo

synthesis of glucose from non-carbohydrate precursors. It accounts for more than half of the total glucose production in humans during the first 22 hours of a fast (Raddatz and Ramadori 2007).

Several enzymes are important controllers of gluconeogenesis, such as pyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphate (FBP) and glucose-6-phosphatase (G6Pase). These enzymes are tightly regulated by availability of substrates and hormones (mainly insulin, glucagon and glucocorticoids). Insulin inhibits these genes in order to suppress gluconeogenesis, whereas glucagon and glucocorticoids induce them to stimulate hepatic glucose output (HGO). Transgenic animal models revealed that overexpression of either PEPCK or G6Pase increases the rate of gluconeogenesis, leading to T2D features, such as hyperglycemia and insulin resistance (Valera et al. 1994; Trinh et al. 1998; Sun et al. 2002).

Fats

The liver takes up NEFA from the bloodstream. They will either be oxidized for energy or esterified to form triacylglyerides (TAG). Fatty acid oxidation takes place mainly in mitochondria, with a small fraction (5-30%) in peroxisomes. Energy generated by fatty acid oxidation is a major energy source for gluconeogenesis. Ketone bodies are also produced during fatty acid oxidation. In the lack of glucose availability, they serve as fuel to the brain and other obligatory glycolytic tissues. Carnitine palmitoyltransferase (CPT) I is an important enzyme in controlling the entry of fatty acids into mitochondria. Its activity is inhibited by the high level of malonyl- coenzyme A (CoA), a substrate for lipogenesis. Esterification of fatty acids produces TAG that serves as a local storage of energy in the liver. TAG is also a main component in very-low-density-lipoprotein (VLDL) particles, which supply fats to the peripheral organs. Since excess intracellular NEFA damage cell functions, it is important to protect tissues from potential lipotoxicity.

In addition, the liver synthesizes fatty acids and cholesterol. Acetyl-CoA carboxylase (ACC) converts acetyl-CoA, an intermediate in glycolysis and amino acid catabolism, to malonyl-CoA. Insulin stimulates the activity of this rate-limiting enzyme. Fatty acid synthase (FASN) then condenses acetyl-CoA and malonyl-CoA to form long-chain fatty acids (LCFA). To form cholesterol, acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Then the rate-limiting enzyme HMG-CoA reductase converts HMG-CoA into mevalonate that will be further processed into cholesterol (Postic et al. 2004; Canbay et al. 2006).

Amino acids

The liver is the major site of amino acid catabolism. The amino groups are eliminated from the body via excretion of urea. First, the amino groups are removed through transamination, resulting in glutamate and keto acids. Glutamate then carries the nitrogen to the urea cycle whereas some keto acids can be oxidized in the muscle. Other keto acids such as pyruvate and oxaloacetate can enter other metabolic pathways. They can serve as substrates for gluconeogenesis, lipogenesis and ketogenesis. Excess keto acids are oxidized to produce energy. About half of the energy required in the liver is supported by amino acid oxidation.

Sex differences in hepatic intermediary metabolism

Most of the studies in hepatic intermediary metabolism have been performed using males. Information is therefore limited regarding possible sex differences in the hepatic metabolism of carbohydrate and amino acids. However, some sex differences in hepatic lipid metabolism have been described.

Fatty acid handling is not the same in male and female liver. In mature rats, the LCFA uptake and incorporation TAG are higher in female livers than in male (Kushlan et al. 1981). These observations can be linked to the concentration of fatty acid binding proteins, which is higher in the female livers. Sex hormones can mimic the level of fatty acid utilization similar to the corresponding sex (Ockner et al. 1979).

The metabolism of plasma chylomicrons is also sex-different. The expression of hepatic apolipoprotein E, a main apolipoprotein of the chylomicron, is higher in female rats than in males (Massimi et al. 1999). Though the rate of disappearance of chylomicrons in the circulation is similar between male and female rats, the female liver takes up more chylomicrons at 10 or 20 minutes after food intake (Staprans and Felts 1989). This suggests that more chylomicrons are "dwelling" on the endothelium of male rats, thus increasing cholesterol deposition and the risk of atherogenesis. A hormone treatment study showed that testosterone accounts for the decrease in chylomicron uptake (Staprans et al. 1990).

Female rats secrete more VLDL but less phospholipid and cholesterol per mole triglyceride (Soler-Argilaga et al. 1975; Soler-Argilaga and Heimberg 1976). Moreover, the amount of VLDL output and the composition of LCFAs are different between sexes after an increase in oleate uptake. Men have lower high density lipoprotein (HDL) levels and smaller HDL size than women (Duvernoy et al. 1999; Pascot et al. 2002). Males have a higher risk in the atherogenesis possibly related to this aspect of the lipoproteins metabolism.

Sex differences in response to metabolic challenges

Post-absorptive state

In the post-absorptive state, the period following absorption of nutrients from the intestines, the plasma levels of metabolites are maintained in homeostasis as a total result of influxes and effluxes in different tissues. Although the plasma levels of most metabolites are similar between the sexes, males and females seem to have slightly different strategies to maintain homeostasis. After overnight fasting, plasma concentration of NEFA and ketone bodies are higher whereas plasma concentration of glucose and insulin are lower in women (Basu et al. 2006). In addition, the rate of VLDL production and secretion are higher in women, in conjunction with a higher VLDL clearance in women (Mittendorfer et al. 2003). At the same time, women utilize more fats and less carbohydrates and amino acids as energy source than men (Tipton 2001; Lamont 2005). These observations fit together and imply that women have a higher rate of VLDL turnover. When the metabolic state is challenged, sex differences sometimes become more apparent. As a major organ for metabolic

control, the liver plays an important role in this context. However, few studies have looked into the molecular responses in the liver.

Postprandial

Women have a higher postprandial plasma glucose level but lower plasma triglyceride level. Using a triple-tracer method, Basu and coworkers showed that women have a higher rate of meal appearance than men after a standard meal (Basu et al. 2006). Since plasma glucose uptake is related to muscle mass, a greater muscle mass in men might account for this difference. A higher postprandial insulin level might mitigate the higher rate of meal appearance. Women have a lower plasma triglyceride concentration than men (Couillard et al. 1999). One explanation can be that adipose lipolysis after a meal is more suppressed in women than in men, especially in the upper body subcutaneous region (Jensen 1995). In the adipose tissue, insulin stimulates lipoprotein lipase (thus lipid uptake) and inhibits hormone sensitive lipase (thus lipolysis). This indicates that women might have a higher insulin sensitivity to the antilipolytic effect. The fat depot is also sex dimorphic. As mentioned above, men store more dietary fat in the upper body whereas women more in the lower body, resulting in a sex-different body composition (Nguyen et al. 1996). The postprandial VLDL production is decreased in both men and women, although the basal VLDL production is higher in women than men (Mittendorfer 2005). Most of the dietary amino acids are either used in protein synthesis or oxidized for energy. Possible sex differences in amino acid metabolism are yet to be discovered.

Hypoglycemia

Insulin secretion can also induce hypoglycaemia. It then leads to counter-regulatory hormonal responses. A few studies report that men and male rats have increased epinephrine and glucagon levels after acute hypoglycaemia (Diamond et al. 1993a; Drake et al. 1998; Davis et al. 2000). With hypoglycaemia clamps of different concentration, Davis and colleagues showed that the degree of neuroendocrine response is higher in men. A lower concentration of glucose is required for women than men to give a certain increase in epinephrine and glucagon. Similar results were observed in rat (Diamond et al. 1993a) but opposite in mice (Karlsson et al. 2002).

Starvation

Both historical famine affecting human populations (such as the period post WWII in Germany and 19th century north-coast populations in British Colombia (Widdowson 1976; Hall 1978)) and animal studies have shown that females have a greater capacity to adapt to a negative energy balance (Hoyenga and Hoyenga 1982; Hill et al. 1985; Hill et al. 1986; Cortright and Koves 2000). Although the empirical data are somewhat incomplete, it seems as if females have a lower plasma glucose level but higher levels of plasma NEFA and ketone bodies during starvation. The lowering of plasma glucose in women is explained by a decrease in endogenous glucose production (Soeters et al. 2007), mainly from the liver. An increase in lipolysis from the adipose tissue might explain the rise in NEFA level in women. A kinetics study suggests otherwise. Women have a higher lipolytic rate at basal state and the

degree of increase during fasting is lower in women than in men (Mittendorfer et al. 2001). Anyhow, a high plasma NEFA level in women leads to higher rate of fatty acid β-oxidation, which in turn increases the formation of ketone bodies (Merimee et al. 1978). The ketone bodies will then be used for energy in the brain. The greater lipid catabolism in women lowers the need for energy from sources such as muscle protein, which could relate to women's lower amino acid efflux from muscle. Since protein is the only long-term source of glucose, increase in utilization of fats for energy will preserve lean mass better. This is clearly demonstrated in an animal study. Although male and female rats lose similar amount of body mass after a 6-day starvation, males have a greater percentage of body mass loss as protein than females (Widdowson 1976). In summary, females utilize more fatty acids and have lower dependence in carbohydrates and amino acids as energy source during starvation.

Regarding hepatic enzymatic activities, Saggerson and Carpenter showed that CPT activity is higher in male rats than female rats. After starvation, CPT activity increases in both sexes and the sex difference disappears, indicating a greater increase in female rats. Starvation decreases CPT's sensitivity to malonyl-CoA but no sex difference is found (Saggerson and Carpenter 1982).

Peroxisome proliferator-activated receptor (PPAR) α regulates the transcription of many enzymes involved in hepatic lipid catabolism. The mRNA and protein level of PPARα are higher in male rats than female (Jalouli et al. 2003). The level of PPARα increases further after fasting, but the degree of increase is similar between sexes. The male gonad, but not the sex dimorphic GH secretory pattern, regulates the sex-different expression of PPARα in the liver. Deficiency of PPARα increases the intracellular lipid content in the liver and hypoglycemia. Under starvation, *Pparα*-deficient mice have elevated hepatic TAG level and the level remained elevated after re-feeding (Sugden et al. 2002). Further inhibiting the mitochondrial fatty acid import, none of the male mice survived the 5-day starvation (Djouadi et al. 1998). However, only 25% of the female mice did not survive. Estrogen treatment rescued the *PPARα*-deficient males from this fatal challenge. Analysis of the plasma glucose level, demonstrated that male animals were unable to increase hepatic glucose production during starvation. The male animals suffered from hypoglycaemia for a prolonged period and died from it. An increase in lipid accumulation in the liver of knockout males suggests that fatty acids were not oxidized to provide energy to produce glucose. Apart from greater hepatic glucose production, female *Pparα*-null mice have higher hepatic secretion of TAG as well as higher serum TAG and apoB levels than male *Pparα*-null mice (Linden et al. 2001). This suggests that the hepatic lipid turnover is nearly abolished in male *Pparα*-null mice.

Endurance Exercise

The metabolic response of endurance exercise is similar to that of starvation. Women have a higher rate of lipolysis and fatty acid oxidation during exercise (Blaak 2001). Men have a higher rate of amino acid oxidation than women both in the resting state and during exercise. During aerobic exercise, around 55% of the energy comes from carbohydrates and amino acids in men, compared to 38% in women (Lamont 2005). The Tarnopolsky group has shown that there are sex

differences in substrate selection during endurance training studies in human. Women proportionally oxidize more lipids and less carbohydrates as compared to men (Tarnopolsky 2000a; Tarnopolsky and Ruby 2001). Although the level of glucose is lower in women than men at the post-absorptive state and post-exercise, there is no sex difference during exercise (Horton et al. 2006). While the resting muscle glycogen content does not show any sex difference, Tarnopolsky and colleagues found that women utilize less muscle glycogen than men during exercise (Tarnopolsky et al. 1990; Tarnopolsky 2000b). This difference can be traced to the liver. After two-hour treadmill running, female rats have lower hepatic glycogenolysis as well as gluconeogenesis than male counterparts and estrogen is believed to be the mediator (reviewed in (Tarnopolsky 2000b)). Interestingly, chronic exercise increases the body mass loss in male rats but not in female rats (Cortright and Koves 2000). In these male rats, fat mass decreased by 17% whereas protein mass by 50%, confirming the sex differences in substrate utilization preference during exercise.

Altogether, females differ remarkably from males in the response to metabolic challenges. There are apparent sex differences in substrate utilization and energy homeostasis. Though the plasma levels of some metabolites and hormones are similar between the sexes, males and females have different levels of glucose, fatty acids, lipoproteins and insulin in the circulation. In short, males and females do use different strategies to maintain their homeostasis.

Table 1. Summary of sex differences under different metabolic states

			Post-absorptive		Postprandial		Starvation/ Exercise	
Plasma Level	**Glucose**	♂ ♀	++ +	(a)	++++ +++++	(a)	+++ ++	(c, e)
	Insulin	♂ ♀	++ +	(a)	+++ ++++	(a)		
	NEFA	♂ ♀	++ +++	(a)	++++ +++	(a)	+ ++	(c, e)
	VLDL	♂ ♀	++ +++	(b)	+ +	(b)	+ +	(b)
	Ketone	♂ ♀	+ ++	(a)			++ +++	(f)
Utilization	**Glucose**	♂ ♀	+++ ++	(c)			++++ +++	(c)
	Amino acids	♂ ♀	+++ ++	(c)			++++ +++	(c)
	Fatty acids	♂ ♀	++ +++	(c)			+++ ++++	(g)

Post-absorptive = 2 hours after meal or overnight fasting, Postprandial = within 60 minutes after meal, Starvation = more than 36 hours without food intake, Exercise = aerobic, conditional training. References from a. (Basu et al. 2006) b. (Mittendorfer 2005) c. (Lamont 2005) d. (Cortright and Koves 2000) e. (Soeters et al. 2007) f. (Merimee et al. 1978) g. (Blaak 2001)

TRANSCRIPTIONAL REGULATION OF HEPATIC SEX-DIFFERENT GENE EXPRESSION

Sex differences in hepatic gene expression concerning drug and steroid metabolism have been studied extensively for more than 20 years (Zaphiropoulos et al. 1989). More recently, microarray screening experiments discovered sex-different hepatic gene expression in other metabolic pathways and suggested that GH secretion patterns is the main modulator of these sex-dependent transcripts (Tollet-Egnell et al. 2000; Gardmo et al. 2002; Ahluwalia et al. 2004). Several transcription factors (TFs) are identified as the mediators of GH-dependent transcriptional regulation leading to sex-specific gene expression in the liver (Gardmo and Mode 2006; Waxman and O'Connor 2006; Laz et al. 2007). Sex-related TFs also modulate the sex-different gene transcription in the liver independent of GH action.

GH-dependent transcriptional regulation

The most studied GH-dependent sex-specific genes in rat liver are the sex-predominant CYP genes. The proposed model of sex-dependent regulation of CYP genes includes four TFs: signal transducer and activator of transcription (STAT) 5b, hepatocyte nuclear factor (HNF) 4α, HNF6 and HNF3β. In male liver, STAT5b is activated by the episodic GH secretion pattern, which also activates HNF4 by an unknown mechanism. STAT5b and HNF4α then bind to the promoter of certain male-specific CYP genes and increase their expression. STAT5b binding sites have been found in the promoter of several male-specific CYP genes in rats, including *Cyp2c11*, *2a2* and *4a2*. In female liver, the expression of HNF6 and HNF3β is increased by the continuous GH secretion pattern. These two HNFs then bind to the promoter region of certain female-specific CYP genes, for example *Cyp2c12*, thereby increasing its transcription. HNF4α, which shows higher activity in male liver, suppresses the expression of HNF6 and HNF3β, which may in-turn contribute to the inhibition of female-specific CYP gene expression in male liver (reviewed in (Wiwi and Waxman 2004)). Some of the GH effect on intermediatory metabolism could be via HNF4 and HNF3β (FOXA2) as it has been shown that they regulate genes involved in glucose and lipid metabolism (Rhee et al. 2003) (Wolfrum et al. 2004; Sampath and Ntambi 2005).

Siganl transducer and activator of transcription 5

The STAT5 protein has two isoforms, STAT5a and STAT5b, which are encoded by two separate genes. Although more than 90% of the amino acid sequences are identical between these two isoforms, the regulation, activation and molecular function are different.

As presented in figure 2, STAT5 proteins are activated directly by the GH pulse in male rats (Choi and Waxman 2000a). In male rats, hepatic STAT5 activity presents a pulsatile pattern similar to the male GH secretion pattern. The STAT5 activity is much lower in female rat livers. It started when Waxman and associates observed the presence of tyrosine-phosphorylated STAT5b proteins in the hepatocyte nucleus from GH-pulse treated hypophysectomised rats (Waxman et al. 1995). Further studies showed that the activity of STAT5b is in phase with the GH secretion pattern

in male mice (Sueyoshi et al. 1999) and rats (Choi and Waxman 1999; Choi and Waxman 2000b; Tannenbaum et al. 2001). Experiments with cultured hepatocytes suggest that phosphotyrosine phosphatase(s) and proteasomal degradation are involved in the desensitization of STAT5 by continuous GH (Fernandez et al. 1998; Waxman and O'Connor 2006).

Recently, Clodfelter and co-workers reported that 23% of the female-predominant genes in mice liver require a normal expression of STAT5a and some of them are regulated by the GH secretion pattern (Clodfelter et al. 2007). The current paradigm becomes that STAT5a plays an important role in the female-specific gene regulation in the liver, whereas STAT5b in male-specific genes. Since both STAT5a and STAT5b can be activated by GH pulses, their activations are similar but not at all the same. And that slim difference seems to decide the sex-different gene expression pattern.

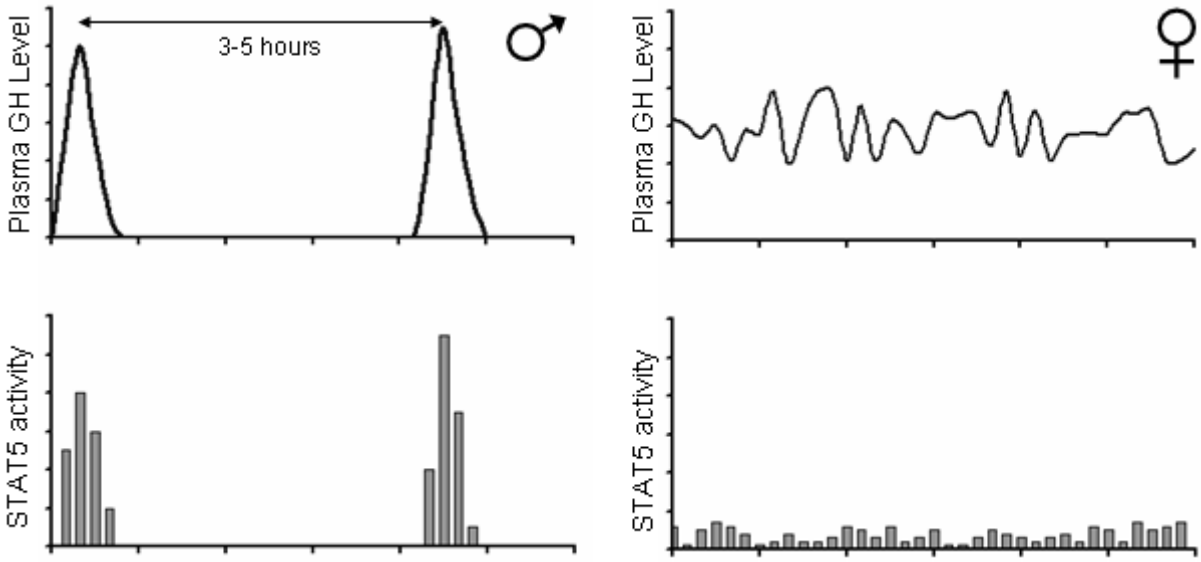

Figure 2. Activation of STAT5b by sex-specific GH secretion patterns
Left: The male-specific pulsatile GH secretion pattern induces repeated cycles of liver STAT5 activation by tyrosine phosphorylation. When GH levels are undetectable during the pulses, little or no active STAT5 is present in adult male rat livers. Right: Continuous presence of GH in the female rats leads to a low level of STAT5 activation in the liver. Adapted from (Waxman and O'Connor 2006).

