Sexually Dimorphic Cytochrome P450 Gene Expression in Wildtype and CYP2C18/CYP2C19 Transgenic Mice

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Cover: Front cover from Drug Metabolism and Disposition. Volume 36, May 2008. FISH analysis result showing the chromosomal location of the inserted CYP2C18/19 gene segment in the CYP2C18/CYP219 humanized mouse investigated in this thesis.

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Abstract

Drugs on the market continuously improve and target directed drugs become more and more common. Animal models are used to predict effects and side effects that may occur when drugs are administered to humans. The use of mice within medical testing is steadily increasing and the use of transgenic mouse models is also expanding. In this thesis, both traditional mouse strains, and a humanized CYP2C18/CYP2C19 transgenic mouse model is investigated.

Initially, we found that cytochrome P450 (CYP) enzymes in the liver of traditional mouse strains are expressed with large strain and sex differences in both protein levels and metabolic activity. These differences contribute to the difficulties when extrapolating data to man from tests performed on animals.

The CYP2C18/CYP2C19 transgenic mouse model investigated contains, in addition to the murine Cyp2c enzymes, the human CYP2C18 and CYP2C19 enzymes. Heterozygous CYP2C18/CYP2C19 transgenic mice were healthy animals, without obvious adverse phenotypic characteristics and were determined to possess approximately 12 tandem copies of the inserted CYP2C18/CYP2C19 gene segment. The inserted gene was translated into CYP2C18 and CYP2C19 mRNA and CYP2C19 protein more efficiently in male than in female mice livers, whereas no sex differences were found in small intestine, lung or brain. The enzymatic activity of CYP2C19 is also higher in males than in females.

The sexually dimorphic, male predominant, *CYP2C18* and *CYP2C19* gene expressions, as well as the expression of female predominant, endogenous, mouse *Cyp2c37, Cyp2c38, Cyp2c39* and *Cyp2c40* genes were found to be dependent on androgens and the release pattern of growth hormone.

In summary, large strain– and sex differences in CYP expression occur in mice. Sex differences in expression of *CYP2C18* and *CYP2C19* are evident in a transgenic mouse model containing human *CYP2C18* and *CYP2C19* genes. The sex dependent differences in expression are affected by gonadal hormones and growth hormone. The transgenic mouse model, may serve as a model for investigation of human *CYP2C18* and *CYP2C19* gene regulation.

Keywords: mouse, CYP2C, liver, transgenic, gender, sex, dimorphic, mRNA, protein, metabolism

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Ur kaoset talade en röst till mig och sade:
"Le och var glad för det kunde vara värre!"
Och jag log och var glad
– och det blev värre!

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Löfgren, S., Hagbjörk, A-L., Ekman, S., Fransson-Steen, R. & Terelius, Y. (2004) Metabolism of human cytochrome P450 marker substrates in nouse: a strain and gender comparison. *Xenobiotica 34, 811-834.*

II Löfgren, S., Ekman, S., Fransson-Steen, R. & Terelius, Y. Few alterations in clinical pathology and histopathology observed in a CYP2C18&19 humanized mice model. (Submitted).

III Löfgren, S.*, Baldwin, RM.*, Hiratsuka, M., Lindqvist, A., Carlberg, A., Sim, S., Schülke, M., Snait, M., Fransson-Steen, R., Terelius, Y. & Ingelman-Sundberg, M. (2008) Generation of mice transgenic for human CYP2C18 and CYP2C19: Characterization of the sexually dimorphic gene and enzyme expression. *Drug metabolism and disposition 36, 955-962.*

IV Löfgren, S.*, Baldwin, RM.*, Mwinyi, J., Terelius, Y., Fransson-Steen, R., Waxman, D. & Ingelman-Sundberg, M. Regulation of Human CYP2C18 and CYP2C19 in Transgenic Mice: Influence of Castration, Testosterone and Growth Hormone (Manuscript).

*, these authors contributed equally to these works.