STAT5-deficient animal models

Stat5b-deficient mice have reduced growth rates in males but not in females. They showed increased plasma GH levels, reduced insulin growth factor (IGF) 1 levels and obesity. Moreover, *Stat5b*-deficinet male mice lose the male-specific gene expression in the liver. The expression levels of male-specific genes are reduced to the levels observed in wild-type females, whereas the expression levels of female-specific genes are increased to the levels intermediate to that of wildtype male and female (Udy et al. 1997). *Stat5a*-deficient mice do not differ in size, weight or fertility from their wildtype littermates (Liu et al. 1997). However, they suffer impaired mammary gland development and female mice fail to lactate after parturition, resembling phenotypes of prolactin receptor-deficient mice (Ormandy et al. 1997). However, disruption of both Stat5 genes causes infertility in female mice. The presence of *Stat5b* in *Stat5a*-deficient mice might save them from infertility, showing a functional redundancy of the STAT5 proteins (Teglund et al. 1998).

Recently, our group has shown that STAT5b does not regulate the most rapid GH-mediated transcriptional effects in rat liver (Vidal et al. 2007). Another study on early GH response genes showed that sex-specific hepatic gene expression is regulated by GH both directly (early response) and indirectly (secondary response) (Wauthier and Waxman 2008). STAT5b is an important transactivator for GH-mediated transcriptional regulation, yet it is apparent that a part of GH-regulated genes rely on activation by TFs other than STAT5b. 61% of the female-specific genes are up-regulated in the *Stat5b*-deficient male mice liver, suggesting inhibitory actions of STAT5b on the female-specific genes (Clodfelter et al. 2006). STAT5b might be the direct regulator of some sex-different genes, including male-specific *Cyp2a2*, but STAT5b protein also regulates the sex-specific gene expression directly or indirectly by inhibiting other TFs.

GHR-JAK-STAT pathway

GH binds to the GH receptor (GHR) on the cell surface and promotes homodimerization of the receptor. It is believed that the binding of ligand induces conformational changes on the receptor, thus promoting the dimerization. This process is sequential, first GH binds to the first GHR, then the first GHR to the second GHR forming the GH-GHR complex (Fuh et al. 1992).

The transformational change in GHR leads to transphosphorylation of Janus kinase (JAK) 2 proteins. It was believed that JAK2 proteins are recruited to the GHR after dimerziation. However, Carter-Su and colleagues showed that JAK2 is constitutively associated with GHR as a JAK-GHR complex, and the ligand-binding stabilizes the complex (Carter-Su et al. 1996). Transphosphorylation of JAK2 activates the kinase domain and phosphorylates GHR at multiple tyrosine residues (Wang et al. 1996). Hence, multiple docking sites are available for Src-homology-2 (SH2) containing signaling molecules or phosphotyrosine binding motifs.

Through the SH2 domain, STAT proteins, especially STAT5a and STAT5b, are recruited to the JAK-GHR complex at the carboxyl terminal of the GHR, where JAK2 phosphorylates the tyrosine residues in STAT5a/b. When phosphorylated, STAT5 proteins dissociate from the complex and form homodimers through the reciprocal phosphotyrosine-SH2 interactions (Horvath 2000). Shortly after dimerization, STAT5 proteins enter the nucleus by facilitated transport such as via importin-α molecules (Levy and Darnell 2002). They then bind to their specific DNA response elements (Wood et al. 1995; Ehret et al. 2001) to regulate gene transcription. In addition to STAT5a/b, GHR activation also triggers multiple parallel signalling pathways, such as MAPK, AKR, PKC. However, the aspect of sex differences is not well-studied.

Suppressors of cytokine signaling

The SOCS family comprises of at least eight proteins, namely SOCS1 to SOCS7 and cytokine-inducible SH2 protein (CIS). They act as negative regulators of many cytokine-activated signaling pathways, including the GHR-JAK-STAT pathway. The central SH2 domain allows SOCS proteins them to interact with the activated receptors (Krebs and Hilton 2000).

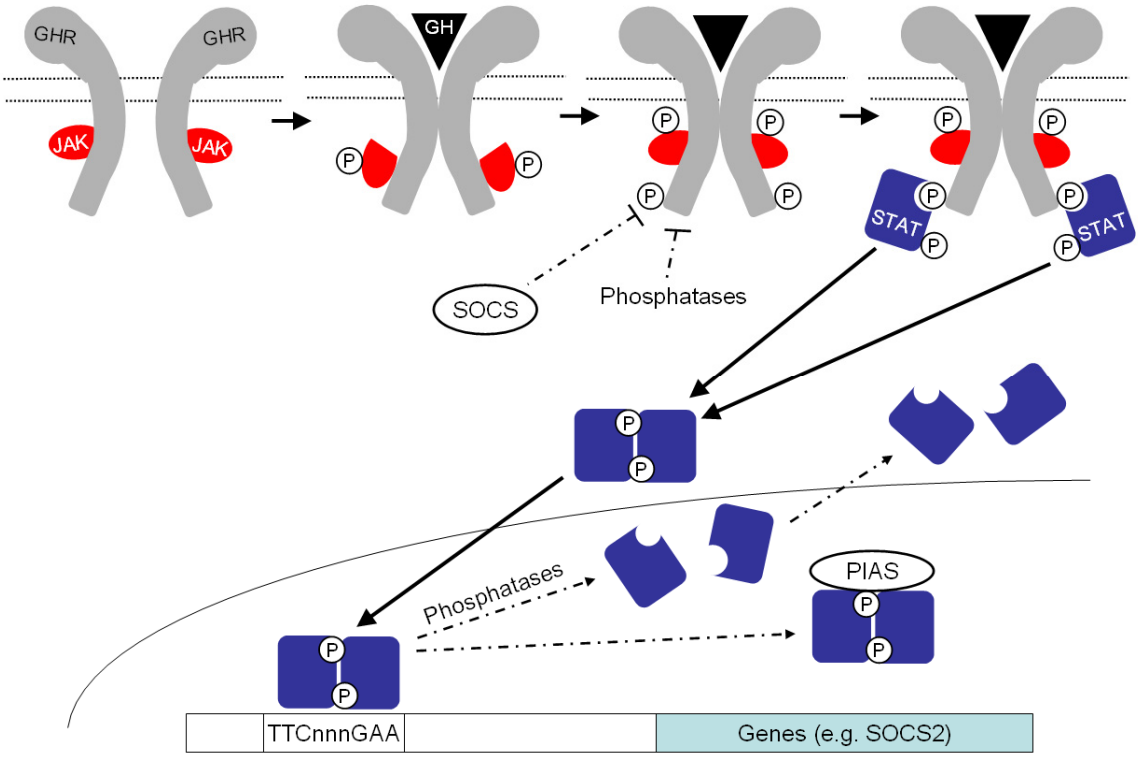

Figure 3. GHR-JAK-STAT pathway
The growth hormone receptor (GHR) dimerizes upon ligand-binding, in turn activates Janus kinases (JAK) for transphosphorylation, following phosphorylation of the receptor tail. Signal transducers and activators of transcription (STAT) proteins are recruited to the phosphorylated GHR through their Src-homology-2 domains and are tyrosine-phosphorylated by JAK. Phosphorylated STATs are activated and form homodimers. After nuclear entry, STAT dimers bind to DNA and regulate gene transcription, such as inducing SOCS2 expression (Vidal et al. 2007). Several mechanisms negatively regulate the STAT activity (dotted lines). Protein inhibitors of activated STATs (PIAS) inhibit the activated STAT dimers by binding. Phosphatases dephosphorylate nuclear STAT dimers and/or the activated GHR-JAK complex. Suppressor of cytokine signaling (SOCS) proteins blocks the activation of STAT close to the cell membrane. Adapted from (Levy and Darnell 2002) with modifications.

The concentration of SOCS proteins is constitutively low but increases rapidly by stimulation with different cytokines or growth factors. SOCS proteins interact with the GHR-JAK complex and down-regulates its signaling, thereby repressing STAT phosphorylation, dimerization, nuclear translocation and transcriptional activity (Flores-Morales et al. 2006; Rico-Bautista et al. 2006). SOCS1 binds with high affinity to JAK2 thereby preventing the binding of other substrates and inducing degradation (Ungureanu et al. 2002). SOCS2, SOCS3 and CIS exhibit GHR-binding activity. SOCS2 shows higher binding affinity to GHR and can competitively inhibit STAT5 binding to the receptor. The binding site of SOCS3 is on the proximal region of GHR and SOCS3 can also bind to JAK2 thereby inhibiting the kinase activity. CIS binds to the distal region of GHR, where STAT5 docking site is situated (Rowland et al. 2005). It suggests a CIS-mediated inhibition in JAK-STAT signal by competitive binding.

The binding of SOCS proteins to GHR or JAK2 leads to ubiquitination and proteasomal degradation (following GHR internalization) (Flores-Morales et al. 2006). However, the detailed mechanisms remain to be investigated. Results from

our group suggest that SOCS2 possesses E3 ubiquitin ligase function and is part of an E3 ubiquitin ligase complex (Greenhalgh et al. 2005). It is hypothesized that upon binding to GHR, SOCS2 also recruits other proteins for poly-ubiquitination such as E2 ubiquitin-conjugating enzymes and other E3 components. The complex then ubiquitinates the SOCS-associated GHR resulting in proteasomal degradation.

Genetically manipulated animals showed that SOCS2, not SOCS1 and SOCS3, is the main negative regulator of GHR signaling. *Socs2*-deficient mice exhibit gigantism without obesity. By crossing giants *Socs2*-/- mice with dwarf mice (mutated GH releasing hormone receptor $GHRHR^{lit/lit}$), results from our group showed that the *Socs2*-/- $GHRHR^{lit/lit}$ mice are more sensitive to GH administration and hepatic GH-dependent gene regulation (Greenhalgh et al. 2005). *Socs2*-/- mice show prolonged activation of STAT5b upon a pulse of GH treatment (Greenhalgh et al. 2002). Except down-regulation of MUPs, these effects do not translate into any major sex differences in hepatic gene expression. In short, SOCS2 is a crucial negative regulator of hepatic GHR-JAK-STAT signaling and GH-activated STAT5 transcriptional regulation.

Cross-talk with STAT5

When STAT5 binds to its response element on the promoters of GH-target genes, it stimulates the transcription of the gene. However, STAT5 can also interact with other TFs and inhibit their transcriptional activity. At the same time, other TFs have also shown to inhibit STAT5 transcriptional activity. STAT5 plays a complex role in modulating gene expression.

GH-activated STAT5b inhibits the trans-activation of HNF6 and HNF3β on the, promoter of Cyp2c12, a female-specific CYP gene (Delesque-Touchard et al. 2000). Yet on the promoter of apolipoprotein CIII, Stat5b synergistically enhances HNF4α activation (Park et al. 2006).

In HepG2 cells, STAT5b tyrosine phosphorylation can be blocked by HNF3β, thus inhibiting STAT5b transcriptional activity on *Cyp2c11* promoter (Park and Waxman 2001). Similarly, HNF4α inhibits STAT5b activity on the β-casein promoter, by blocking GH-dependent tyrosine-phosphorylation of JAK2 and subsequently STAT5b activation (Park et al. 2006).

Glucocorticoid receptor (GR) acts as a co-activator when STAT5 is bound to the promoters of STAT5-regulated genes such as β-casein in COS7 cells (Stoecklin et al. 1997). Engblom and colleagues showed that the GR acts as a co-activator to STAT5 by interacting with the N-terminus of STAT5 in the mouse liver (Engblom et al. 2007). On the other hand, STAT5 has been shown to inhibit GR's binding activity to GR-regulated genes (Stocklin et al. 1996; Biola et al. 2001).

PPARα expression level is inhibited by GH independent of the secretory pattern (Carlsson et al. 2001; Flores-Morales et al. 2001; Jalouli et al. 2003). This inhibition might be mediated by STAT5b since GH-activated STAT5b must be nuclear active in order to inhibit the AF1 transactivited PPARα transcription (Zhou and Waxman

1999a; Zhou and Waxman 1999b). Moreover, SOCS3 inhibition of STAT5b can abolish the suppression of PPARα expression (Zhou and Waxman 1999b).

Other TFs that interact with STAT5 affecting gene regulation include PPARγ, PPARδ (Zhou and Waxman 1999b), thyroid hormone receptor (Luo and Yu-Lee 2000), nuclear factor kappaB (Doppler et al. 2000), ERα (Faulds et al. 2001), cAMP response element binding protein (CREB)-binding protein (Gewinner et al. 2004) and Smads 2,3 and 4 (Cocolakis et al. 2008).

Hepatocyte nuclear factors

HNFs regulate many hepatocyte-specific genes via promoter and enchancer regions. Though none of them are entirely liver-specific, they are important in maintaining hepatocyte-specific gene expression. They include the POU-homeodomain-containing protein HNF1α, several winged helix HNF3s (α, β and γ), the orphan nuclear receptor HNF4α, the one-cut homeoprotein HNF6 and several of the basic region leucine zipper (bZIP) CAAT/enchancer binding proteins (C/EBPs) (Gonzalez and Lee 1996).

The importance of HNFs is demonstrated in the knockout mice models, among many die prematurely. *Hnf1α*-/- mice die during weaning due to a massive loss of glucose and amino acids via urine. *Hnf3β*-/- mice fail to undero gastrulation and die in utero by day 10. *Hnf4α*-/- animals also die in utero by day 6.5 due to impaired gastrulation. *Hnf6*-/- mice die postnatally from diabetes due to disruption of pancreas. *C/EBPα*-null animals suffer impaired glucose homeostasis. They die within 8 hours after birth from hypoglycemia and failure to store lipids (Costa RH 2001).

These liver TFs form an interactive network by direct, feed-back and auto-regulatory loops. Positive auto-regulation of HNF3β (FOXA2), HNF4 and C/EBPα and negative auto-regulation of HNF1α have been described previously (Rastegar et al. 2000). HNF6 can up-regulate the expression of *HNF4α* and *HNF3β* (Hayashi et al. 1999). Liver-specific deficiency of *HNF4α* in mice reveals that it up-regulates HNF1α, C/EBPα and C/EBPβ but down-regulates HNF3α and PPARγ coactivator-1α (Wiwi et al. 2004). HNF4α induces the expression of *HNF1α*, which in turn represses the expression of *HNF4α* (Wiwi and Waxman 2004). GH induces the expression of HNF3β, HNF4α, HNF6 and C/EBPα (Eleswarapu and Jiang 2005). In response to GH, the HNF6 expression is up-regulated by Stat5 and HNF4 (Lahuna et al. 2000). C/EBPα represses the HNF6 expression by interacting with the HNF6 promoter, an effect that disappear after a GH injection (Rastegar et al. 2000).

HNF4α (NR2A1) has two zinc finger motifs that are involved in protein-protein and protein-DNA interactions, allowing it to bind to DNA as a homodimer. Fatty acyl-CoA thioesters are the HNF4α ligands and induces the transcriptional activation (Hertz et al. 1998). In other cases, HNF4α can be transactivated without exogenous ligand. HNF4α regulates many important hepatic enzymes involved in fatty acid, cholesterol and glucose metabolism, urea and apolipoprotein synthesis. Although the relationship between GH signalling and HNF4α is largely unknown, its ability to positively regulate male-predominant genes and negatively regulate female-

predominant genes suggests that HNF4α is involved in sex-related, especially masculine, transcription regulation in the liver.

The expression level and the DNA binding activity of HNF6 are 2 to 3-fold higher in female than male (Lahuna et al. 1997; Wiwi et al. 2004). GH increases the HNF6 level by transactivating STAT5 and HNF4 (Lahuna et al. 2000) and by repressing C/EBPα inhibition (Rastegar et al. 2000). Together with HNF3β, it synergistically activates the expression level of female-specific *Cyp2c12*. These imply that HNF6 might be involved in the transcriptional regulation of hepatic female-predominant genes.

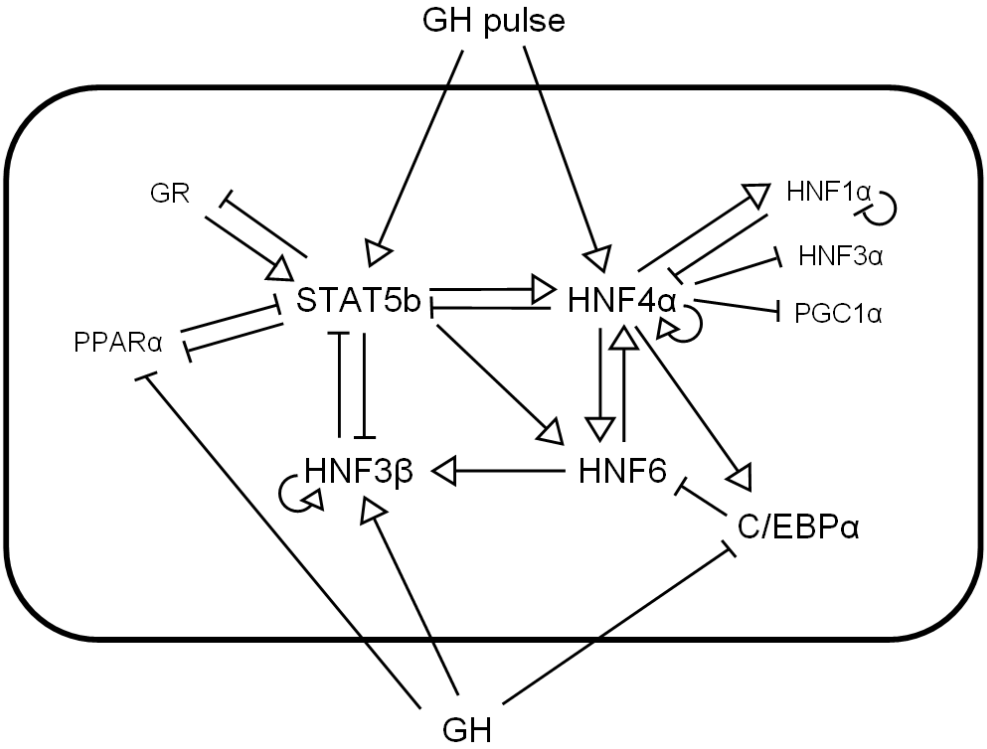

Figure 4. The interactive network of STAT5b and hepatocyte nuclear factors (HNFs) in liver cells.

Sex limited protein (Slp) and Regulator of Slp (Rsl)

Slp is expressed predominantly in liver. Testosterone up-regulates the hepatic expression of *Slp* via the pituitary GH pulsatile secretion (Georgatsou et al. 1993). Phosphorylated STAT5 protein are found on the enchancer region upstream of the Slp gene, suggesting that intermittent GH might increase *Slp* expression via STAT5 (Varin-Blank et al. 1998).

Rsl suppresses the expression of *Slp* independently of GH and androgen (Tullis et al. 2003). Expression of *Slp* in homozygous recessive-rsl female mouse livers is increased, together with male-specific mouse MUPs and *Cyp2d9*. However, female-predominant *Cyp2a4* expression is unchanged in both homozygous male and female, suggesting that Slp would be an important regulator of male-specific gene expression. One possible mechanism of Rsl inhibition is chromatin remodeling. The promoter of *Cyp2d9* is more extensively demethylated in male mice than in female. GA-binding protein (GABP) and Slp bind specifically to the demethylated sites, thereby transactivating the gene (Yokomori et al. 1995a; Yokomori et al. 1995b).

Peroxisome proliferators-activated receptor α

PPARα is a nuclear receptor, NR1C1, whose ligands include long-chain unsaturated fatty acids and their derivates. It plays an important role in lipid homeostasis, in particular fatty acid oxidation and lipoprotein metabolism. Its expression level is high in liver, heart and skeletal muscle, where fatty acid oxidative activity is high.

Gonadal steroids and GH affect PPARα expression and protein level in liver (Jalouli et al. 2003). Male rat livers contain more PPARα than females. Estrogen administration to male rats or castration decreases hepatic PPARα expression. Testosterone administration to female or ovariectomy increases the expression. The mode of GH administration regulates peroxisomal β-oxidation (Sugiyama et al. 1994). However, GH regulates the level of hepatic PPARα independent of its sex dimorphic secretion pattern (Jalouli et al. 2003). GH injection to female hypophysectomized rats decreases PPARα expression. In addition, continuous GH administration to male rats decreases *Cyp2c11* mRNA level as expected but it does not alter *PPARα* level. Fasting positively regulates the expression of PPARα with no sex difference.

Other GH-related transcription factors

The expression and transcription activity of oncogenes c-fos and c-jun are higher in male rat livers than in female. Males treated with continuous GH resemble the levels found in females (Hallstrom et al. 1989).

Recently, three female-predominant TFs are identified from mouse and rat liver. They are thymus high-mobility group box protein (Tox), tripartite motif-containing 24 (Trim24)/transcription initiation factor-1α (TIF1α) and cut-like 2 (Cutl2)/cut homeobox 2 (Cux2). Cutl2 and Trim24 are GH-regulated whereas Tox is less responsive to the female secretory pattern of GH. The level of Cutl2, Trim24 and Tox are up-regulated in Stat5-deficient male mice and *Hnf4α*-deficient male mice. Cutl2 and Trim24 exhibit repressor activity and putative binding sites of Cutl1, which has similar DNA-binding specificity as Cutl2, are overrepresented in Stat5b-dependent male-specific mouse gene promoters (Laz et al. 2007).

Sex steroid-related transcriptional regulation

Androgen Receptor (AR)

AR, also known as NR3C4, was isolated from the rat liver and studied by different research groups (Gustafsson et al. 1975; Levinson and Decker 1985). The level of AR in liver is similar to those in classical androgen target tissues (Eisenfeld and Aten 1987). Maturation and aging affects the hepatic level of AR, suggesting a possible regulaiory role by gonadal steroids and GH. AR binds to androgens, becomes activated, translocates to the nucleus and dimerizes. The activated AR regulates gene transcription either by direct binding to the hormone response element on target gene promoters or by interacting with other TFs (Heinlein and Chang 2002). In HepG2 cells, estrogen via ER induces the transcription of LDL receptor (LDLR) but AR antagonizes this effect (Croston et al. 1997).

Estrogen receptors (ER)

There are two specific estrogen receptors, ERα (NR3A1) and ERβ (NR3A2), which have different tissue distribution. ERα is mainly expressed in the uterus, liver, kidney and heart where ERβ in ovary, prostate, lung, gastroinstestinal tract, bladder and hematopeietic and central nervous system (Matthews and Gustafsson 2003). Though ERα and ERβ have similar binding-affinity to endogenous estorgen (17β-estradiol), the receptors bind differently to some phytoestrogens and other novel ligands (Kuiper et al. 1998; Sun et al. 1999). Moreover, synthetic estrogens tamoxifen and raloxifene trigger ERα partially but are antagonists for ERβ (Barkhem et al. 1998).

The expression of ERα is auto-regulated by estrogen. Pituitary hormones are also important for the normal expression of ERα. Hypophysectomy abolishes hepatic ER which can be restored by administration of GH, prolaction and glucocorticoids. The promoter of ERβ has been studied and two alternative transcripts of ERβ are expressed. However, the regulatory mechanism remains unclear. (Reviewed in (Lax 1987; Zhao et al. 2008)).

Knockout animal studies showed that ERβ antagonized ERα when both receptors are co-expressed. In the absence of ERα, ERβ replaces ERα in the regulation of about one-third of estrogen responsive genes in the liver (Lindberg et al. 2003). ERα-null female mice suffer from impaired glucose homeostasis and insulin insensitivity whereas ERβ-null animals are normal (Bryzgalova et al. 2006). In the liver, abolishment of ERα causes up-regulation in lipid biosynthesis and down-regulation in lipid transport. The hepatic expression of leptin receptor is lower in the ERα-null mice, although they exhibit lower plasma leptin level and higher adiponectin level. ERα plays an important role in hepatic insulin sensitivity and glucose and lipid homeostasis (Bryzgalova et al. 2006).

In summary, GH is the most influential factor of the sex-differentiated gene expression pattern in the liver, with STAT5 mediating most of the GH's regulatory effects. Several studies show that hepatic TFs form interactive networks and the cross-talk "traffic" is as busy as the signaling pathways. STAT5b affects the transcription and/or transactivation of many TFs that are regulated by hormonal and nutritional status. In addition, novel GH-regulated and sex-different TFs are recently discovered, suggesting that the GH-related regulatory network is extensive and complex.

POST-TRANSCRIPTIONAL REGULATION BY MICRORNA

Recent research has uncovered the importance of small untranslated RNA, called microRNA (miRNA). miRNAs are short RNAs of 19-25 base pairs in length. In the latest release of miRBase (11.0, April 2008), a sequence database of miRNA hosted by the Sanger Institute (http://microrna.sanger.ac.uk/) (Griffiths-Jones et al. 2008), there are 678 identified and known miRNAs in human, 472 in mouse and 287 in rat. Most of the well-defined miRNAs are highly conserved across species, suggesting their importance in cellular regulation.

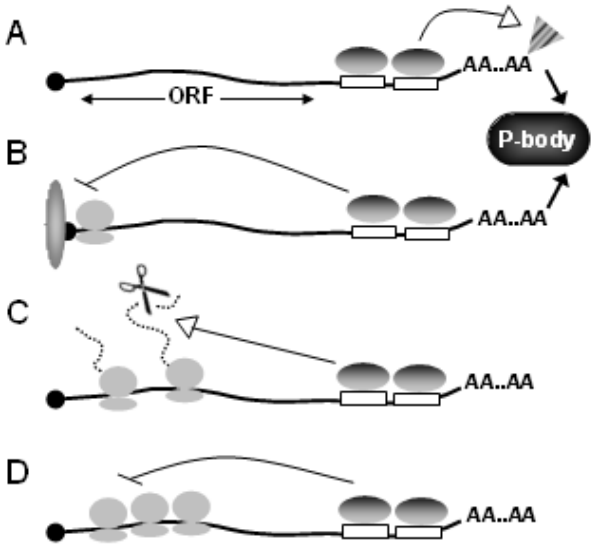

Figure 5. Suggested mechanisms of the microRNA-mediated post-transcriptional gene repression. Adapted from (Filipowicz et al. 2008).

These small RNAs are processed from precursor molecules that are either transcribed as ordinary genes (pri-miRNA) or generated as by-products from splicing. The maturation takes place in the cytoplasm when the miRNAs are assembled into miRNA-ribonuclear protein complexes, which in turn selectively bind to a target mRNA and modulate its translatability. At least five possible mechanisms for this miRNA-dependent regulation of translation have been suggested (Shyu et al. 2008). If the sequences of the miRNA and the target mRNA are perfectly complementary to each other, it will trigger endonucleic cleavage of the target mRNA. Otherwise, as illustrated in figure 5, imperfect match between the miRNA and the target mRNA will instead lead to either (A) deadenylation of the poly(A) tail, or (B) inhibition of the assembly of ribosomal complex required for translation initiation, or (C) degradation of the most recently translated peptides, or (D) reduction in the speed of elongation. Deadenylation or failure to assemble ribosomal complex will cause mRNA to move to P-bodies for storage or degradation (Filipowicz et al. 2008). Regardless of their mechanisms of action, miRNAs are significant controllers in gene regulation by negatively affecting mRNA expression and translation.

Some miRNAs are ubiquitous, such as let-7b and miR-22 (Tang et al. 2007), while others are highly tissue specific. miR-1a is heart-specific and miR-124a is exclusively found in nervous tissues such as brain and spinal cord (Lagos-Quintana et al. 2002; Tang et al. 2007). miR-122a is abundant in liver, where it contributes with 70% of the total pool of hepatic miRNA. Studies have shown that miR-122a is a regulator of hepatic lipid metabolism, since inhibition of this miRNA lowers the serum cholesterol levels in normal mice and mice with diet-induced obesity. At the cellular level, miR-122a increases hepatic fatty acid oxidation and decreases lipogenesis (Esau et al. 2006). Although the molecular mechanisms behind these effects are still unclear, these findings have paved the way for a new concept within the field of metabolic research.

INTRODUCTION TO FATTY ACID TRANSLOCASE/CD36

Fatty acid translocase/CD36 (FAT/CD36) is a cell-surface glycoprotein involved in various metabolic pathways. The protein is also known as scavenger receptor class B, member 3 (Scarb3) but it should not to be confused with scavenger receptor class B, member 1 (SR-B1) (Zhang et al. 2003). FAT/CD36 is found in various tissues, including liver, adipose tissue, muscle, monocytes/ macrophages, endothelial cells, retina, brain and tongue (reviewed in (Febbraio and Silverstein 2007)). The molecular weight ranges from 78 to 88 kDa, depending on the cell type and glycosylation.

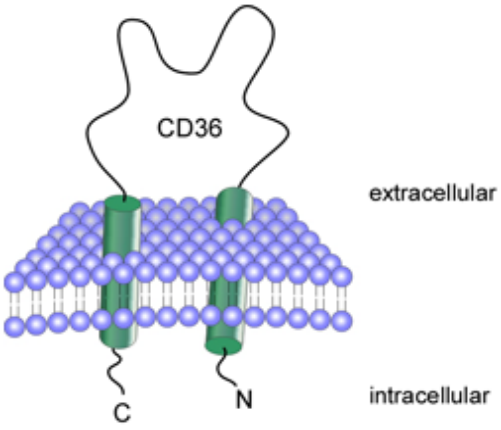

Figure 6. The protein structure of fatty acid translocase/CD36 protein (Courtesy of Victor Drover)

Function of FAT/CD36

It was long believed that fatty acid transport across the cell membrane was independent on any membrane protein. Although it is biochemically feasible, evidences showed that fatty acid uptake in cells is indeed facilitated (Abumrad et al. 1981; Abumrad et al. 1984). After more than a decade, a translocase protein, FAT/CD36, was first cloned (Abumrad et al. 1993). The main ligands of FAT/CD36 are long chain fatty acids. Unlike other fatty acid binding proteins, modified lipids and apoptotic cells can also be bound to FAT/CD36 (Coburn et al. 2001; Ibrahimi and Abumrad 2002).

Genetically manipulated animals revealed that FAT/CD36 plays a role in fuel metabolism and homeostasis (reviewed in (Febbraio et al. 2002)). FAT/CD36 is found to be one of the mutated genes in the spontaneously hypertensive rat (SHR), an animal model containing symptoms of human metabolic disease (Aitman et al. 1999; Pravenec et al. 2004). Overexpression of FAT/CD36 improves the insulin sensitivity and blood lipids (Aitman et al. 1999; Pravenec et al. 2003). In mice, FAT/CD36 deficiency does not directly impair insulin sensitivity. The muscle insulin sensitivity is increased. However, these animals develop hepatic insulin resistance (Goudriaan et al. 2003). When challenged with a high-fat diet, both wild-type and CD36-null mice develop glucose intolerance. However, *Cd36*-null mice but not wild-type develop insulin resistance under a high-fructose diet (Hajri et al. 2002). A recent report described that diet-induced obesity causes elevated hepatic triglyceride storage and secretion and increases the FAT/CD36 level in the mouse liver. Overexpression of hepatic FAT/CD36 mimics this impaired hepatic lipid handling (Koonen et al. 2007). Since the expression level of hepatic FAT/CD36 varies depending on sex and genetic background (Zhang et al. 2003), it might play an important role in energy homeostasis and pathogenesis.

Studies in muscle also showed that FAT/CD36 facilitates stransport of long-chain fatty acid to mitochondria (Campbell et al. 2004; Holloway et al. 2006). Although its

level correlates to fatty acid oxidative potential (Bonen et al. 1998; Bonen et al. 1999; Coburn et al. 2000), FAT/CD36 does not affect the mitochondrial oxidation directly (Holloway et al. 2007; King et al. 2007).

In human, FAT/CD36 deficiency is found in Asian and African population. The absence of a functional FAT/CD36 can predispose to metabolic syndrome (Hirano et al. 2003; Yamashita et al. 2007). Moreover, several common polymorphisms in *FAT/CD36* gene's non-coding region have been linked to insulin resistance, diabetes, and atherosclerosis (Lepretre et al. 2004a; Lepretre et al. 2004b; Love-Gregory et al. 2008).

Gene structure of *FAT/CD36*

FAT/CD36 is located in human chromosome 7 q11.2, mouse chromosome 5 A3 and rat chromosome 4 q11. It consists of 15 exons, of which exon 3 to exon 14 are translated (Armesilla and Vega 1994). Transmembrane domains are found in exon 3 and exon 14. Extracellular domains can bind to long chain fatty acid, thrombospondin, collagen, oxidized LDL and apoptotic cells (Rac et al. 2007).

Alternative splicing of *FAT/CD36* takes place in both translated and untranslated region, generating many splice variants or isoforms. Exon 4 and 5 are excluded in one of the splice variants in human (Tang et al. 1994) and exon 4, 9 and 11 are skipped in CD36-deficient patients (Hanawa et al. 2002). Alternative first exons are identified in both human and rodents. Sequencing information revealed that there are at least 5 alternative first exons in human (Kuriki et al. 2002; Andersen et al. 2006) and 3 in mouse tissues (Sato et al. 2002; Sato et al. 2007). Andersen and colleagues also showed that the expression level of alternative first exons varies in different tissue.

Transcriptional regulation of *FAT/CD36*

Despite the apparent importance of FAT/CD36 in metabolic diseases, few studies were performed to investigate the transcription regulation of *FAT/CD36*. Before the identification of the alternative first exons, Motojima and co-workers reported that PPARα agonist increases *FAT/CD36* mRNA level in liver and intestine whereas PPARγ agonist is more potent than PPARα's in raising the mRNA level in adipose tissue (Motojima et al. 1998). PPAR response elements in the gene promoter have also been located (Teboul et al. 2001). After the alternative first exons have been sequenced, the Japanese team found that different alternative promoters have different degree of responsiveness to PPARα and γ activators (Sato et al. 2002; Sato et al. 2007). Interestingly, FAT/CD36 mediated fatty acid uptake increases the expression level of PPARα (Drover and Abumrad 2005), suggesting a possible feed-forward loop. Recent studies discovered that FOXA2, Nur77, PXR, C/EBP and LXR also affect the expression level of FAT/CD36 (Wolfrum et al. 2004; Maxwell et al. 2005; Zhou et al. 2006; Qiao et al. 2008; Zhou et al. 2008).

AIMS

The overall aim of this thesis was to extend the knowledge of sex differences in hepatic fuel metabolism at the molecular level using high-throughput technologies.

The specific aims for each paper were:

- To identify genes involved in hepatic fuel metabolism with a sex-differentiated expression and to determine which of these might be regulated by the sex-specific secretion of GH.

- To extend our knowledge about regulatory mechanisms behind a sex-differentiated gene, *FAT/CD36*. Since alternative CD36 transcripts have been described in mouse and human tissues, we investigated whether there are alternative transcription start sites in rat, and whether they might be involved in FAT/CD36 gene regulation by sex, GH and nutrient availability.

- To examine how the male and female rat livers respond differently to a change in nutrient availability or to insulin treatment.

- To screen for sex-differentiated miRNA in the liver and whether they are regulated by hormones or nutrient availability.

- To test the possibility that metabolomics can be used to translate changes in hepatic gene expression into changes in the pattern of low molecular compounds in the circulation.

METHODOLOGIES

The general strategy in this thesis is screening through transcript profiling together with further investigations of specific genes of interest. Norwegian rats (*Rattus norvegius*) were chosen to be the animal model for these studies due to their renowned sex difference in growth and liver metabolism.

In paper I, we generated hepatic transcript profiles for male, female and male rats treated with GH administrated by mini-pump. The results were confirmed using solution hybridization assay and real-time quantitative polymerase chain reaction (PCR). Further studies on FAT/CD36 were performed and described in paper II. In paper III, sex differences in insulin-regulated genes were mapped using transcript profiling and confirmed with quantitative real-time PCR. Downstream physiological changes were thereafter examined with relevant methodologies.

In order to further explore the molecular differences between males and females, high throughput screening of miRNA in liver and metabolites in the serum were performed. These types of studies are often referred as omics research, which will be further discussed below.

OMICS RESEARCH

The development of high-throughput 'omics' technologies has promised a dramatic advancement in our understanding of biology. Massive experimental data are generated using these technologies. Therefore bioinformatics tools are needed to analyze and integrate the data from different experiments and from different sources.

Omics technologies are applied to understand biology at different levels. These are named according to the studied subjects – genomics, transcriptomics, proteomics, metabolomics and beyond (Figure 7).

At the DNA level, genomics research investigates changes in chromosomal structure and gene sequences which may influence gene expression or activity. Examples are 1) the assessment of chromosomal activation or inactivation by imprinting, development stages and disease-induced chromosomal methylation; 2) chromosomal alteration in cancer; and 3) on the DNA associated disease risk such as single nucleotide polymorphisms (SNPs). The most common technologies in genomic research are DNA sequencing, comparative genome hybridization using in situ hybridization, genotyping and/or microarray.

At the RNA level, transcriptomics research study the changes in expression levels of, messenger RNA (mRNA), miRNA and other RNA molecules. With the increasing availability of microarray, almost all types of experiments involving changes in expression levels would benefit from the high-throughput data collection. The most common technologies used in transcriptomics are microarray and real-time PCR based screening.

At the protein level, proteomics consists of research on protein expression and post-translational modifications. Analyses can focus on certain fraction of the cell such as membrane-bound proteins or focus on certain protein structure such as hydrophobic chains. In addition, certain post-translational modification such as phosphorylation and ubiquitination can be studied. The most common technologies are two-dimensional gel electrophoresis and liquid chromatography-mass spectrometry detection and protein sequencing.

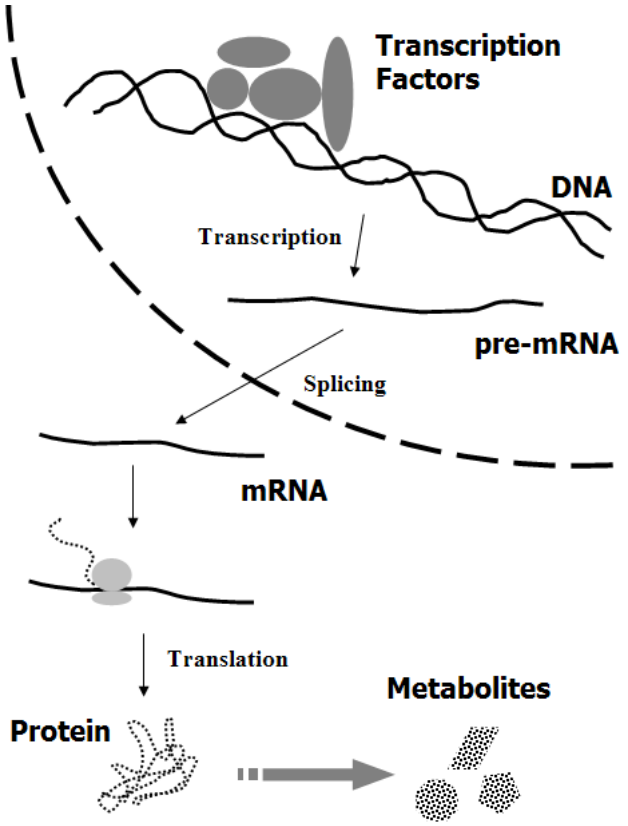

Figure 7. The central dogma of molecular biology

Metabolomics involves studies on the composition and concentrations of metabolites in different biological systems, such as body fluids, tissues or organisms. This method is widely used in plant biology in order to identify novel plant hormones. In medical science, it is commonly used in toxicity research as well as in pharmacodynamic studies. The most common technologies are gas or liquid chromatography-time of flight (TOF)-mass spectrometry (MS) detection, coupled to metabolite identification.

In this thesis, transcriptomics and metabolomics was used in studying the molecular difference between male and female livers. Further explanation and discussion around these two technologies will follow.

TRANSCRIPTOMICS

Transcript profiling is often performed with microarray, which are commonly commercially available nowadays.

Platform

Different types of microarrays are usually referred as platforms. The base material used has developed from nitrocellulose membrane to glass slides. Probes on the microarray have been derived from complementary DNA (cDNA) clone or chemically synthesized oligonucleotides. Most of the current available platforms are glass slides with printed oligonucleotides or oligonucleotides generated by on-chip synthesis.

Our group has developed the techniques of generation and analysis of microarrays in the past years. We have used printed microarrays due to their readiness and flexibility.

In paper I, in-house cDNA microarrays representing 3200 genes were used to screen sex differences in the liver. In paper III, we have increased the number of genes to approximately 27,000 with the use of Operon rat oligo library. The oligo microarrays were generated by the Department of Biotechnology, Royal Institute of Technology Sweden. In paper IV, miRCURY^{TM} LNA (Locked Nucleic Acid) microRNA Arrays (208000V8.0) from Exiqon (Danmark) representing 500 miRNA were used to screen sex differences in hepatic miRNA population.

Labelling and hybridization

The labeling and hybridization of microarrays are specifically designed for the different types of analysis and/or for the types of platforms. RNA samples are labeled with fluorescent-incorporated nucleotide (cyanine 5 or cyanine 3) during reverse transcription. The labeled samples are then hybridized to the specific probes on the microarray.

In the early years, background noises on the arrays were high and specificities of probes were not always satisfactory. RNA samples from each group were pooled, labeled and hybridized to microarrays. In order to minimize false positives, as well as the potential dye preferences, the hybridization were repeated with the same pooled sample but labeled with a dye-swap.