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Abbreviations

7-EFC	7-ethoxy-4-trifluoromethyl coumarin
ACN	acetonitrile
ATP	adenosine triphosphate
BAC	bacterial artificial clone
BSA	bovine serum albumin
CAR	constitutive androstane receptor
CAR-RE	constitutive androstane receptor-responsive element
cDNA	complementary DNA
CYP	cytochrome P450
DAPI	4', 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dUTP	2'-Deoxyuridine 5'-Triphosphate
ECL	enhanced chemiluminescence
EMSA	electrophoretic mobility shift assay
EROD	7-ethoxyresorufin O-deethylation
ES cells	embryonic stem cells
FAM	6-carboxyfluorescein
GAPDH	glyceralaldehyde-3-phosphatedehydrogenase
gDNA	genomic DNA
GH	growth hormone
HAc	acetic acid
HE	haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNF	hepatocyte nuclear factor
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine guanine phosphoribosyltransferase
HRP	horseradish peroxidase
IL-2	Interleukin-2

LC	liquid chromatography
mRNA	messenger RNA
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
PAGE	polyacrylamide gel electrophoresis
pH	negative logarithm of the hydrogen ion concentration of an aqueous solution
Poly (dI-dC)-Poly (dI-dC)	polydeoxyinosinic-deoxycytidylic acid
PVDF	polyvinylidene fluorid
PXR	pregnane X receptor
RNA	ribonucleic acid
rtPCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
STAT5b	signal transducer and activator of transcription 5b
SYBR Green	an asymmetrical cyanine dye used as nucleic acid stain
TBS	tris-buffered saline
UV	ultraviolet
VIS	visible
YAC	yeast artificial clone

Introduction

Background

Available drugs continuously improve- and with medical treatments today we can cure previously untreatable and fatal diseases. Analytical methods develop and target-directed drugs become more and more common. However, knowledge about factors influencing the fate of the administered drugs in the body is essential to avoid undesired and toxic effects. Adverse effects are not always dose dependent; they may depend on the patients' diet, drinking- and smoking habits as well as interactions with other drugs. Polymorphism in the cytochrome P450 (CYP) system may also give unexpected and toxic effects.

In modern drug development, animal models are used to predict drug kinetics and toxicity in humans. Interpretation of the results and extrapolations to man of data from studies performed on animals may be difficult due to broad interspecies and interindividual variations among cytochrome P450 enzymes (Gonzalez, 1990). Different species may express different levels of related enzymes/subfamilies and these differences can give rise to species specific toxic effects. Differences in expression of CYP enzymes have been observed between genders (Kato & Yamazoe, 1992; Werner *et al.*, 1995), and expression may also vary with age (Wauthier *et al.*, 2007). It is therefore important to take CYP enzyme differences into consideration when using animal species as models to predict kinetics and toxicity in humans (Pearce *et al.*, 1992).

Over recent years, more and more experiments have been performed in mice, which are used both as disease models and for drug safety testing.

Mice, like other species, have evolved complex mechanisms for metabolizing compounds and other molecules that enter the body. Innoxious molecules should be put to some use and toxic compounds should be neutralized. The means to accomplish this has been solved in slightly different ways in different species and strains. Despite the growing use of mice, relatively few studies have been performed to examine specific reactions catalyzed by different mouse CYP isoforms and their relevance to humans. When extrapolating data from mouse models to humans, it is important to compare the drug metabolism of mouse to that of humans (Perloff *et al.*, 2000).

Optimized animal models where expected (and unexpected) human pathology is mimicked and where pathogenic biological effects are identified are desirable to predict adverse compound effects in humans. Expectations have risen that humanized mice will work as tools to accomplish more predictive animal models.

Elimination of drugs from the body is accomplished by metabolism of the drug, usually to a more water-soluble state, and finally by excretion. How quickly the drug is eliminated from the body is highly dependent on the rate with which the compound is metabolized. The rate of the transformation affects the concentration of the compound in different organs as well as how long the drug stays in the body. The cytochrome P450 system in the liver is central in oxidation of a wide variety of structurally unrelated compounds, including exogenous compounds such as pharmaceutical agents, chemicals and other lipophilic xenobiotics, as well as endogenous steroids, fatty acids, prostaglandins and vitamin D3 (Bièche *et al.*, 2007; Gonzalez, 1990; Ryan & Levin, 1990). However, CYP enzymes are also expressed in other tissues (Bièche *et al.*, 2007).