In paper I, equal amounts of total RNA from five animals within in the same experimental group were pooled before cDNA labelling (Figure 8a). Although pooling of samples might lead to the signals being confounded by mixed individuals, it also minimizes the biological noise. This method is criticized since it produces technological replica that ignore the individual differences between samples (Churchill 2002).

As the quality and the analysis technique of microarray have improved, technological replica is no longer needed to assure quality of results. Individual RNA samples are labeled and hybridized to microarrays using several designs

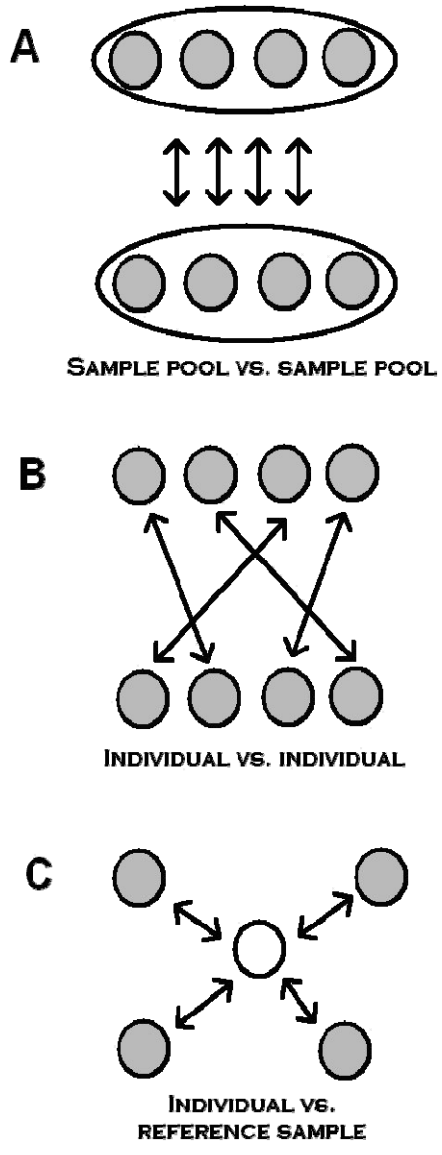

Figure 8. Different designs for microarray experiment

(Figure 8b). These biological replicas represent the individual differences, which have higher experimental values. The results are analyzed with improved statistical methods in order to minimize false positives. If the experiment compares two groups, sample-to-sample hybridization is a direct and cost-effective method. Dye-swapping should be used to minimize the possible dye bias. If more than two groups will be compared to each other, a group can be determined as reference, those RNA will be labeled by one color whereas RNA from other studied groups is labeled by another color. Alternatively, an external control group can be used. Then all studied groups are labeled in another color and hybridized against the external control (Figure 8c). This method is useful for studies involving time course, dosage response and multiple drug treatments. However, it requires more microarrays and thereby increasing the cost.

In paper III, we studied the insulin response in male and female rats and in paper IV, we studied the sex differences in miRNA levels. Therefore, sample-to-sample hybridizations were used. In the preliminary data, male was set as the control group. RNA samples from female and estrogen-treated male livers were labelled and hybridized to labelled RNA samples from male.

Data analysis

Each experiment was performed in at least triplicate. The signals were detected and quantified using GenePix software. Then the signals were normalized with locally weighted scatterplot smoother (LOWESS) algorithm. A data-point would be neglected if it was detected in less than 75% of the hybridizations. Data-points with more than 75% presence were considered to be a consistently expressed transcript. In most cases we used four microarrays per comparison, i.e. detectable signals in three out of four hybridizations. The significance of the expression ratios were estimated using the significance analysis of microarrays (SAM) statistical technique developed at the Standford University (Tusher et al. 2001; Zhang 2007). Missing value would cause error in the algorithm of SAM. Therefore if there is a missing value, it will be replaced by an average value from the same data-point in other hybridizations. After the analysis, a q-value was assigned for each expressed transcript in the array. Q-value represents the percentage of probability for a false positive, similar to p-value. Normally, transcripts with a q-value less than 5% were considered significantly differentially expressed. In paper III, q-value cut-off was set at 10% in order to capture all potentially sex-different miRNAs. A value of 1.5 was chosen to denote differences (increased or decreased expression) in the level of hybridization between control and experimental sample in all the studies. This cut-off was empirically chosen based on previous validation studies using different independent techniques to assess the changes in gene expression.

Real-time quantitative PCR

In order to confirm the results from microarray analyses, real-time quantitative PCR is the most commonly used method today. Northern blot was previously the main method but it is less sensitive and less quantitative. Solution hybridization is a quantitative technique but its sensitivity is not desirable for transcripts at low level. Real-time PCR technique amplifies transcripts hence increasing sensitivity for low

level transcripts. Real-time PCR array, a screening technique based on real-time PCR assays, has recently been developed. It provides higher sensitivity than microarray but less number of total genes can be studied.

In short, cDNA are reverse-transcribed from the sample RNA, and then gene-specific PCR reactions are performed. With the use of fluorescent molecules, each cycle of replication can be monitored by sensors.

There are two approaches in real-time quantification (illustrated in Figure 9). The TaqMan™ technique uses a specially designed gene-specific probe which has a flourophore on one end and a quencher on the other end. Before the PCR amplification, the probes bind to the target gene and the close proximity of flourophore and quencher on an intact probe will not give off any signal. Upon amplification of the specific gene, the polymerase will digest the bound probe by 3'-to'5' exonuclease activity and the fluorophore will become detected by its specific wavelength. The more cDNA copies, the more fluorophore are released resulting in a higher signal. If multiple probes with distinct fluorophores are used, the level of multiple genes at the same time can be detected (multiplex reaction).

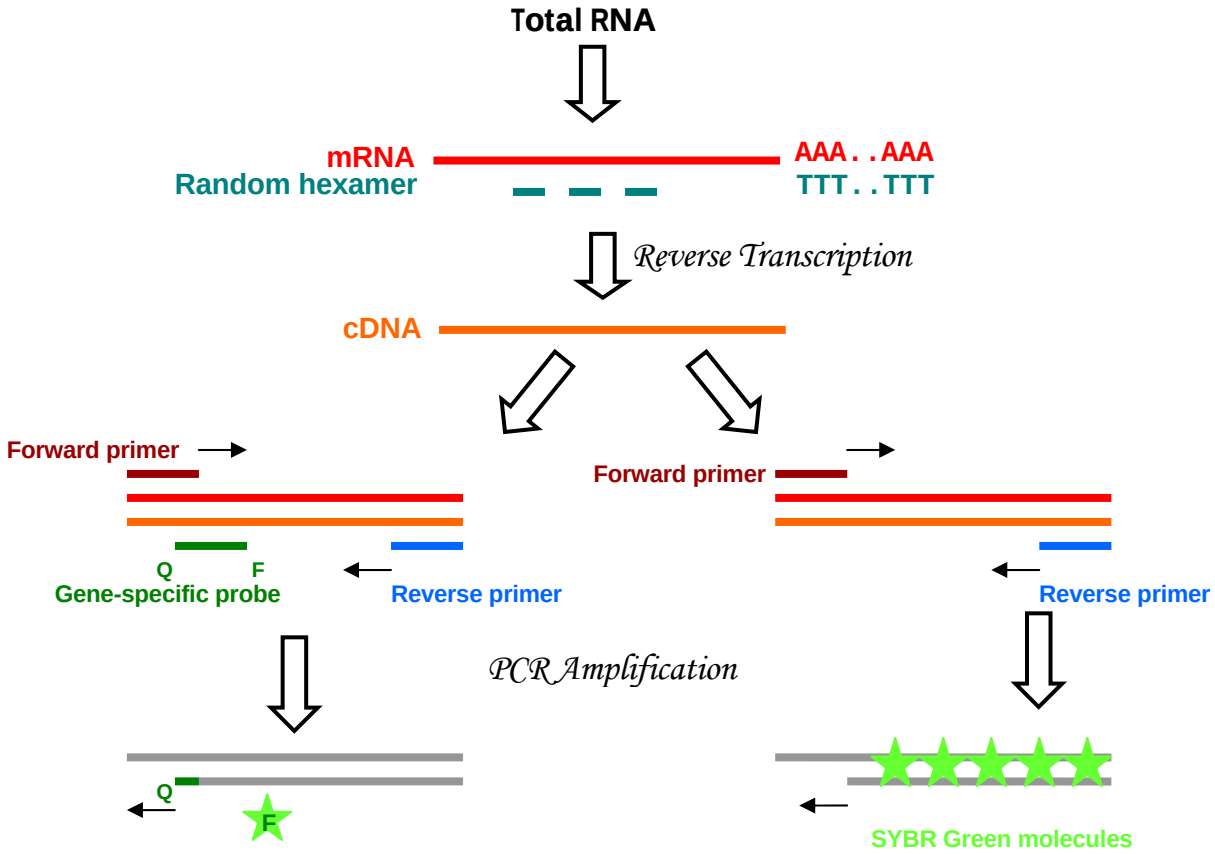

Figure 9. Two approaches in quantitative real-time PCR
cDNA are reverse transcribed from total RNA extracted from cells or tissues, with random hexamer and/or poly oligo d(T). Left: The TaqMan™ technique uses a specially designed gene-specific probe which has a flourophore (F) on one end and a quencher (Q) on the other end. Close proximity of flourophore and quencher prevents signal emission. Upon amplification, the bound probe is digested by the 3'-to'5' exonuclease activity if the polymerase. Fluorophores are released and can be detected by specific wavelength. Right: During PCR amplification, double-stranded DNA is produced. SYBR® Green binds to the product double stranded DNA, making it detectable.

SYBR® Green is a molecule that binds to double stranded DNA. During PCR amplification, double-stranded DNA is produced. Upon binding with SYBR® Green, the products can be detected. The product length also affects the strength of the signal and the longer the product is, the stronger the signal will be. However, multiplex reaction cannot be performed with this technique since the molecule binds to all double-stranded PCR products.

Real-time PCR performed in this thesis used SYBR® Green technique. In paper II, exon-specific primers for FAT/CD36 were used in order to monitor the alternative first exon usage. In order to monitor the primer efficiencies, relative standard curves were constructed using serial dilutions of pooled cDNA representing all individual hepatic RNA samples.

The transcript level was determined by the cycle threshold, at which the amplification rate is switched from lagging phase to the exponential phase. The detected levels were normalized with the level of the corresponding reference gene in each sample, and the obtained data were analyzed using Student's T-test.

The detection of miRNA requires another reverse transcription approach due to its short length. Biotech companies have developed different methods for miRNA reverse transcription with high specificity (Figure 10). Applied Biosystems's assays use miRNA-specific primers with a 5'-loop structure which increases the stability of primer hybridization. QIAGen's approach is to synthesize poly(A) tail in vitro, followed by normal reverse transcription using random hexamer and/or oligo-d(T). Exiqon's technique uses LNA-primers which bind to the specific miRNA with high stability and high specificity in shorter length. In paper III, the real-time PCR reactions were performed with Exiqon's assays.

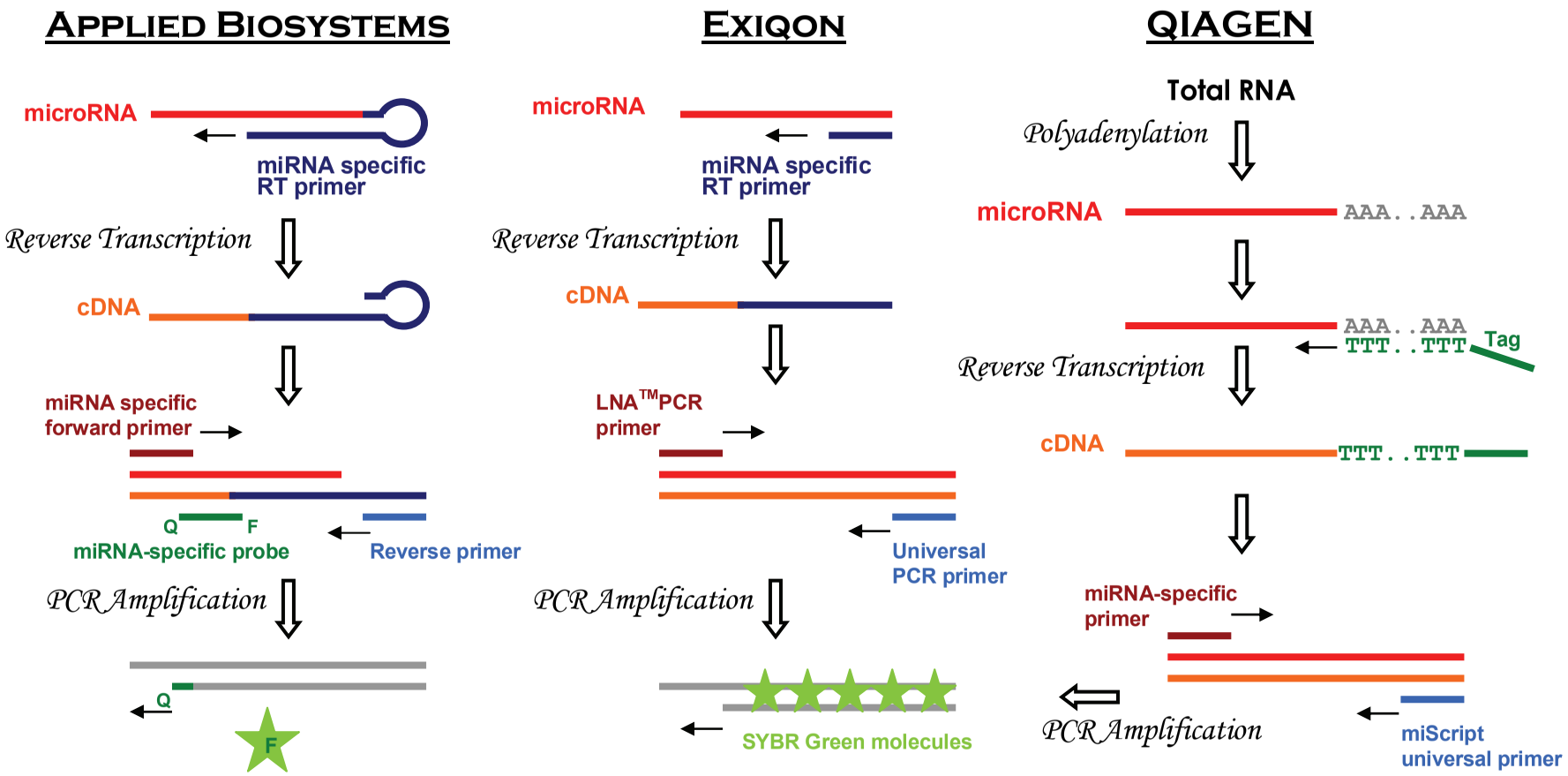

Figure 10. Overview of different real-time PCR strategies in quantatfication of microRNA
Applied Biosystems's assays use miRNA-specific primers with a 5'-loop structure which increases the stability of primer hybridization. miRNA specific primers and miRNA specific probes are used in a TaqMan™ real-time PCR reaction. Exiqon's technique uses LNA-primers which bind to the specific miRNA with high stability and high specificity in shorter length. With LNA PCR primers specific to the miRNA, the target is amplified, thus becomes detectable by binding of SYBR®Green molecules. QIAGen's approach is to synthesize poly(A) tail in vitro, followed by normal reverse transcription using random hexamer and/or oligo-d(T). With miRNA specific primers, the target is amplified and quantified with SYBR®Green molecules. Adapted from product manuals from Applied Biosystems, Exiqon and QiAgen.

METABOLOMICS

Metabolomics is a novel technology to profile metabolites in a system. The sample preparation and the detection methods will determine the sensitivity of measurements. The methods described below were used for the studies in this thesis (Figure 11).

Reference compounds

Since this method is very sensitive and detects low molecular weight compounds, a small deviation in the detection might be misinterpreted as another metabolites. Reference compounds serve as internal controls for normalizing the technical variation between samples. In the present study, 11 stable isotope reference compounds were used. [$^{2}H_4$]succinic acid, [$^{13}C_5$, ^{15}N]glutamic acid, [$^{2}H_7$]cholesterol, [1,2,3-$^{13}C_3$]myristic acid, [$^{13}C_5$]proline, and [$^{13}C_4$]disodium 2-oxoglutarate were purchased from Cil (Andover, MA), [$^{13}C_6$]glucose, [$^{13}C_{12}$]sucrose, [$^{13}C_4$]hexadecanoic acid, and [$^{2}H_4$]-1,4-butane-diamine 2HCl from Campro (Veenendaal, The Netherlands), and 2-hydroxy-[$^{2}H_6$]benzoic acid from Icon (Summit, NJ).

Extraction and derivatization

Extraction and derivatization protocols were essentially as described in (A et al. 2005). In summary, metabolites were extracted under vigorous shaking with methanol::H_2O (8:2, v:v), which contained the internal standards mentioned above. After 2 hours on ice, the solution was centrifuged. 200 µl of the resulting supernatant was transferred to a gas chromatography (GC) vial, and evaporated to dryness in a Speed-vacc Concentrator (Savant instrument, Framingdale, NY, USA). Methoxyamine in pyridine was added to re-dissolve and derivatize (methoxyamination) the samples. After 16 hours methoxyamination, MSTFA with 1% TMCS were added as catalyst for another derivatization (silylation). Heptane (containing 0.5 µg methyl stearate) was added as another internal standard for the GC analysis. In order to remove sample bias, all samples were prepared and analyzed in to two different randomized orders, one for sample preparation and the other for GC/MS analysis.

GC/TOF-MS analysis

1 µl of derivatized sample was injected splitless by an Agilent 7683 Series Autosampler (Agilent; Atlanta, GA) into an Agilent 6980 GC equipped with a 10 m×0.18 mm ID, fused silica capillary column chemically bonded with 0.18 µm DB5-MS stationary phase (J&W Scientific; Folsom, CA). The injector temperature was set at 270°C. Helium was used as carrier gas at a constant flow rate of 1 ml per minute through the column. For every analysis, the purge time was set to 60s at a purge flow rate of 20 ml per min and an equilibration time of 1 min. The column temperature was initially kept at 70°C for 2 min, and then increased from 70°C to 320°C at 40°C min^{-1}, where it was held for 2 min. The column effluent was introduced into the ion source of a Pegasus III TOFMS (Leco Corp.; St Joseph, MI). The transfer line temperature was set at 250°C and ion source temperature at 200°C. Ions were generated by a 70 eV electron beam at a current of 2.0 mA.

Masses were acquired from m/z 50 to 800 at a rate of 30 spectra per sec, and the acceleration voltage was turned on after a solvent delay of 170 s.

Analysis of GC/TOF-MS data

To evaluate the data, non-processed MS-files from GC/TOF-MS analysis were exported in NetCDF format to MATLAB software 6.5 (Mathworks; Natick, MA), where all data-pretreatment procedures, such as base-line correction, chromatogram alignment, time-window setting and hierarchical multivariate curve resolution (H-MCR) (Jonsson et al. 2005) were performed using customized scripts. ChromaTOF™ 2.00 software (Leco Corp.; St Joseph, MI) was used for calculating peak areas of internal standards and specific compounds. To obtain accurate peak areas for the internal standard and specific compounds, two unique quantification masses for each component were specified. Mass spectra of all detected compounds were compared with spectra in the NIST library 2.0 (as of January 31, 2001), in-house mass spectra library database established by Umeå Plant Science Center and the mass spectra library maintained by the Max Planck Institute in Golm (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html).

Multivariate data analysis

To analysis the massive amount of data, multivariate analysis was used. All data analysis and modelling were done using Simca 11 (Umetrics, Umeå, Sweden). Principle component analysis (PCA) and partial least square modeling with discriminating data (PLS-DA) were used to relate the peak areas of all resolved GC/TOF-MS peaks to sample class, e.g. sex or hormone treatment (Y matrix). Cross-validation with seven cross-validation groups was used throughout to determine the number of components. For all models, the variables were both centered and scaled to unit variance. Factors that did not improve the PLS-DA model according to cross-validation, established by Wold (Wold 1978), were removed before interpretation. The following statistics for the regression models are discussed in paper III. R^2X is the cumulative modelled variation in X, R^2Y is the cumulative modelled variation in Y and Q^2Y is the cumulative predicted variation in Y, according to cross-validation. The range of these parameters is 0-1, where 1 indicates a perfect fit.

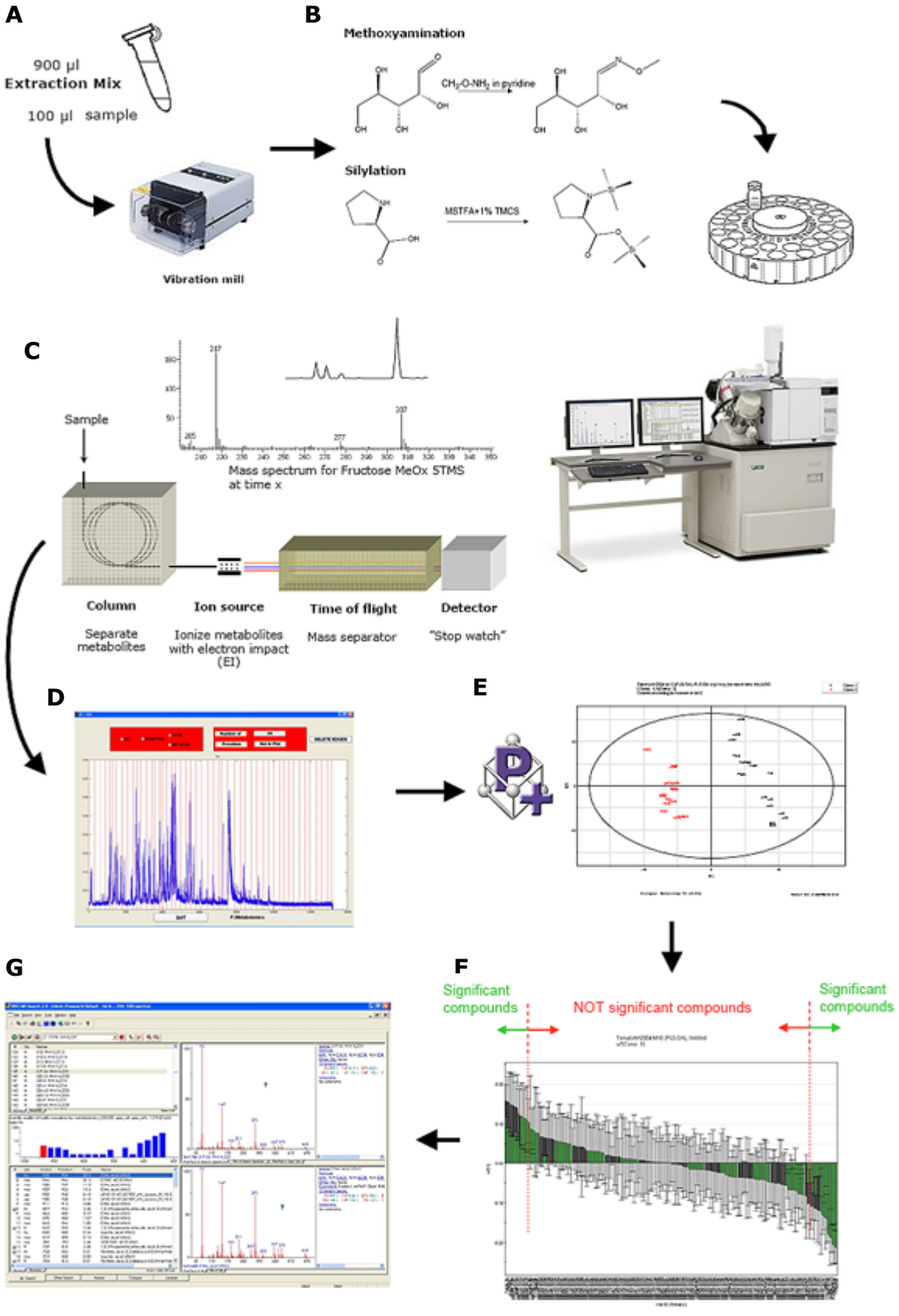

Figure 11. Workflow of experiement and analysis of a metabolomic study
A. Sample extraction B. Derivatization C. GC/MS analysis D. Data preparation in MATLAB E. Multivariate data analysis in SIMCA F. Defining significant compounds, which confidence intervals does not cross at zero. G. Compound identification with mass spectra libraries.

BIOINFORMATICS

High-throughput omics technologies generate massive amount of data that require proper data analysis as well as data storage. The Journal *Nucleic Acid Research* dedicated a whole issue in January 2007 to describe most of the databases in the field of molecular biology. It covers the many renowned databases such as those hosted by National Center of Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI). Most of these databases describe the basic and static information, i.e. sequences. However, there are also increasing amount of dynamic information incorporated to these databases, such as SNPs linked to different diseases and expression levels in different tissues and diseases.

in silico promoter analysis

One important mechanism in the regulation of a gene is through its promoter. Different bioinformatics tools have been developed to make use of the vast information from the databases. Gene expression mediated by the polymerase complex is under the control of gene specific TFs. The TF binds selectively to their corresponding sequences (TF binding sites, TFBS), which can be found upstream or downstream of the genes. TFBSs that can successfully bind to its TF do not have exactly the same sequences. Certain positions are more stringent while others can be any of the four nucleotides. Therefore, TFBS prediction is made via position weight matrix (PWM) which is built for the specific TF. PWM is generated from experiments, including promoter-reporter system in cells and in vitro TF-DNA binding assays from genomic sequences or randomly generated sequences. PWM describes the probability of each nucleotide to be present in that particular position on a functional TFBS. Searching for sequences that can fulfil the constraint of the PWM with the prediction algorithm, putative TFBS can be identified. Many of the bioinformatics methods assume that evolution imposes selective pressure so that mutation on the functional regions of genes will accumulate slower than the non-function regions (Wasserman and Sandelin 2004). Limiting the TFBS search to the conserved regions in a gene sequence could reduce the number of false positives, which is the constant limitation of bioinformatics prediction.

In paper I and II, orthologous promoter sequences from human, mouse and rat *FAT/CD36* were extracted from the UCSC genome browser and [GenBank:AF317787]. The sequences are then aligned with the ORCA algorithm and the conserved regions were identified using CONSITE program (Sandelin et al. 2004). Putative TFBS were predicted in the conserved regions of the promoters by CONSITE program in paper I and by Match™ program public version 1.0 in paper II (Kel et al. 2003). Match™ program uses a library of mononucleotide weight matrices from TRANSFAC® 6.0 (Matys et al. 2003). In paper I we used all PWM available at that time whereas in paper II, the analysis was limited to TFs known to be expressed in liver (GATA3, NF1 and PBX1) or known to be sex- or GH-dependent (AP-1, C/EBPβ, HNF3β, SOX9, SRY and STAT5). The PWM for STAT5 compiled by Ehret *et al.* (Ehret et al. 2001) was analyzed separately in both papers using the corresponding prediction program.

In paper III, we used the Prometheus software application (developed in (Vidal et al. 2007)) to screen for TFBS in the regulated genes. The software obtains promoter sequences from the Ensembl Database Sequence, aligns them using the pair-wise alignment program, LAGAN (Brudno et al. 2003) and searches for TFBS on the evolutionary conserved regions of the promoters. Using NorduGrid (a Grid computing paradigm), Prometheus can perform these intensive algorithms on large datasets in a shorter time. The PWM for KLF15 (paper III) was built by aligning genomic sequences known to bind this TF (5'-CG/TCCCC-3'), as identified in the published literature (Otteson et al. 2005).

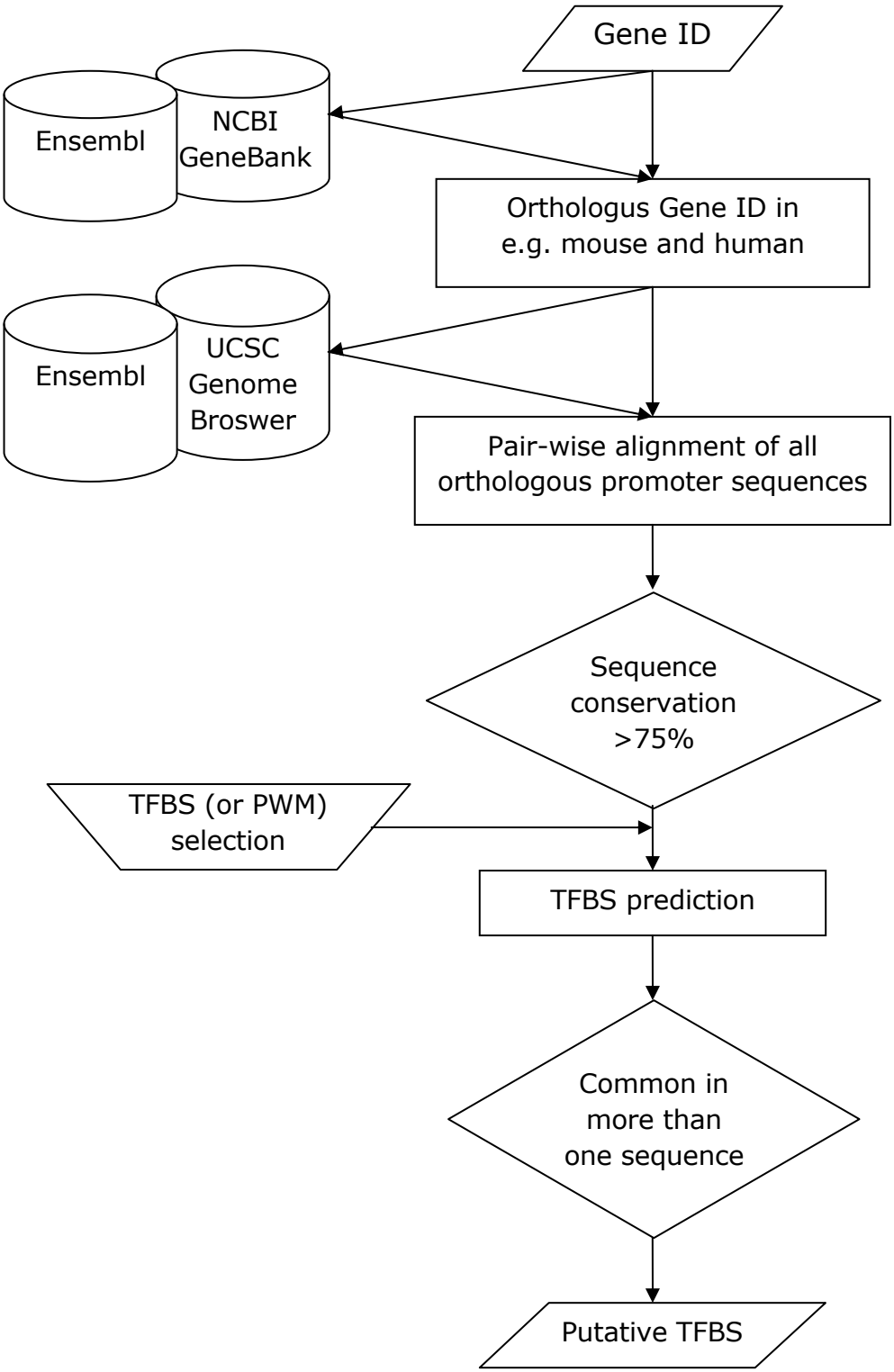

Figure 12. Workflow of *in silico* promoter analysis

RESULTS

FAT/CD36 as a female-predominant gene in the liver (Paper I)

To begin the search for sex differences in hepatic fuel metabolism, we screened for genes with sex-different expression pattern in the liver using microarrays and determined which of these might be regulated by the sex-specific secretion of GH in the liver.

Out of 3200 transcripts represented on the arrays, approximately 1800 were detected in the liver. Among these, 246 transcripts were expressed in a sexually differentiated manner; 69 transcripts (4%) were a statistically significantly higher (at least 1.5-fold) in females, whereas 177 (10%) had a higher level in males. 1-week of GHc in male rats changed the expression levels in 16% of the total number of hepatic genes. 21 female-predominant genes were induced by GHc in male rats and 51 male-predominant genes were reduced by GH. These genes might be regulated by GH-dependent TFs.

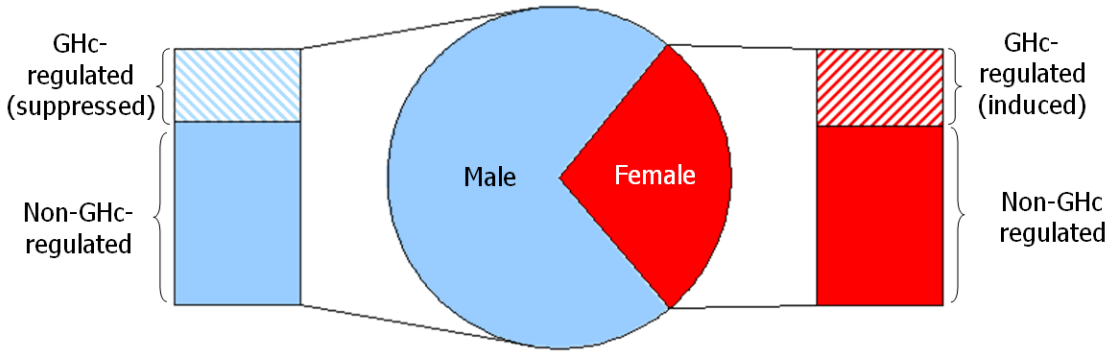

Figure 13. Sex-differentiated genes (the middle circle) and the proportions (the columns) of male- or female-predominant genes that are regulated by continuous GH infusion (GHc)

Next we grouped gene products that might be involved in fuel metabolism into functional categories. The male liver was found to have a higher expression of several genes involved in glucose, lipid, and amino acid metabolism compared with the situation in females.

Most notable among the female-predominant transcripts was *FAT/CD36*, which was shown to have 18-fold higher mRNA levels in the female liver and 3.75-fold higher mRNA levels in the male liver after GH treatment. Previous reports have concluded that FAT/CD36 is only expressed in the liver at low levels (Zhang et al. 2003). However, we confirmed our observation by RNase protection/solution hybridization assay and immunoblotting. FAT/CD36 can facilitate fatty acid uptake into cells, therefore the effect of sex and GH-treatment was determined on liver triglyceride and cholesteryl ester contents. The hepatic triglyceride content was not different between the sexes, but increased after GH treatment. Similarly, no sex difference was detected for total cholesterol. The hepatic content of cholesteryl ester was significantly lower in the females, but was not affected by GH-treatment.

in silico analysis of the *FAT/CD36* promoter revealed that rodent and human FAT/CD36 promoters share regions of conserved sequences. These regions contain

more than 50 different conserved putative DNA-binding elements including HNF3β, c-Fos and nuclear factor-kappa B. These TFs have previously been shown to be involved in sex-differentiated gene regulation (Hallstrom et al. 1989; Legraverend et al. 1994; Delesque-Touchard et al. 2000; Kono et al. 2000).

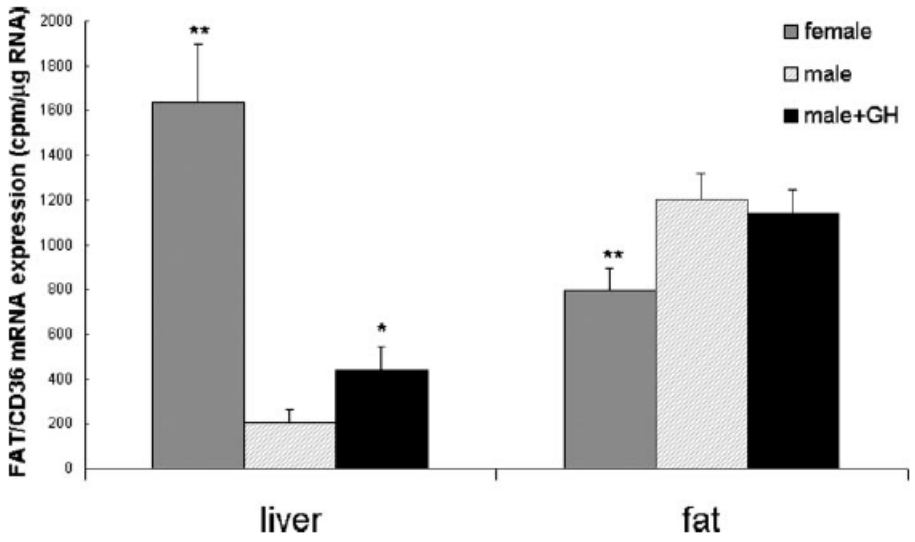

Figure 14. FAT/CD36 mRNA expression in liver and adipose tissue from male rats, female rats and male tats treated with continuous GH, determined by RNase protection/solution hybridization analysis.

Regulation of hepatic *FAT/Cd36* via preferential exon usage (Paper II)

In the second paper, we extended our knowledge about regulatory mechanisms behind hepatic *FAT/CD36* expression. Since alternative *FAT/CD36* transcripts have been described in mouse and human tissues (Kuriki et al. 2002; Sato et al. 2002; Andersen et al. 2006; Sato et al. 2007), we investigated whether alternative transcription start sites might be involved in differentially regulating *FAT/CD36* mRNA abundance in rat liver in different hormonal and nutritional states.

First, we extended the genomic information of rat *FAT/CD36*. Two alternative first exons of rat *FAT/CD36* were identified and named according to the nomenclatures in mouse and human. From the cDNA sequencing results, it was apparent that there are two alternative first exons in rat and the use of exon 1a and exon 1b is mutually exclusive in rat liver ([GenBank:EF116601] and [GenBank:EF116602]) and the gene structure in rats is similar to that in human and mouse. In order to allow further investigation of the regulatory mechanism of rat *FAT/CD36*, exon 1a promoter was also sequenced and it showed high similarity to the corresponding sequences of human and murine *FAT/CD36*.

Tissue-specific expression patterns of alternative first exons of *FAT/CD36* have been described in mouse (Sato et al. 2002; Sato et al. 2007) and human (Andersen et al. 2006), suggesting that the alternative first exons of the gene are regulated individually. RNase protection/solution hybridization assay performed in paper I showed higher expression level of *FAT/CD36* in female rat liver using the same probe present on the microarray. However, the probe does not correspond to the 5'-

UTR. In this study, we therefore analyzed the expression levels of exon 1a and 1b in male and female livers from rats and mice by quantitative real-time PCR and the results were compared to the level of exon 3–4, representing the protein coding region. The exon 1a-3 transcript was significantly higher expressed in females as compared to males, in both rat and mouse. Although similar results were obtained for exon 1b-3 and the coding region (exon 3–4), the sex-difference was most pronounced for exon 1a-3.

Hormonal treatments also affect the expression levels of exon 1a and 1b. The largest feminizing effect was observed in exon 1a-3, suggesting that that EE and continuous GH (GHc) treatment might induce *FAT/CD36* expression preferentially through the exon 1a promoter. When old male rats were treated by episodic administration of GH (GHi), *FAT/CD36* gene expression was repressed and restored to the same level as in young adult males with no significant difference between the two alternative exons. We speculate that regulatory DNA sequences mediating the

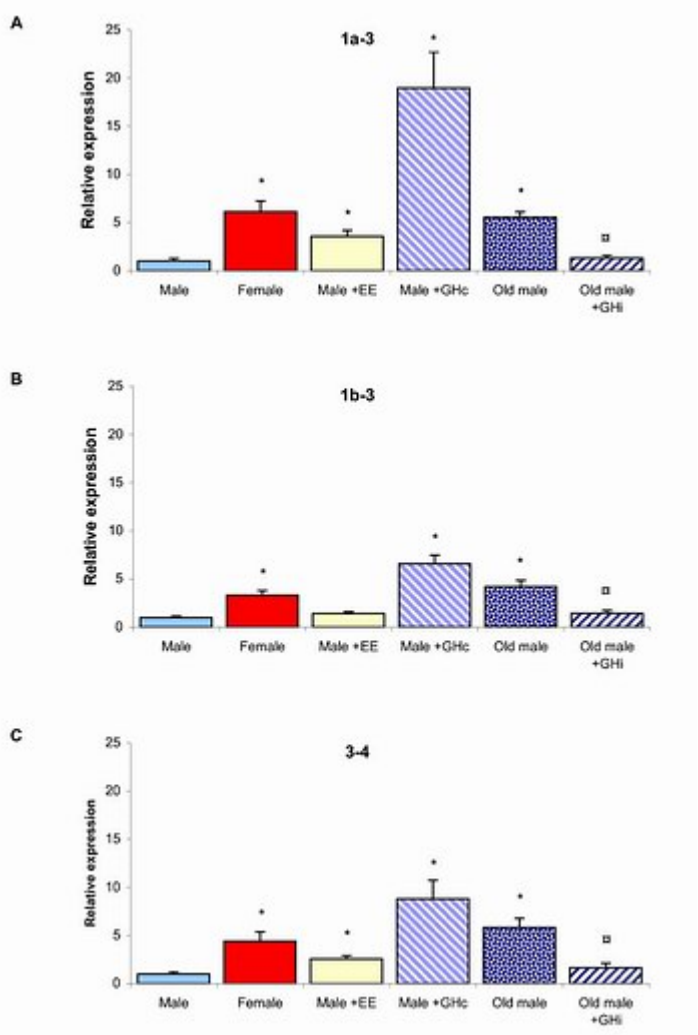

Figure 15. Effects of different hormonal treatments on hepatic *Cd36* mRNA expression in rats. mRNA expression of *Cd36* exon 1a-3 (A), exon 1b-3 (B) and exon 3–4 (C) was quantified by real-time PCR in livers from males, females, males treated with continuous infusion of GH (GHc), males treated with injections of estradiol, old males or old males treated with twice daily injections of GH (GHi). Asterisks indicate statistically significant differences versus male (*) whereas circles (¤) indicate significant effects of GHi in old males ($p < 0.05$)

inhibitory action of pulsatile GH are present in both exon 1a and 1b promoters, whereas those important for the stimulatory effect of continuous GH are specific for exon 1a.