Drug metabolising enzymes

The metabolism and elimination of xenobiotic compounds is traditionally divided into two phases. The so-called Phase I is usually effected by P450 monooxygenases, also called cytochrome P450s (CYPs) or mixed-function oxidases. The most common primary outcome of Phase I activity is the addition of one atom of oxygen to the substrate, although many other reactions are also catalyzed by cytochrome P450 enzymes (Isin & Guengerich, 2007). The metabolites formed by the Phase I reactions are generally more hydrophilic than the parent compound. Furthermore, the

insertion of an oxygen atom into the parent molecule allows the resulting metabolites to easier undergo other metabolic transformations, known as Phase II, or conjugation reactions. Phase II reactions are enzymatic reactions that covalently link an endogenous compound to the product derived from the cytochrome P450 oxidation. Such conjugates are usually water-soluble, and therefore easily excreted in either bile or urine (Kyle & Farber, 1991).

The combination of Phase I and Phase II metabolism converts lipophilic xenobiotics into forms that can readily be excreted from the body, hence this metabolism promotes elimination of compounds that would ultimately prove toxic if allowed to accumulate in the body. For this reason, oxidation is widely regarded as a detoxifying mechanism. However, there is little doubt that CYP dependent oxidation can also convert some foreign compounds into reactive metabolites. The biotransformation sometimes produces species that are clearly more toxic, mutagenic or carcinogenic than the original substrate (Kyle & Farber, 1991). Furthermore, extensive and variable biotransformation by cytochrome P450s can sometimes result in the formation of one or more metabolites with pharmacological activity, which may be equal to, or even higher than that of the parent drug (Schellens *et al.*, 2000).

Cytochrome P450

The CYP enzymes are heme containing, membrane bound proteins. The original term cytochrome P450 was coined at the time when the protein was given its provisional name (Omura & Sato, 1962). The figure, 450, originates from the peak absorbance at 450 nm. The drug metabolizing CYPs are commonly localized in the membranes of the smooth endoplasmatic reticulum, but mitochondrial (Omura, 2006) and nuclear activities (Madra *et al.*, 1996) have also been described. However, the drug metabolizing activities of the last two compartments are low compared to that of the system in the endoplasmatic reticulum.

To be enzymatically active, the CYPs require oxygen and a supply of electrons. The first electron is derived from NADPH via an accessory flavoprotein (NADPH cytochrome P450 reductase). Either NADPH cytochrome P450 reductase or cytochrome b_5 donates a second electron to yield a reduced complex with two electrons (Guengerich, 2001). The reductive activation of oxygen is followed by cleavage of the oxygen-

oxygen bonds. One atom of oxygen is transferred to the substrate, while the other atom is incorporated into water.

The members of the P450 superfamily are divided into families, subfamilies and individual members. Members within a gene family have >40% homologous amino acid sequence, and the families are enumerated with Arabic numbers. Members within a subfamily share >55% of their amino acid sequences, and are grouped with Latin letters (Gonzalez, 1990). Finally, the individual isoforms are identified by again using Arabic numbers (Nelson *et al.*, 1996).

There are three main cytochrome P450 gene families involved in drug metabolism in the human liver, namely CYP1, CYP2 and CYP3 (Rang *et al.*, 1999). These families are also important in many extrahepatic tissues including lung, kidney, heart, brain, gut and nasal epithelium (Jeffery, 1991).

Cytochrome P450-CYP2C family

In humans the CYP2C family has four members, namely CYP2C8, CYP2C9, CYP2C18 and CYP2C19 (Goldstein, 2001), and they contribute to biotransformation of about 30% of all drugs in the liver (Guengerich, 2003). Despite the >80% amino acid sequence identity shared by CYP2C8, 9, 18, and 19, considerable differences in substrate specificity exist between them. Diclofenac, tolbutamide, and *S*-warfarin are typical CYP2C9 substrates, and *S*-mephenytoin and *R*-omeprazole are characteristic substrates for CYP2C19. CYP2C8 metabolizes a few known drugs such as paclitaxel, troglitazone, and zopiclone (Goldstein & de Morais, 1994; Pelkonen *et al.*, 1998). CYP2C8 and CYP2C9 are also involved in the endogenous metabolism of arachidonic acid (Rifkind *et al.*, 1995; Daikh *et al.*, 1994). To date, no CYP2C18 protein or drug substrate has been found *in vivo* (Läpple *et al.*, 2003).

The mouse Cyp2c subfamily is one of the largest and most complex in the cytochrome P450 super family and contains 15 genes and four pseudogenes (Figure 1) all located to chromosome 19 (for an update, see http://drnelson.utmem.edu/CytochromeP450.html). Cyp2c29 was the first mouse Cyp2c member identified (Matsunaga *et al.*, 1994), followed by Cyp2c37, Cyp2c38, Cyp2c39, and Cyp2c40 (Luo *et al.*, 1998). Thereafter, ten more mouse Cyps have been found, namely Cyp2c44 (DeLozier *et al.*,

2004), Cyp2c50, Cyp2c54 Cyp2c55 (Stewart & Strother, 1999) and Cyp2c65-70 (Nelson *et al.*, 2004; Stewart & Strother, 1999).