Starvation in the form of 12 h of food deprivation significantly reduced *FAT/CD36* mRNA expression in livers from females, without any differences in alternative exon 1 transcripts. No effect was observed in male rat livers. In contrast, the expression of *FAT/CD36* in skeletal muscle was increased in both males and females. This stimulatory effect of food withdrawal was highest for exon 1a-3, suggesting that fasting might induce *FAT/CD36* expression preferentially through the exon 1a promoter in muscle.

We observed that the expression level of exon 1a is preferentially regulated by sex and hormones. Since the sequence information has been extended, we performed another in silico promoter analysis with a modified approach and updated bioinformatics data, as compared with paper I. Most of the putative TFBS were conserved between rat and mouse and some could also be predicted in the human promoters. Some TFBS were found to be common between the two promoters, such as those for HNF3β, whereas others were promoter-specific. The two putative binding sites for HNF3β were both found to be conserved between rat and mouse and might be of special interest since constitutively active HNF3β was previously shown to increase hepatic *FAT/CD36* expression (Wolfrum et al. 2004). However, no putative binding site for STAT5b, a GH-mediated male-specific TF (Waxman et al. 1995), were revealed.

Sex differences in the control of gluconeogenesis and hepatic glucose output in rats (Paper III)

Based on our finding in paper I that important genes for lipid synthesis and fatty acid oxidation have a male-predominant expression in rat liver (Stahlberg et al. 2004), it might be speculated that hepatic lipid turnover is higher in males as compared to females. The hepatic TAG content, FA oxidation rate and ketone bodies in blood from males and females at either 4 or 12 hours of food deprivation were determined and no sex-difference was observed in these analyses. Similarly, no sex difference in lipogenic gene expression was found at either 4 or 12 hours of food deprivation, though mild starvation reduced their mRNA level.

Before starvation, male rats had higher glycogen content, higher rates of glucose production as well as higher expression levels of the gluconeogenic genes, *G6Pase* and *PEPCK*, confirming the higher expression of genes involved carbohydrate turnover in paper I. Upon mild starvation, the males had increased levels of gene products involved in FA oxidation, gluconeogenesis and glycogen synthesis, indicating that starved male rats might be more efficient in producing glucose and glycogen. Along with this, the HGO was also higher in the males than in females.

Starved females responded more to insulin-induced hypoglycaemia, as judged from gene expression profiles. In particular, transcripts encoding proteins involved in

amino acid catabolism, mitochondrial import of amino acids and gluconeogenesis were induced to a greater extent in the females.

We are then interested in the transcriptional regulatory mechanism in the gluconeogic genes that responded to insulin. A recent report showed that KLF15, a member of the Krüppel-like family of TFs, is an important regulator of gluconeogenesis (Gray et al. 2007). By immunoblotting, we observed a female-predominant protein expression of KLF15 in the rat livers. Moreover, a three-fold stronger signal (KLF15 related to β-actin) was obtained from insulin treated females as compared to the saline-treated. We next addressed the question whether the genes whose expression was more affected by the insulin-induced counter-regulatory response in females could be direct transcriptional targets of KLF15 using *in silico* promoter analysis. We identified a total of 33 genes with putative KLF15 binding sites. Among them, 25 (76%) were more responsive in female livers, including orphan nuclear receptors NR4A1 (Nur77). Overexpression of Nr4a1 in liver induces gluconeogenic genes, stimulates glucose production and raises blood glucose levels (15). The female-specific increase in KLF15 by insulin might induce the expression of Nr4a1 and thus increasing glucose production and the expression of gluconeogenic genes.

Sex-different and GH-regulated expression of miRNA (Paper IV)

In this paper, we further studied the hepatic sex differences in transcript levels, but in another group of RNA, namely (miRNA). These small RNA can regulate gene expression by inhibiting protein translation efficiency. First we generated hepatic miRNA profiles from normal male and female rats using microarray. Out of 324 unique probes on the array, 254 (78%) were expressed in the liver and 14 of those expressed miRNA (5%) were found to be sex-different. We were able to validate a female-predominant miRNA, miR-29b, using quantitative real-time PCR and believe that miR-122a also had higher expression level in female than male liver.

To address the question whether the sex-different hepatic miRNAs could be regulated by GH, we determined their expression levels in GHc-treated male rats. Interestingly, GHc drastically down-regulated miR-29b levels by 21-fold. However, miR-122a expression was unaffected by this treatment.

Since mild starvation changes the sex differences in the liver, we examined the expression levels of the selected miRNA in male and female rats after 4 hour or 12 hour of food deprivation. After 4-hour food deprivation, miR-122a displayed a significantly higher level in females than males, which was not obvious in animals without any starvation. Mild starvation raised the levels of miR-451, miR-122a and miR-29b in both sexes. Sex differences observed before mild starvation in miR-29b and miR-122a were diminished after 12 food deprivation.

As a reference, 5S rRNA was used due to the similarity in size to miRNA. Similar levels of 5S rRNA were observed in male and female liver. Though U6B is the most commonly used reference, it is not well characterized in rat. We did not detect U6B in our rat samples using assays designed for human U6B quantification.

A potential miR-29b target, insulin-induced gene 1 (INSIG1, or growth response protein CL-6), was identified in a recent study (He et al. 2007). Both mRNA and protein levels of Insig1 were shown to be inhibited by over-expression of miR-29 in cultured adipocytes. We have previously shown that Insig1 mRNA expression is highly up-regulated by continuous GH-treatment in male rat livers (Tollet-Egnell et al. 2001), which might be related to the drastic decrease in miR-29b observed in this study. We thus compared the protein levels of Insig1 in livers from untreated and GH-treated male rats by immunoblotting. GH-treatment led to increased levels of Insig1. Since Insig1 inhibits the maturation process of sterol regulatory element binding protein (SREBP) (Loewen and Levine 2002), we then further examined whether the same treatment would also lead to retention of immature SREBP. The molecular weights of the detected proteins correlate with those of the mature form of SREBP (68kDa) and its precursor (125kDa). There was an increase in the levels of SREBP1's precursor and INSIG1, suggesting that GHc-treatment impairs SREBP1 processing. These results also suggest another level of gene regulation by sex and hormones.

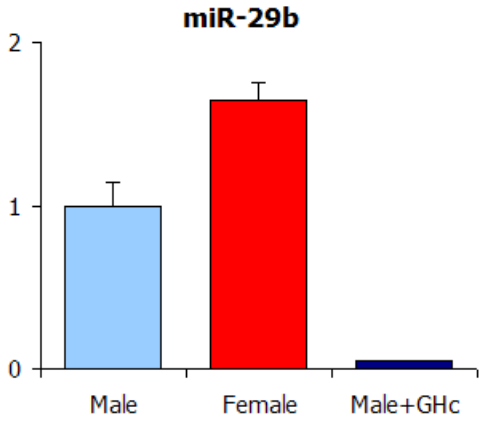

Figure 16. Expression level of miR-29b in males, females and males treated by GH infusion (GHc) under 1 week.

Serum metabolomic profiling to study hormone actions (Unpublished data)

From paper I, we learned that there are sex differences in different metabolic pathways at the transcript level. Further investigating the metabolic turnover in paper III, we observed that sex differences at transcript level were translated into the metabolite level in terms of carbohydrate turnover. Therefore, we performed this pilot study to screen for sex-different metabolites in blood and in the liver, and attempted to relate transcript and metabolite levels to each other.

The metabolic profiles from individual rat serum and liver lysate samples were obtained by GC/TOF-MS. The levels of low molecular compounds were recorded and analyzed by multivariate data analysis. In order to assess if estrogen (17α-ethinylestradiol) could affect the metabolite pattern in serum and in liver, partial least squares (PLS) models for sex differences were built (Figure 17). Then data points for estrogen-treated individuals were predicted into the sex-differentiating model. Serum metabolites from estrogen-treated males showed a tendency leaving the male group towards the female group. In the liver, metabolic profiles from estrogen-treated males were dispersed between the male and female groups with high individual variation. The models showed that there were sex differences in metabolic composition. Also, the models indicate that estrogen had an impact on changing the metabolic composition in liver and serum, with a higher effect in the liver.

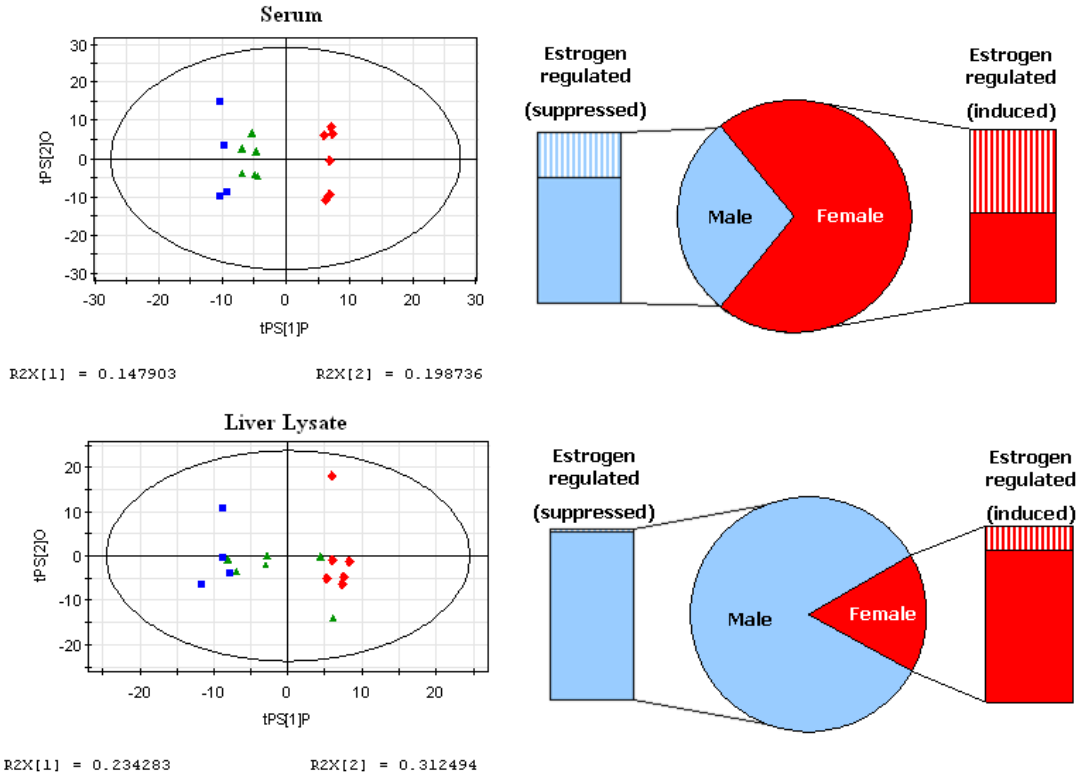

Figure 17. Partial Least Squares with discriminating data (PLS-DA) model of serum (upper left) and liver lysate (lower left) metabolite profiles discerning between males and females, with prediction of estrogen-treated males. (Square=male, diamond=female, triangle=estrogen-treated male) The relative proportion of male- or female-predominant metabolites in serum (upper right, circle) and liver lysate (lower right, circle) that are affected by estrogen treatment (right, stripped sections of the columns).

Narrowing down to the metabolites which contributed to the models, 15% (78 of 507) of serum metabolites and 16% (43 of 277) of liver metabolites were found to be sex different and thus the key metabolites to differentiate between male and female samples. Estrogen could mimic 38% of these differences in serum and 9% in liver. Of 56 female-predominant metabolites in the serum, 27 were increased by estrogen treatment, and six of 22 male-predominant serum metabolites were suppressed. In the liver, the level of only one out of 36 metabolites in males and one out of 27 in females were affected accordingly by estrogen. Due to the limited number of known spectra for metabolites in the reference libraries, the identities of many detected metabolites are unknown. However, urea was found to be higher in

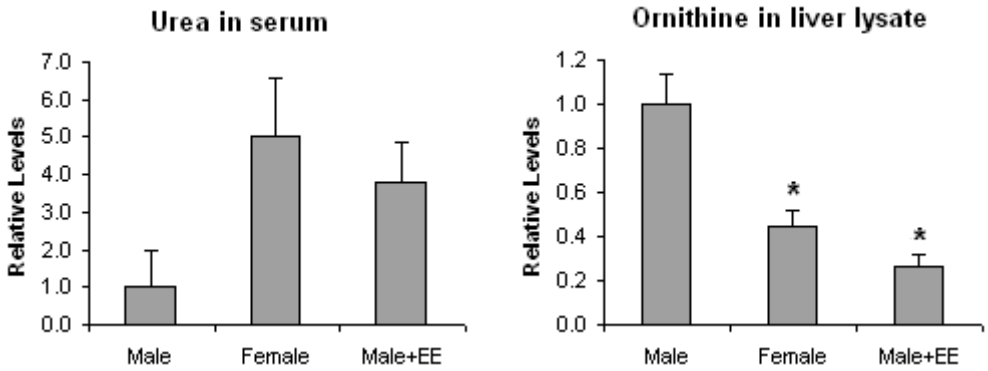

Figure 18. The relative levels of urea in serum and ornithine in liver lysate of male, female and estrogen (EE)-treated male rats, determined by GC/TOF-MS. * = $p<0.01$

sera from females and estrogen-treated males than that from males. In the liver lysates, ornithine was found to be higher in males than in females or estrogen-treated males (Figure 18).

At the level of gene expression, 6% of the hepatic transcripts were sex different using whole-genome microarray and estrogen could micmic 25% of these (Figure 19). Functional categorization revealed that protein catabolism might be higher in female liver as well as in estrogen-treated males. Since the two metabolites found by metabolic profiling are also involved in protein catabolism, we examined the mRNA level of two key enzymes, arginase 1 (Arg1) and ornithine aminotransferase (Oat). Arginase converts arginine into ornithine and urea as part of the urea cycle. Urea will then be excreted whereas ornithine can take part in other metabolic pathways. One possible pathway is transamination by Oat into glutamate, although it is supposed that ornithine generated from the urea cycle is more likely to be recycled within the urea cycle than to be transaminated (Morris 2002).

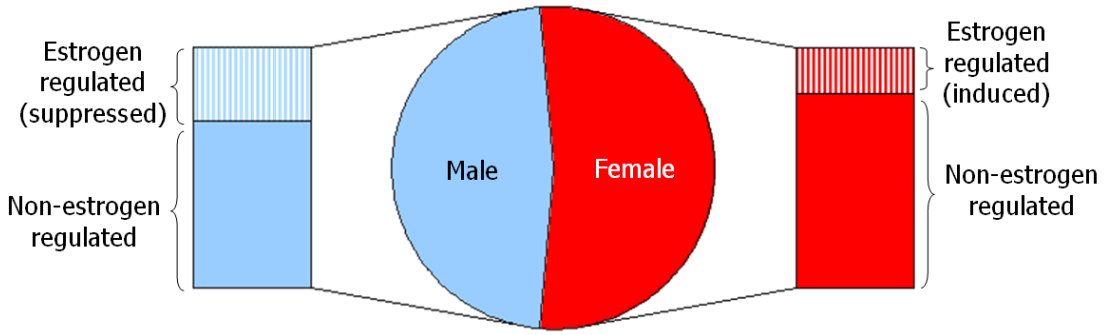

Figure 19. Sex-differentiated genes (the middle circle) and the proportions (the columns) of male- or female- predominant genes that are regulated by estrogen.

From the microarray, the expression level of Oat was significantly higher in female. This was then confirmed by real-time PCR (Figure 20). However, no sex difference was found in the expression level of *Arg1*. Estrogen did not affect the expression level of *Oat* nor *Arg1*.

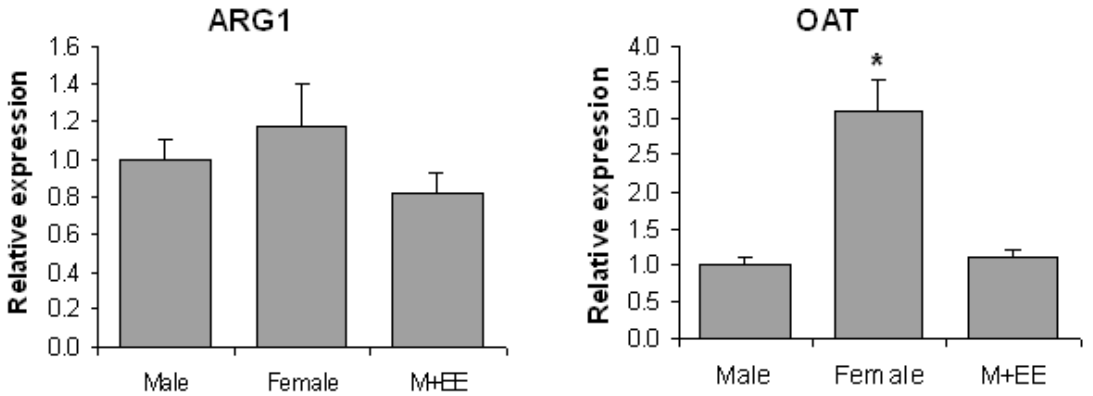

Figure 20. mRNA expression of hepatic Arg1 and Oat in male, female and males treated with estrogen (EE) was quantified by real-time PCR, normalized to the expression of ribosomal protein RPLP0. * = $p<0.01$

If expression levels would be translated into protein level and enzymatic activity, the production of ornithine and urea from arginine might be the same between male and female rats, whereas the high level of ornithine in male might be due to the low

level of Oat. Liver might not necessarily be solely responsible for a high urea level in female serum since the role of sex differences in the uptake rate of urea in the kidney is unknown. Since the number of metabolites detected by GC/TOF-MS in the liver lysates is lower than in the serum, further improvement in the protocol for liver lysate preparation might be needed. Further studies on enzymatic activities of Arg1 and Oat should also be performed to verify the findings on mRNA level. Moreover, enzymatic activities are usually substrate and/or product dependent, therefore it is relevant to examine the hepatic levels of arginine, urea, ornithine, pyrroline-5-carboxylic acid, proline and glutamate using target-specific assays, in order to understand whether the studied biochemical reactions are sex different in the liver.

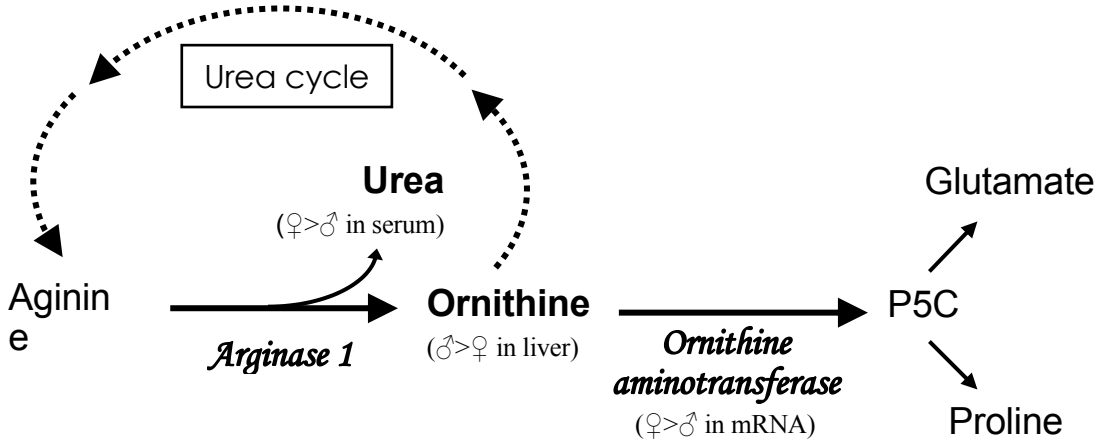

Figure 21. An overview of the part of arginine metabolism studied. Bold style represents metabolites and enzymes examined. P5C= pyrroline-5-carboxylic acid.

DISCUSSION AND FUTURE PERSPECTIVES

Two characteristics of life are homeostasis and adaptation. Since males and females have different functions in the life cycle and in the social setting, they do not have the same metabolic needs such as energy storage or muscle strength. In many mammals, the male bodies are more adapted to impulsive exercise such as hunting and fighting whereas the female bodies have to last through the bearing and nurture of offspring. Consequently, they respond differently to the same metabolic challenge such as impulsive exercise and long-term starvation. Both males and females need to maintain the optimal physiological status (homeostasis) using different strategies.

Since males and females show differences in metabolism, some of these differences might be reflected at the level of gene expression. A genome-wide screening of sex-dimorphic genes in mice showed that thousands of genes are sex-differentiated in metabolic organs (liver, muscle and adipose tissue) compared to hundreds of sex-different genes found in the brain (Yang et al. 2006). This thesis focused on hepatic metabolism because liver is one of the well-studied organs regarding sex-differences. The examined molecular differences between male and female livers and the related factors in the circulation will be discussed from a homeostasis perspective, in particular carbohydrate and lipid metabolism and gene regulation.

SEX DIFFERENCES IN THE LIVER

The liver is a key metabolic organ. We and others have shown that some of the sex-related hepatic metabolic differences are reflected in the blood and urine. Using GC/TOF-MS metabolic profiling, we obtained different metabolic profile patterns from both liver and serum samples of male and female rats. Due to the limited number of known mass spectra for metabolites in the reference libraries, the identities of many detected metabolites are unknown. However, the level of ornithine was found to be higher in female liver extracts, along with a higher hepatic mRNA expression of Oat, a transaminase. A few years ago, it was shown that urinary metabolite profiles are sex-different in Han Wistar rats. The British scientists found that male rats excrete more of the sulfate conjugate of m-hydroxyphenylpropionic acid whereas urine from female rats contain more trimethylamine-N-oxide (TMAO), N,N'-dimethylglycine (DMG), m-hydroxyphenylpropionic acid (m-HPPA), N-acetylglycoprotein (NAC), and cholate (Stanley et al. 2005). Four of the female-predominant metabolites (TMAO, DMG, NAC and cholate) are bile acids or their derivatives, and thus related to hepatic metabolism. The higher levels of urinary bile acids reflect a higher sterol synthesis in females. Although less than 4% of the urinary metabolites and only 15% of the serum metabolites were distinctly different between males and females, sex differences in hepatic metabolism might still contribute to this.

Our results indicate that male rats have a higher expression of genes encoding important proteins for carbohydrate, lipid and amino acid turnover. This observation might explain why male rats have a higher metabolic rate than females and is in line with the fact that males have a greater muscle mass and higher growth rate. Our group has previously observed that genes involved in hepatic fatty acid oxidation

and glycogen production are induced by intermittent GH treatment in old male rats (Tollet-Egnell et al. 2001). As this type of treatment is believed to mimic the male-specific episodic secretion of GH, the results suggested that this set of genes might be male-predominant. As the higher growth rate in males is also dependent on episodic GH secretion, this might be a way to ensure that increased body growth is always coupled to increased utilization of fatty acids for energy expenditure.

Regarding hepatic intermediary metabolism, most observed sex-differences were related to lipid and carbohydrate turnover. Some sex differences were found in relation to protein turnover using the microarray and metabolomics analyses, but these observations require further confimatory studies. Furthermore, a newly characterized group of RNA, miRNA, also shows a sex-different expression pattern. The regulation of two miRNAs studied in this thesis will be discussed later.

Carbohydrate homeostasis

There were higher hepatic expression levels of the gluconeogenic genes G6Pase and PEPCK, in normal male rats, as shown with microarray analysis in paper I and real-time quantitative PCR in paper III. Moreover, we observed that male rats had higher plasma insulin levels, higher hepatic glycogen content and higher rates of glucose production. HGO was higher in male rat liver in conjunction with expression levels of the gluconeogenic genes. However, the plasma level of glucose was similar between male and female rats, indicating there are sex differences in other tissues that might contribute to glucose homeostasis.

Upon mild starvation (12 hours of food restriction), the males had increased levels of gene products involved in FA oxidation, gluconeogenesis and glycogen synthesis, indicating that starved male rats might be more efficient in producing glucose and glycogen. One of liver's main tasks during starvation is to supply glucose to obligatory glycolytic tissues. Since males are more dependent on carbohydrates as energy source during starvation and exercise, it is logical that HGO is higher in males. In order to increase HGO, an increase in beta-oxidation will supply enough energy for gluconeogenesis and an increase in gluconeogenic genes will ensure a faster production of glucose.

Lipid homeostasis

No sex-differences were observed regarding hepatic TAG content or FA oxidation rates at either 4 or 12 hours of food deprivation in paper III. Similarly, no sex-difference in lipogenic gene expression was found though mild starvation reduced their mRNA levels. This was in contrast to the microarray findings in paper I, in which lipogenic gene expression was shown to be higher in male liver. One possible explanation is that the animals in the latter study were food-deprived for 4 hours; therefore they were more homogenous in their metabolic state (food-deprived).

Higher level of FAT/CD36 in female livers

In paper I and II, we reported that *FAT/CD36* is a female-predominant gene, regulated by GH secretion pattern – induced by continuous GH infusion but

repressed by pulsatile GH injection. FAT/CD36 is a cell surface glycoprotein as a receptor/transporter for LCFAs. It is expressed in various tissues; however the liver was not believed to be a major site for FAT/CD36 expression. The FAT/CD36 expression level in rat livers was reported to be low (Van Nieuwenhoven et al. 1995; Pelsers et al. 1999), but Zhang and coworkers showed that it actually varies with sexes and strains (Zhang et al. 2003). With quantitative real-time PCR, we observed a distinctly higher level of *FAT/CD36* mRNA as well as protein in Sprague-Dawley female rat livers.

… results in higher fatty acid uptake in liver

In paper I, we showed that female and GHc-treated males have increased hepatic content of TAG in parallel with higher *FAT/CD36* mRNA levels. Female rat liver import and utilize more LCFA than male livers (Kushlan et al. 1981). One explanation could be the higher concentration of fatty acid binding proteins in the female livers (Ockner et al. 1979). However, Sorrentino and co-workers later reported that when there is no sex-difference in the amount of plasma fatty acid binding protein, female hepatocytes still have a greater oleate uptake than male hepatocytes (Sorrentino et al. 1992). Since oleate is one of the main ligands of FAT/CD36, the higher level in female hepatic FAT/CD36 might explain the above observation.

Furthermore, in paper II, we observed a greater increase in *FAT/CD36* expression in males treated with GHc compared to estrogen treatment. It might be explained by other indirect effects of GH treatment. GH is well-known to stimulate adipose lipolysis, leading to increased levels of circulating free fatty acids (Kastin et al. 1975), which might reprogram hepatic gene expression through various lipid-modulated TFs. In line with this, hepatic TAG levels and *FAT/CD36* expression are upregulated in $LDLR^{-/-}$ $apoB^{100/100}$ male mice (a model of human familial hypercholesterolemia) fed trans-10, cis-12 conjugated linoleic acid (Degrace et al. 2006). Other studies also showed that high levels of hepatic FAT/CD36 coincide with increased hepatic lipid content (Stahlberg et al. 2004; Degrace et al. 2006; Zhou et al. 2006) and LCFA uptake (Soler-Argilaga et al. 1975; Kushlan et al. 1981). Whether this is a direct effect of higher levels of fatty acids in the circulation or indirect through altered hepatic lipid homeostasis is not clear, but points to a role of FAT/CD36 in hepatic fatty acid uptake.

… might lead to an increase in VLDL production

The liver secretes TAG via VLDL particles. Higher TAG might lead to increases in VLDL production. Women have higher basal VLDL production and female rats secrete more VLDL (Soler-Argilaga et al. 1975; Soler-Argilaga and Heimberg 1976). Female-predominant expression of FAT/CD36 seems to be accompanied by higher rates of hepatic LCFA uptake (Soler-Argilaga et al. 1975; Kushlan et al. 1981), esterification (Ockner et al. 1979), VLDL TAG formation and output (Soler-Argilaga and Heimberg 1976) in female rats as compared to males. A higher level of hepatic FAT/CD36 level might lead to an increase in VLDL production for fat distribution to the subcutaneous fat tissue.

… does not affect hepatic beta-oxidation

Apart from transport of LCFA across the cell membrane, it has been proposed that FAT/CD36 facilitates transport of LCFA to mitochondria in muscle cells (Campbell et al. 2004; Holloway et al. 2006) and its level correlates to muscle beta-oxidation (Bonen et al. 1998; Bonen et al. 1999; Coburn et al. 2000). It is uncertain if the same holds for FAT/CD36's function in the liver. Firstly, two reports suggested that FAT/CD36 does not affect muscle beta-oxidation directly (Holloway et al. 2007; King et al. 2007). Secondly, a rate-limiting enzyme in beta-oxidation, CPT, has higher activity in male livers than female, opposite to what is observed for *FAT/CD36* expression. Similarly, liver CPT activity increases after starvation (Saggerson and Carpenter 1982), when beta-oxidation is also increased. While, in paper II, we found that the mRNA level of hepatic *FAT/CD36* decreased after mild starvation.

One way to interpret this finding could be that hepatic FAT/CD36 is involved in anabolic actions (re-esterification of fatty acids and VLDL TAG formation), in contrast to the situation in muscle where it has been linked to catabolic and ATP-producing processes (Bonen et al. 2004; Schenk and Horowitz 2006). Further studies are required to elucidate whether hepatic FAT/CD36 levels affect beta-oxidation.

… might change the transcription of other genes

Since fatty acids also act as signals in regulating gene expression, a greater capacity to import these molecules might lead to a sexually differentiated gene expression in situations when the availability of fatty acids increases. Several genes – pyruvate kinase, *G6Pase*, *FASN*, spot 14, and stearyl-CoA desaturase 1 - are down-regulated by fatty acids. These proteins are involved in the lipid and carbohydrate metabolism. Interestingly, all of the mentioned gene products were expressed at a lower level in female liver in microarray experiments in paper I. It is tempting to speculate that the higher level of FAT/CD36 observed in female rats and female humans might lead to an increased capacity to repress hepatic lipogenesis when circulating levels of LCFAs increase. Whether this observation is related to the higher level of FAT/CD36 in female livers must await further investigations.

… might affect risks for various metabolic complications

Animal models and human genetic studies point out that defect or deficient *FAT/CD36* is related to metabolic complications, such as dyslipidemia, insulin resistance, diabetes, and atherosclerosis. (Aitman et al. 1999; Hirano et al. 2003; Pravenec et al. 2003; Lepretre et al. 2004a; Lepretre et al. 2004b; Pravenec et al. 2004; Yamashita et al. 2007; Love-Gregory et al. 2008).

At least two studies have pointed out the importance of hepatic FAT/CD36. *Cd36*-deficient mice do not suffer from whole-body insulin resistance and their muscle insulin sensitivity is increased. Yet these animals develop steatosis and hepatic insulin resistance (Goudriaan et al. 2003). On the other hand, over-expression of hepatic *FAT/CD36* increases hepatic lipid uptake, storage and secretion (Koonen et al. 2007). Seemingly, the normal liver monitors the FAT/CD36 at a "healthy" level by some unknown mechanism. Interestingly, Griffin and co-workers revealed that

the glucose level can regulate the translation efficiency of *FAT/CD36* in monocytes through its 5'-UTR (Griffin et al. 2001). Alternative first exons, which are part of the 5'-UTR in rodent and human *FAT/CD36*, have been identified by us and others (Sato et al. 2002; Andersen et al. 2006; Cheung et al. 2007; Sato et al. 2007). It is plausible that transcripts carrying these alternative first exons would exhibit different translation efficiency, thus increasing the complexity of *FAT/CD36* regulation. Altogether these findings indicate that the level of FAT/CD36 is important and from gene to protein it is regulated at several levels by hormonal and nutritional status, emphasizing its importance in metabolic homeostasis. Our finding that FAT/CD36 expression is sex-different leads us to speculate that this gene might be involved in the sexually dimorphic development of diseases resulting from or characterized by disturbances in lipid metabolism. In summary, a higher level of hepatic FAT/CD36 in females would increase the level of TAG that might consequently be secreted as VLDL for distribution in the subcutaneous fat tissue and setting aside circulating fats. Thus, it might be protective against the development of metabolic complications.

GH-regulated miR-29b might be a key regulator in hepatic lipogenesis

In paper IV, we reported a higher concentration of miR-29b in female livers. The expression is also inhibited remarkably by GHc in males and induced by starvation in both males and females. By immunoblotting, we found that a potential target of miR-29b, Insig1, displays a higher level in GHc-treated males. This increase in Insig1 also correlates to the delay in maturation of SREBP1 in the treated animals. However, GHc is also known to increase the mRNA level of SREBP1, increasing hepatic TAG secretion and expression of lipogenic genes (Frick et al. 2002). Similarly, both *Insig1* (Diamond et al. 1993b) and *Srebp1* (Osborne 2000) are induced by insulin. In the adipocytes, insulin raises the level of miR-29 (He et al. 2007). In paper III, the microarray experiments showed that the expression level of Insig1 in starved males is up-regulated by insulin. Whether or not GHc and insulin action on SREBP1 and INSIG1 is directly or indirectly mediated by miR-29b, it suggests a fine-tuning in the regulation of lipogenesis that require further investigation from a time perspective.

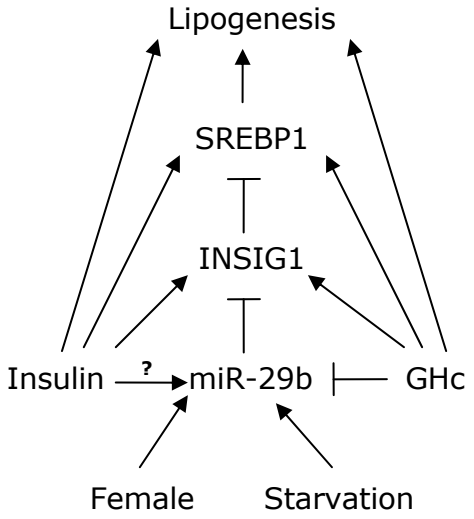

Figure 22. Relationship of miR-29b, INSIG1 and SREBP1 with different stimuli

miR-122a is part of the hepatic lipid turnover

miR-122a is a liver-specific miRNA which is involved in lipid homeostasis as well as the development of hepatitis. With the use of anti-sense oligonucleotides (aka antagomir), Esau and colleagues showed that miR-122 promotes lipid synthesis and inhibit fatty acid oxidation in the liver. Silencing hepatic miR-122 in vivo lowers plasma cholesterol level, regulates several lipolytic and lipogenic genes in the liver

(Esau et al. 2006). These include some genes that have a female-predominant expression in the liver, such as ATP citrate lyase (*ACLY*), *SREBP1* and *FAT/CD36*. Since the hepatic FAT/CD36level is higher in females, which is related to a higher TAG level and an increase in VLDL production, it is conceivable that a female-predominant expression of miR-122a (paper IV) also takes part in a highe$r lipid turnover in female liver.

It is exciting that pharmaceutical companies, Isis Pharmaceuticals, Inc. and Santaris Pharma A/S, are developing miR-122 antagomir to help fighting metabolic disease and hepatitis. However, since miR-122a was found to have a higher level in female rat livers, it is important to find out whether the level is also higher in women, hence requiring a higher effective dosage. Furthermore, disrupting miR-122a leads to changes in female-predominant genes involved in hepatic lipid metabolism, which is also sex-different. The metabolic consequences of the product antagomir might display differently in men and women. Therefore, in my opinion, sex differences should be evaluated in the early stages of development of potential drugs.

GENE REGULATION

GH-mediated gene regulation in liver

Decades of research have proven that the sex-different GH secretion pattern in rats is the main factor determining the sex-different gene expression in rat liver. A large part of these sex differences are mediated through STAT5b transcriptional activity. Comparing our microarray profiles to those generated by the Waxman group, we found that 16% of the sex-different genes were under the regulation of GHc, whereas they showed more than 50% (Ahluwalia et al. 2004). The disparity can be due to the differences in platform, analysis tools and/or threshold cut-off. In the article of Ahluwalia *et al.*, GHc in male rats induces 27 of 37 female-predominant genes and represses 44 of 49 male-predominant genes, compared to 21 of 69 female-predominant and 51 of 177 male-predominant genes in paper I. Since we reported a larger number of sex-different genes, the percentage of GH regulation was subsequently lowered. However, the actual number of GH-regulated sex-different genes is similar.

In paper II, we described a dual regulation of *FAT/CD36* by GH depending on the mode of treatment. GHc treatment of male rats induced *FAT/CD36* expression preferentially through the exon 1a promoter. Old age was also associated with increased FAT/CD36 expression in male rats, albeit without any preferential first exon usage. GHi treatment in old male rats reversed (reduced) this effect with no difference between the two alternative first exons. We therefore suggest that the effects of continuous GH secretion in females (which is stimulatory) and intermittent GH secretion in males (which is inhibitory) explains the sex-different expression of this gene. It has been described before that GH-mediated STAT5b induces the expression of male-predominant genes and suppresses the expression of female-predominant genes in male liver (Udy et al. 1997). However, we failed to locate any putative STAT5 binding sites in the promoters of the two alternative first exons. STAT5b might still have an indirect function in the regulation of *FAT/CD36* through

the regulation of other TFs, such as HNF3β whose putative binding sites are found in both promoters of exon 1a and 1b.

Based on the sex-differentiated CYP genes, Wiwi, O'Connor and Waxman have proposed two GH-mediated transcriptional regulation models, consisting of two TFs each, STAT5b + HNF4 as male-specific and HNF6 + HNF3β as female-specific. We performed an in silico promoter analysis using the Prometheus software and our lists of sex-differentiated genes. Preliminary results (unpublished data) suggest that the male-specific model (STAT5b + HNF4) can only be predicted in 5% of the promoters of rat hepatic male-predominant genes. For the female-predominant genes, 15% of these gene promoters contain the female-specific model (HNF6 + HNF3β). Therefore GH-activated STAT5b is the main factor for the sex-dimorphic gene expression in the liver, most of its actions are apparently indirect.

The use of alternative promoters

In paper II, we investigated the promoter structure of rat *FAT/CD36* and revealed that there are two alternative first exons similar to the human and murine *FAT/CD36*. These alternative first exons have different tissue expression pattern (Andersen et al. 2006) and respond differently to PPAR-agonists (Sato et al. 2002; Sato et al. 2007). We found that the alternative first exons of rat *FAT/CD36* are also regulated differently. The exon 1a displays a greater sex-specific expression difference. Furthermore, estrogen or GHc treatment of male rats induced preferentially through this promoter. The stimulatory effect of food withdrawal in muscle *FAT/CD36* expression was highest for exon 1a-3, suggesting that fasting might induce *FAT/CD36* expression preferentially through the exon 1a promoter. This is in line with the notion that PPARα, which would be more activated in fasted animals, induces exon 1a promoter more than that of exon 1b in skeletal muscle (Sato et al. 2007).

Two independent studies showed that more than 50% of the human genes contain putative alternative promoters (Cooper et al. 2006; Kimura et al. 2006). The use of alternative promoters has been reported in cell-type or developmental stage specific expression. Recently, it has also been suggested that alternative promoters might influence alternative splicing (Xin et al. 2008). If the alternative promoters are dys-regulated, it can lead to a disease state. For example, several imprinted genes such as IGF2 lose their imprinting in tumor cells (Davuluri et al. 2008). Furthermore, in a study of 16,735 genes, 38% of the cancer-related genes have a single promoter compared to 50% of the non-cancer genes. Though not all cancerous genes have multiple alternate promoters, they still have an average of two alternate promoters per oncogene but only 1.5 promoters per non-oncogene (Davuluri et al. 2008). Gene aberrations such as polymorphisms in the alternative promoters have been associated with diseases. It is interesting to note that one SNP, rs2151916, is located within the exon 1a promoter region of *FAT/CD36* (Ma et al. 2004), which might inactivate a putative binding site of the transcriptional repressor growth factor independent 1 (GFI1). Although GF1 is not expressed in our liver samples, our preliminary data suggested that the binding activity of that promoter segment

tended to be higher with male nuclear proteins than female ones (unpublished data).