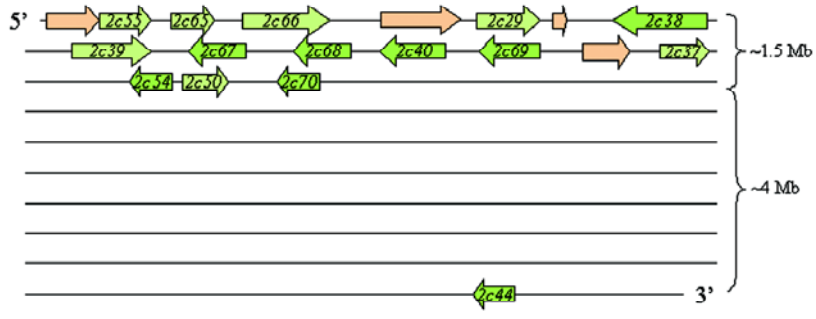

Figure 1. Mouse Cyp2c locus on chromosome 19. Genes are marked with green arrows, while pseudogenes are marked with apricot arrows. Cyp2c44 is located 3.8Mb downstream from the main Cyp2c cluster (1.5Mb).

The mouse Cyp2c: s differ in their tissue distribution and catalytic function (Luo *et al.*, 1998; Tsao *et al.*, 2000; Tsao *et al.*, 2001; DeLozier *et al.*, 2004; Stewart & Strother, 1999) but many of the mouse Cyp2c isoenzymes metabolize the same substrates, like arachidonic acid, but to different regio- and stereo specific products.

Transgenic mice

An important step in the development of transgenic mice was reached by Rudolf Jaenisch in 1974 (Jaenisch & Mintz, 1974). Viral DNA was successfully inserted into mouse blastocysts (preimplantation embryos) and the inserted DNA persisted to adult life. Another important milestone was when Gordon in 1981 injected foreign DNA into mouse pronuclei (Gordon & Ruddle, 1981). The genetic material was successfully integrated into the genomes of the newborn mice, and maintained through further development to adulthood. Thereafter, it would just take one more year until expression of inserted DNA segments were shown (Costantini & Lacy, 1982).

Today there are two main strategies for developing transgenic mice: In the so-called pronuclear injection, the foreign DNA is inserted into one of the pronuclei (usually the male which is larger) just after fertilization of the egg, see Figure 2. The injected transgenic DNA tends to integrate randomly into the genome, and often many tandem copies of the insert are integrated

head-to-tail. The resulting mouse is only partially transgenic, but if the transgenic cells contribute to the germ line some transgenic eggs or sperms will be produced and the next generation of mice will be fully transgenic (Voncken & Hofker, 2005).

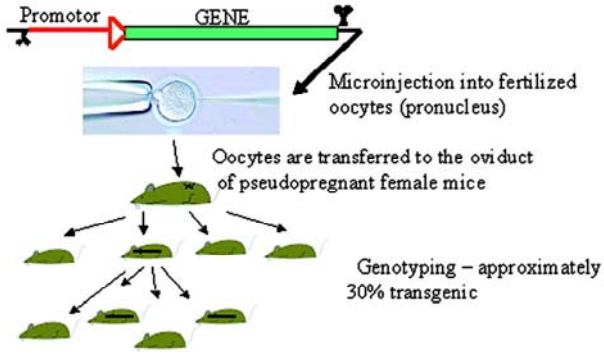

Figure 2. Pronuclear Injection

The second strategy to incorporate foreign DNA is by using embryonic stem cells (ES cells), see Figure 3. These cells are isolated from blastocysts and they are pluripotent, i.e. they are undifferentiated and have the capacity to differentiate into any cell type when introduced into an embryo. The foreign DNA is introduced into the ES cells *in vitro* and may introduce randomly, but if the foreign DNA possesses DNA sequences that are homologous to mouse DNA a single shift of DNA may occur. In that case a single copy of the transgene is inserted at a specific site. The transgenic ES cells are injected into the blastocyst of a donor mouse and the resulting blastocyst is transferred into the uterus of a foster mouse. ES cells colonize the host embryo and often contribute to the germ line, with the result that some sperm carry the transgenic DNA. When these sperm fertilize a normal egg, a transgenic heterozygous mouse containing foreign DNA is produced (Voncken & Hofker, 2005).

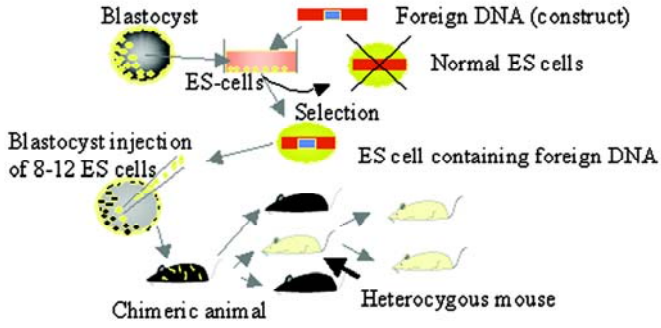

Figure 3. Generation of mice using embryonic stem (ES) cells.

Generation of mice containing the human CYP2C genes

The cytochrome P450 transgenic mouse project within AstraZeneca was originally divided into two parts. The first part was to generate a mouse in which the human *CYP2C* genes were added. The second included the use of ES cells to target the mouse *Cyp2c* genes to make a mouse line devoid of its *Cyp2c* gene cluster. Ultimately, once both types of genetically modified mice had been generated, they would be crossbred to make a line whose only source of *CYP2C* genes were the introduced human genes.

Several strategies were explored to achieve this goal and initially it was believed that using pronuclear microinjection to introduce a YAC (yeast artificial clone) spanning the complete human locus would be the best approach. Unfortunately a suitable YAC was not found and an alternative approach using a bacterial artificial clone (BAC) was suggested. However, the human locus is ~437kb and thus too large to be contained on a single BAC. The strategy adopted was therefore to identify two BACs, each encoding two CYP2C genes of the human cluster, and inject each of the two BACs separately. An attempt to include all four genes by simultaneous coinjections of both BACs was also performed but was unsuccessful.

A BAC clone named 466J14, containing the human *CYP2C18* and *CYP2C19* genes was identified. The 5' end of the BAC is located at position –5,828 bp from the start of exon 1 of *CYP2C18* and the 3' end of the BAC is located at position +30,869bp from the end of exon 9 of *CYP2C19*, see Figure 4. BAC DNA was prepared by digestion to release the desired *CYP2C18* and *CYP2C19* genes and the gene fragment was purified. The purified BAC DNA was injected into C57BL/6 eggs

(pronuclear injection) and founders were identified by genotyping of DNA extracted from tail or ear biopsies.

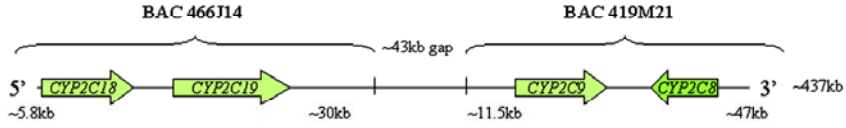

Figure 4. Human CYP2C locus, located to chromosome 10, and regions contained within BAC 466J14 and 419M21.

A second BAC clone named 419M21 (Figure 4) has been used to generate a humanized mouse containing human *CYP2C8* and *CYP2C9* genes. No knockout model devoid of the mouse *CYP2C* genes has been generated to date.

Genetic regulation of CYP2C genes

Endocrine cells synthesize steroid hormones, such as glucocorticoids, mineralocorticoids, androgens and estrogens. Once produced, they enter the blood stream to reach their target cells, where they diffuse through the cellular membrane and bind to their receptors. The receptors are located either in the cytoplasm or within the nucleus in an inactive state. Once the receptors bind their ligands (hormones), they transform into their active state. The activated hormone-receptor complex can effectively bind to DNA elements, so called hormone responsive elements and thereby regulate gene transcription (Tsai & O'Malley, 1994), see Figure 5.