The promoter to exon 1b of *FAT/CD36* can be defined as the core promoter since it showed least variation between cell types in mouse (Sato et al. 2002; Sato et al. 2007), human (Andersen et al. 2006) and rats (Cheung et al. 2007). We and others found that the alternative promoter to exon 1a seems to be more sensitive to regulation (Sato et al. 2002; Cheung et al. 2007; Sato et al. 2007). The use of alternate promoters and consequent alternative use of first exons allows more controlled gene regulation according to cell type or condition. It grants the complexity required for the highly elaborated molecular systems in mammals, particularly in humans.

microRNA

The discovery of miRNA changed the landscape of research on regulation of gene expression. It fills part of the gap between mRNA levels to protein levels. Some miRNAs are transcribed and possess promoters while others are derived as secondary products from gene splicing. However, little is known about the targets of miRNAs and how their expressions are regulated. The two miRNAs studied in this thesis, miR-29b and miR-122a, belong to the former type. Interestingly, the promoter regions of these two miRNAs are poorly conserved albeit the mature functional sequences of these miRNAs are identical across species. On the other hand, it is imaginable that the functions of these miRNAs are similar but their actual tasks are dissimilar in e.g. rodents and humans. Therefore the regulatory machineries are not conserved as they might not carry any evolutionary value. It poses another problem to the understanding of miRNA action, i.e. a larger limitation in the use of animal models or animal cell lines to study miRNA regulations and functions.

In this rising field of miRNA research, there are only a handful of publications about hormonal regulation. Gauthier and Wollheim summarized the importance of miRNAs in the insulin secretion of pancreatic β cells. It was shown that miR-375, miR-124 and let-7b can inhibit the expression of myotrophin (MTPN), hence suppressing insulin secretion (Gauthier 2006 nature med). In paper IV, we found that GHc suppresses the level of miR-29b, which might be related to an increase of a potential target, INSIG1, and a change in the protein pattern of an interacting protein of INSIG1, SREBP1. The involvement of miRNA in the development of insulin resistance, diabetes and other metabolic imbalances deserves further investigation.
Recent studies indicate that tissue-specific miRNAs may function at multiple hierarchical levels of gene regulatory networks, such as controlling the level of regulators for transcription and pre-mRNA splicing (Makeyev and Maniatis 2008). Shalgi and co-workers unveiled an interactive gene regulation network consisting miRNA and TFs. Bioinformatic tools enabled them to predict miRNA targets as well as putative TFBS. By analyzing the potential targets of miRNA and the putative binding sites of all genes including miRNA, they proposed five network designs where miRNA and TF jointly regulate the gene transcription (Shalgi et al. 2007). A few biological studies have observed an interaction of miRNA and TF involved in cell

differentiation (Fujita et al. 2008; Hino et al. 2008; Sun et al. 2008). This multilevel regulation would allow individual miRNAs to profoundly affect the gene expression programming.

In summary, gene expression is a finely monitored cellular event that involves regulatory networks consisting of regulators including TFs and miRNAs. There has been a pursuit on the master switch(es) for the sex-differentiated gene expression in the liver. GH secretory pattern and STAT5 could be potential master switches. As the complexity of gene regulation reveals, it is likely that the master switch(es) could as well be a component of a regulatory network.

TECHNOLOGICAL DISCUSSION

Quantity versus quality

The development of 'omics' technologies increases the amount of experimental data exponentially. These high-throughput screening technologies such as large quantity cloning-sequencing and microarray analyses have a higher possibility to generate false positive or false negative data than using conventional methods. Therefore other established techniques are often required to confirm such a discovery. At the same time, the vast amount of data also allows bioinformatics tools such as data integration, correlation analysis and data mining to blossom.

Even with the current technologies and the current genomic information, no microarray can claim to cover the entire genome. Each probe on a microarray is only a representative part of a gene, in most cases a coding region. Since most of the mammalian genes have isoforms form such as splice variants, most of the microarray platform cannot distinguish them.

This thesis provides an example of such genes with isoforms. *FAT/CD36* was first identified as a female-predominant gene through microarray analysis in paper I. On the microarray, it showed 18-fold higher in mRNA level in female liver than in male. The sequence on the microarray corresponds to exon 15, a part of the 3'-untranslated region. Solution hybridization assay and quantitative real-time PCR detecting specifically that region confirmed that females displayed a higher level of this transcript, but failed to show as high fold changes as the microarray results. In the genome database, the human and mouse homologues of *FAT/CD36* has multiple alternative first exons. We then in paper II showed that one of them (exon 1a) is more than two-times higher in female than in male. Exon 1a also responds to GHc more than three-times higher than the other alternative first exon (exon 1b). Although microarray analysis has developed into a technique with improved specificity and reproducibility, it still has limitations in detailed studies of gene expression.

High-throughput data analysis

In this thesis, we attempted to analyze the same sample at different 'omics' levels (miRNA, mRNA and metabolite levels) and integrate the results. However, it shows

that more effort will be needed in integrating the data translation from one level to another. In this case, the limited number of known spectra for metabolites in the reference libraries has put a hold on the identification of sex-different metabolites. Likewise for miRNA, the knowledge of biological functions of miRNAs and the algorithm for rational prediction of miRNA targets is of limited utility.

Apart from screening, 'omics' technologies provide data for correlation studies. This method is often referred as data mining. From microarray results, genes showing similar expression patterns can be clustered with the help of clustering programs. The co-regulated genes can then be studied with in silico promoter analysis to test if they are under the same transcriptional mechanism. Alternatively, genes can also be grouped according to their properties, such as molecular function or biological pathways. Gene Ontology (GO) provides a good source of annotation for this type of analysis. Genes that regulate metabolite levels in the circulation can be correlated to the metabolite profiles. Genes that show kinase activity can be correlated to phosphorylation of novel proteins detectable with 2-D gel electrophoresis.

The biggest obstacle in this type of research is discrepancies between technological platforms. Initiatives and efforts are made from international scientific consortia at all different 'omics' levels. MIAME (minimum information about a microarray experiment) was developed to ease the comparison between different microarray platforms at the transcriptomics level (Brazma et al. 2001). MIAPE (minimum information about a proteomics experiment) was put forward last year for standardizing the reporting of proteomics data, so that differences in techniques such as gel electrophoresis and mass spectrometry would no longer hamper data exchange (Taylor et al. 2007). At the metabolomics level, MIAMET (minimum information about a metabolomics experiment) has been put forward (Bino et al. 2004) and standardized data exchange guidelines is being prepared by the Metabolomics Standards Initiative. Then, the next level will be to allow data translation between different levels through these minimum information and related annotation.

TOWARDS SYSTEMS BIOLOGY

An interdisciplinary approach – systems biology – has recently emerged by combining high-throughput biological data with novel computational and mathematical tools to understand the specific design rules and principles of biological systems. Metabolic networks described by this approach usually display bow-tie "architecture" (Figure 23). Between the input nutrients and the building-blocks such as amino acids and fatty acids, there are only a handful of carriers (e.g. ATP) and precursor metabolites (e.g. glucose). Similarly in the transcription and translation processes, a few universal polymerase modules and a universal codon usage protocol determine the production of a huge variety of proteins (Csete and Doyle 2004). Unfortunately, this type of robust design is inherently fragile to hijacking. For example, viruses and tumor cells hijack the host's transcription and translation machinery for pathological proliferation.

Diseases such as the metabolic syndrome can also be viewed as a breakdown of robustness. Kitano and colleagues proposed that a drifted equilibrium between energy intake and consumption, primarily glucose, triggers the feedback mechanisms to handle the accumulation of excess energy. When a certain threshold is reached, the feedback mechanisms unfortunately collapse and turn into a vicious cycle, resulting in elevated levels of plasma NEFA and glucose (Kitano et al. 2004). The high-throughput data collected in this thesis, after refinement and further analysis with appropriate bioinformatic tools, would have the potential to describe the sex differences at a systems biology persepctive allowing better understanding in how these differences would affect disease development.

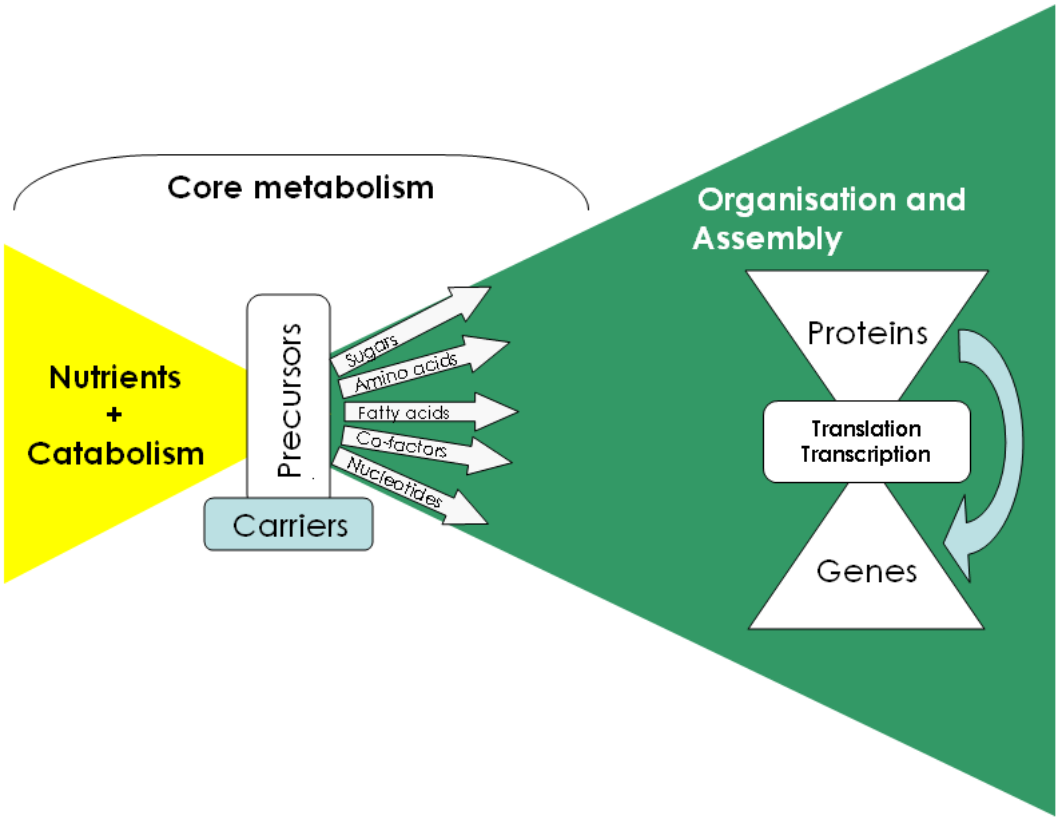

Figure 23. The nested bow-tie architectures of metabolism. Adopted from (Csete and Doyle 2004)

CONCLUDING REMARKS

This thesis explored sex differences in hepatic fuel metabolism at the molecular level and the findings that are summarized below.

- Hepatic transcript profiles were found to be sex-different. Healthy male rats with free access to food had a higher expression of genes encoding important proteins for glucose oxidation, glycogen production, lipid synthesis, fatty acid oxidation, and amino acid turnover. GHc (continuous GH infusion) treatment in males changed the mRNA levels for 16% of the genes expressed in the liver. 21 female-predominant genes were induced by GHc in male rats and 51 male-predominant genes were reduced by GH.

- A sex-differentiated and GH-regulated gene, *FAT/CD36*, were shown to have two alternative first exons and corresponding promoters. GH had a dual action on the regulation of this gene. GHc (a female secretory pattern) induced the expression of *FAT/CD36* preferentially through the promoter of exon 1a, whereas GHi (GH injections) repressed the expression through both alternative promoters.

- Hepatic transcript profiles were found to be sex-different after mild starvation and after insulin-induced hypoglycemia. After mild starvation, the male livers had lower expression of lipogenic genes and higher expression of gne for fatty acid oxidation than the females. Moreover, a larger number of hepatic genes were regulated by insulin-induced hypoglycemia in females than in males.

- Two miRNAs, miR-29b and miR-122a, exhibited sex-different expression in rat liver. In addition, the level of miR-29b is suppressed by GHc in parallel with an increase in protein level of its potential target, INSIG1.

- Sex-different metabolic profiles were obtained using extracts derived from liver lysates or serum. The level of urea was higher in male serum whereas the level of ornithine was higher in female liver lysates.

POPULAR SCIENCE SUMMARY

Men and women are not "created equal". Male and female individuals differ in sex by the genetic makeup. A combination of XY defines a male and XX a female. Furthermore, the major observable sex differences are behaviour, body composition, genome, hormones and metabolism. They are orchestrated by multiple factors such as developmental stages, nutritional condition, endocrine status and environmental factors. These discrepancies in turn also account for differences in cell and tissue functions, circulating metabolite levels and other physiological parameters.

One important aspect of metabolic control in the human body deals with fuel metabolism, which balances the energy intake and output. Imbalance in fuel metabolism can cause diseases such as obesity, diabetes and cardiovascular disease. One of the risk factors for these diseases is central obesity (commonly referred to as belly fat), which is more often found in men than women before menopause.

The liver is a key metabolic organ coordinating various physiological processes. Males and females have slightly different metabolic needs. Therefore, this key metabolic organ is adapted to its sexual makeup and responds differently to the same metabolic challenge. More than 20 years ago, scientist found that the sex-dimorphic pattern of growth hormone secretion in rats causes the liver to display a sex-different metabolism, including drug metabolism and glucose and lipid homeostasis. Although most of these studies were conducted in rodents, similar sex differences might also exist in humans.

In order to obtain a deeper understanding about the sex-differentiated activities of the liver, we screened for the molecular sexual differences (messenger RNA/mRNA, microRNA and metabolites) using high-throughput techniques. We have found that the metabolic activities in male and female rats are indeed different. Also in the serum, the metabolic composition is different between male and female rats. Some of these differences might be contributed by the liver.

Regarding fuel metabolism, the liver of a male rat has a higher expression of genes encoding important proteins involved in glucose, fatty acid and amino acid metabolism at the basal state. It means that males normally might have a higher metabolic rate than females. During starvation, one of the crucial functions of the liver is to synthesize and export glucose. This process requires a lot of energy that is usually obtained by degradation of fats. We found that, after mild starvation, the male livers had lower expression of the genes involved in fatty acid synthesis and higher expression of those involved in fatty acid degradation. These suggest that male livers might consume more fats than females, so that they can supply their bodies with more glucose.

Since males and females are different in body composition and metabolism, it is possible that there also exist differences in the way they develop metabolic imbalance and related diseases. An increased understanding about any sex differences in fuel metabolism should be useful in efforts to improve the diagnosis and treatment of both male and female patients suffering from those diseases.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Män och kvinnor är olika. De skiljer sig åt avseende kromosomuppsättning, vilket bland annat leder till att män och kvinnor ser olika ut. En kombination av XY definierar en hane och XX en hona. De synliga könsskillnaderna är även påverkade av andra faktorer som utvecklingsstadie, nutrition, hormoner och miljöfaktorer. Andra könsskillnader innefattar beteende, kroppskomposition och ämnesomsättning. Dessa skillnader är relaterade till skillnader på molekyl-, cell- och vävnadsnivå.

En viktig del av ämnesomsättningen i kroppen är energiomsättningen (omsättningen av glukos, fett och aminosyror). Den styr tillgång och efterfrågan på energi samt ser till att överskott av energi lagras för senare bruk. Obalans kan orsaka sjukdomar som fetma, diabetes och hjärt-kärlsjukdomar. En av riskfaktorerna för dessa sjukdomar är bukfetma (folklig. Eftersom bukfetma är vanligare hos män än fertila kvinnor, har detta kopplats samman med den ökade risken för män att utveckla metabola sjukdomar. Detta kan i sin tur bero på könsskilld ämnesomsättning.

Levern är ett viktigt metabolt organ som samordnar olika fysiologiska processer. Män och kvinnor har något annorlunda metabola behov och därför kan man tänka sig att levern är anpassad till sitt kön och att mäns och kvinnors levrar kanske reagerar något annorlunda på samma fysiologiska förändring. För mer än 20 år sedan upptäckte forskare att han- och honråttor har olika utsöndringsmönster av tillväxthormon. Detta ger upphov till skillnader i leverns ämnesomsättning. Som exempel kan nämnas läkemedels-metabolism samt glukos och lipid omsättning. Trots att många av dessa studier huvudsakligen har utförts på gnagare, så har liknande könsskillnader också observerats hos människor.

För att få en djupare förståelse av könsdifferentierade aktiviteter i levern, så utforskade vi eventuella könsskillnader på molekylär nivå (budbärar-RNA/mRNA, mikroRNA och metaboliter) med hjälp av olika ”high-throughput” metoder. Vi har visat att han- och honråttor uppvisar delvis olika metabola aktiviteter i levern. Dessutom skiljer sig hanars och honors metabola sammansättning åt i blodplasman. Några av dessa könsskillnader kan vara en följd av olikheter i leverns metabolism.

Vad gäller könsskillnader i ämnesomsättning, så uppvisar levern hos hanråttorna ett högre uttryck av gener som är inblandade i metabolismen av glukos, fett och aminosyror. Det betyder att en hanlever normalt har högre ämnesomsättning än en honlever. Under svält är en av de viktigaste funktionerna för levern att producera glukos. Denna process kräver mycket energi, vilket normalt genereras genom att förbränna fett. Vi fann att efter en mild svält, hade hanlevern en lägre nivå av genuttryck för fettproduktion och en högre nivå av genuttryck för fettnedbrytning. Hanlevern tros därför konsumera mer fett än honlevern och därmed kan den vid behov generera mer glukos.

Eftersom män och kvinnor har olika kroppskomposition och ämnesomsättning är det möjligt att män och kvinnor kanske utvecklar metabol obalans och relaterade sjukdomar på något olika sätt. En ökad insyn i de molekylära mekanismerna bakom könsskillnader i ämnesomsättning och relaterade sjukdomar kan i framtiden komma att förbättra diagnostik och behandling av patienter som lider av metabola störningar.

科 普 摘 要

男人和女人並不是“平等地造成的”。其不同的遺傳基因定義男性和女性。基因型 XY 定義為男性，而 XX 為女性。此外，性別差異也受到發育階段、營養狀況、內分泌水平和週邊環境多種因素所影響。這些主要差異導致男女間行為舉止、身體構造、激素水平和新陳代謝有所不同，從而也導致了細胞和組織功能、代謝物水平和其他生理參數出現分歧。

生物體裡其中一個重要的新陳代謝環節是能量的代謝。這代謝平衡了能量的需求和吸收，並把過剩的能量儲存起來供日後使用。能量代謝失衡可導致很多疾病，如肥胖症、糖尿病與心血管疾病。這些疾病的其中一個危險因素是中央肥胖，俗稱「大肚腩」，而這種身體特徵較普遍在男性身上出現。

肝臟是一個重要的代謝器官。它協調各種生理過程。男性和女性對新陳代謝的需要略有不同。因此，對相同的生理刺激，男性和女性的肝臟會作出不同的回應。二十多年前，科學家發現男性和女性的生長激素的分泌模式並不相同，從而導致了肝臟的某些代謝呈現不同，例如藥物代謝和血糖及血脂的平衡。這方面的研究主要是在動物體進行的，但類似的情況也可能在人類中存在。

為了對肝臟活動的男女差別有更深刻的概念，我們利用生物晶片技術從分子水平上篩選性別的差異（如信使核糖核酸 mRNA 、微核糖核酸 microRNA 和代謝物）。我們的實驗發現男性和女性肝臟的新陳代謝的確有所不同。此外，在血清樣本中，代謝物組成也呈現性別不同。我們猜想血清樣本的差別有可能與肝臟活動的性別差異有關。

在基礎代謝狀態下，涉及重要葡萄糖，脂肪酸和氨基酸代謝的基因在雄性大鼠的肝臟裡表達比雌性較多。這意味著雄性能量的代謝率通常高於雌性。在飢餓狀態下，肝臟最重要的功能是合成葡萄糖供應生理需求。這一個化學反應需要大量能源，而這些能源通常源於脂肪消耗。我們發現在飢餓狀態下，涉及脂肪酸消耗的基因在雄性的肝臟裡表達較低，而涉及脂肪酸合成的基因表達較高。這意味著雄性比雌性的肝臟消耗更多的脂肪，使他們可以產生更多的葡萄糖供身體其他器官使用。

由於男性和女性的身體構造及能量代謝有所不同，他們很可能從不同的方式導致代謝失衡及相關的疾病。了解能量代謝上的性別差異不僅有助於防止代謝失衡在男性和女性中發生，而且可以改善相關疾病在不同性別上的診斷和治療。

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