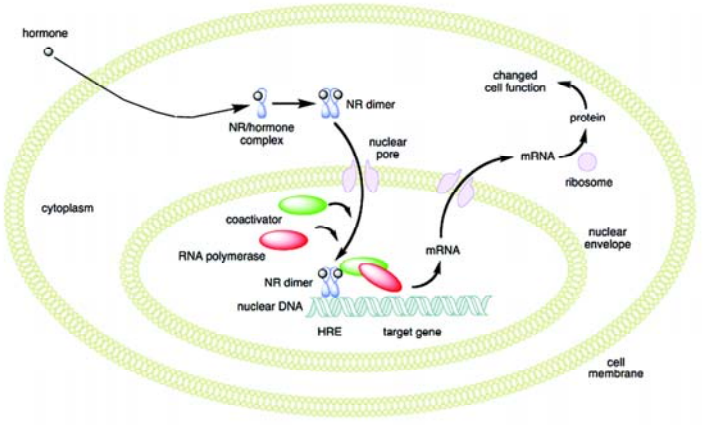

Figure 5. General overview of the mechanism for nuclear receptor action. Hormone binds to its nuclear receptor (NR), dimerizes, and translocates to the nucleus where the NR binds to its specific sequence of DNA, the so-called hormone responsive element (HRE). The nuclear receptor DNA complex in turn recruits other proteins that are responsible for transcription of downstream DNA into mRNA, which is eventually translated into protein, resulting in cell function changes. Picture modified from http://en.wikipedia.org/wiki/Nuclear_receptors.

Growth hormone (GH) is released from the pituitary gland in a sexually dimorphic manner. In many species, including rats (Jansson *et al.*, 1985) and mice (MacLeod *et al.*, 1991) males secrete GH in a highly pulsatile manner causing interpulse intervals of little or no detectable GH in plasma, while the female release is characterized by more frequent pulses, giving an almost continuous presence of GH in plasma. The GH free interval observed in males is required for expression of some male-specific CYPs, such as rat *CYP2C11* (Waxman *et al.*, 1991). Rats treated with continuous exogenous GH over several days abolish the expression of male-specific liver genes and dramatically induce female-specific genes, such as rat *CYP2C12* (Thangavel *et al.*, 2004).

In rats, the female specific *CYP2C12* gene is regulated by signal transducer and activator of transcription 5 (STAT5) together with hepatocyte nuclear factors (HNF) -4 and –6 (Sasaki *et al.*, 1999), as well as HNF3α and HNF3β (Delesque-Touchard *et al.*, 2000). The female-specific pattern is proposed to be stimulated by the liver enriched HNF: s and dominantly inhibited by the GH-activated STAT5b that is manifest in males (Delesque-Touchard *et al.*, 2000). Another hypothesis is that STAT5 (and other transcription factors) do not have access to their target elements in

male rats which causes a silencing effect of the *CYP2C12* gene (Endo *et al.*, 2005). The expression of the GH-pulse inducible, male-specific *CYP2C11* is regulated by STAT5 as well as liver nuclear factors (Park & Waxman, 2001). STAT5-DNA complexes are formed in male rats but not in female rats. The *CYP2C11* STAT5 site is flanked by a HNF3 consensus sequence, and HNF3β can suppress GH-inducible STAT5b tyrosine phosphorylation and thereby also *CYP2C11* expression (Park & Waxman, 2001).

STAT5b is a transcription factor, which responds to sexually dimorphic GH release. When GH binds to the GH-receptor, the GH-receptor-associated tyrosine kinase (JAK2) is activated. JAK2 in turn phosphorylates tyrosine in the cytoplasmic domain of the GH-receptor at multiple sites, generating docking sites for STAT5b which is activated and dimerizes (Waxman & O'Connor, 2006). The activated complex translocates from the cytosol into the nucleus and induces transcription of the target gene (Waxman *et al.*, 1995). STAT5b undergoes repeated activation in direct response to successive GH pulses in adult male rats, while the activation of STAT5b is desensitized by the more persistent pattern of GH exposure in females (Tannenbaum *et al.*, 2001).

The genetic regulation of the human *CYP2C* genes is partially known. In the promotor regions of *CYP2C8*, *CYP2C9* and *CYP2C19,* binding sites for the constitutive androgen receptor (CAR-RE) have been identified (Chen *et al.*, 2003; Dvorak *et al.*, 2003; Gerbal-Chaloin *et al.*, 2002) and in all three genes the CAR-RE binds CAR as well as the pregnane X receptor (PXR). Glucocorticoid-responsive elements have also been identified in the promotor regions of *CYP2C8, CYP2C9* (Gerbal-Chaloin *et al.*, 2002; Dvorak *et al.*, 2003) and *CYP2C19* (Chen *et al.*, 2003). CYP2C9 appears to be a primary glucocorticoid-responsive (GR) gene, which could also be induced through CAR/PXR activation (Gerbal-Chaloin *et al.*, 2002), while CYP2C19 may be upregulated equally by CAR/PXR and GR (Chen *et al.*, 2003).

Aims

The overall aims of this thesis were to examine differences in cytochrome P450 metabolism in commonly used mouse strains and to characterize a transgenic humanized CYP2C18 and CYP2C19 mouse model and explore the usefulness of this model in giving a more human-like metabolism in pharmacokinetic and toxicological studies.

The specific aims of this thesis were:

- To study differences between commonly used mouse strains and genders regarding cytochrome P450 activities and protein levels.

- To characterize a heterozygous *CYP2C18* and *CYP2C19* gene-addition mouse to obtain information regarding clinical status, clinical pathology and anatomical pathology.

- To characterize a heterozygous *CYP2C18* and *CYP2C19* gene-addition mouse to obtain information regarding gene expression, enzyme expression and incorporation of the transgene segment.

- To elucidate sexually dimorphic gene expression observed in the *CYP2C18* and *CYP2C19* gene-addition mouse with main emphasis on GH and androgen effects on mRNA expression and *CYP2C19* promoters.

Materials and Methods

Animals

Mice (Paper I)

In paper I, livers from five different commonly used mouse strains were analyzed. The strains analyzed were CBA, C57BL/6, 129/SvJ, NMRI and CD1. In addition a crossbreed mouse (C57BL/CBA) and a transgenic mouse (Muta™Mouse) were analyzed. The CBA, C57BL/6, C57BL/CBA and 129/SvJ strains were all 8 weeks old at organ sampling, the NMRI mice were 4-5 weeks, the Muta™Mouse were 12 weeks and finally the CD1 mice were 24 weeks old. Liver microsomes from both males and females (except Muta™Mouse where only males were available) were prepared. Livers were pooled for each strain and sex separately. Pools prepared from CBA, C57BL/CBA and C57BL/6 mice contained 10 animals per gender; CD1 pools contained livers from five animals per gender. The 129/SvJ and NMRI pools were prepared from nine males and 10 females, each. The Muta™Mouse pool contained 11 males.

Mice (Paper II-IV)

In papers II-IV, transgenic mice, heterozygous for the human *CYP2C18* and *CYP2C19* genes, and their wildtype littermates were used. The transgenic model was developed at AstraZeneca Transgenic Centre in Mölndal, Sweden. The model was generated by nuclear injection of a human bacterial artificial chromosome (BAC clone) RP11-466J14, which contains both complete *CYP2C18* and *CYP2C19* genes. In all studies with this mouse model the founders were genotyped using genomic DNA (gDNA) extracted from tail or ear biopsies.

In paper II, six animals per group were examined from each gender and genotype. All mice were observed clinically one week prior to necropsy at an age of approximately 11 weeks old. During that week, clinical signs were recorded and the body weight gains and food consumptions were measured. Individual body weights were recorded four and six days before necropsy for males and three and six days before necropsy for females. Six males and six females from each genotype (wildtype and tg-CYP2C18 & 19) were examined.

In paper III animals of different ages were used for the different experiments. For mRNA analysis, 6 males and 7 females were used, all being 26-31 weeks old. For the FISH analysis, two heterozygous mice were crossed and 13-day embryos were collected to obtain primary mouse embryonic fibroblasts. For *S*-mephenytoin activity measurements, liver microsomes from 9 weeks old transgenic and wildtype mice were used. Six wildtype mice and eight transgenic mice from each gender were used. For measuring omeprazole metabolism, liver microsomes from 12 weeks old male wildtype (n=10) and transgenic (n=4) mice were used. Finally, when measuring protein expression, the same liver microsomes from 12 weeks old male mice were used as for the omeprazole analysis. Due to limitations in material availability, microsomes from four transgenic males were still used, but microsomes were only available from nine (out of ten) wildtype mice.

In paper IV, two separate in-life studies were conducted. In the first study, transgenic male mice were castrated or underwent sham surgery at 3 weeks of age. Female transgenic mice had a testosterone capsule or a placebo capsule implanted subcutaneously at an age of 3 weeks. All transgenic mice and their wildtype littermates were euthanized at an age of 11 weeks. All groups contained five animals, except the sham surgery group, which contained only four mice. In the second in-life study, adult (8-11 weeks old) male transgenic mice had a GH capsule or a placebo capsule inserted subcutaneously. The transgenic males (GH and placebo) were euthanized four or seven days post surgery. Male wildtype and female transgenic mice were used as controls. All groups contained 4-6 individuals.

Genotyping (Paper II-IV)

In papers II-IV, the mice were genotyped to ascertain the possession of the inserted human genes. In paper II, the possession of *CYP2C18* and *CYP2C19,* as well as the 3' and 5' ends of the inserted BAC segment, was investigated. In papers III and IV, only the possession of the *CYP2C19* gene was investigated and IL-2 was used as an internal control. Genomic DNA was extracted from ear or tail biopsies using commercial kits (DNeasy Tissue, Qiagen) or established protocols (Laird *et al.,* 1991; Sambrook & Russel, 2001). The primers transcripts were amplified by PCR and the amplified products visualized on ethidium/agarose gels.

mRNA expression (Paper III and IV)

Total RNA from liver, kidney, small intestine, brain (papers III and IV) heart and lung (paper III) was extracted using commercially available kits (Qiagen) and the purity and concentration of the extracted RNA was assessed spectrophotometrically. The integrity of the RNA was visualized via formaldehyde-agarose electrophoresis. The RNA was reverse transcribed into cDNA before real time analysis. The primer pairs used for the amplification of *CYP2C18* and *CYP2C19* were designed to show low homology with endogenous murine Cyp2c isoforms using a multiple sequence alignment (ClustralW). Combinations of murine hypoxanthine guanine phosphoribosyltransferase (HPRT), glyceralaldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as normalization factors for the various analyses. The quantitative expression of the *CYP2C18* and *CYP2C19* genes, as well as the murine endogenous *Cyp2c29, Cyp2c37-40, Cyp2c54-55* and *Cyp2c70* were determined by real time PCR. In paper IV. a large number of known sexually dimorphic genes (Holloway *et al.,* 2006) were used as positive controls. The amplified products were measured directly by monitoring the fluorescence of the reporter dye, FAM (TaqMan analyses), or monitoring the increase in SYBR®Green bound to the amplified double-stranded DNA (SYBR analyses)

Protein determinations (Paper I and III)

Microsomes were prepared by homogenization of livers (paper I and III) and kidneys (paper III) followed by centrifugation for 10 minutes at 10 000 *g*. The supernatants were then centrifuged and washed twice at 105 000 *g* for 70 minutes. The washed microsomes were suspended in 50 mM potassium phosphate buffer (0.4 mg/g original weight) and stored at approximately -80°C until use. The liver microsomes were used for both protein determination (western blots) and activity measurements.

In papers I and III, western blot analyses were performed on microsomal proteins separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to PVDF membranes. The membranes were incubated with primary antibodies (see Table 1 for paper I). In paper III, an α-hCYP2C antipeptide targeted against the four C-terminal amino acids of human CYP2C8/9/18/19 and murine Cyp2c70 was used. Secondary antibodies were applied after washing of the membranes. The membranes were again washed and the complexes were detected with ECL™ detection kit and Hyperfilm-ECL (paper I) or with horseradish peroxidase conjugation and SuperSignal West Pico chemiluminescent substrate (paper III).

Table 1. Antibodies used for protein detection in Paper I

Primary antibody (polyclonal)	
Antirat 1A1	Antirat 2C11
Antirat 1A2	Antirat 2C13
Antihuman 1B1	Antirat 2D1
Antihuman 2A6	Antirat 2D4
Antirat 2B1	Antihuman 2D6
Antihuman 2B6 and Antirat 2B1/2	Antirat 2E1
Antirat 2C6	Antirat 3A
Antihuman 2C8/9/19 and Antirat 2C12	Antirat 4A

In paper III kidney microsomes and Huh-7 and COS-7 cells transiently transfected with recombinant *CYP2C18* were also analyzed using western blot with the same α-hCYP2C antipeptide.

Activity measurements (Paper I and III)

In paper I, classical marker substrates for human CYP enzymes were incubated with the mouse liver microsomes and the activities of the microsomes were measured with fluorescence, high performance liquid chromatography (HPLC) or liquid chromatography/mass spectrometry (LC/MS). For substrates and detection systems used, see Table 2.

In paper I, the total CYP content, CYP reductase activity and NADPH oxidation were also measured spectrophotometrically. In the case of total CYP content, the concentration was measured from the CO-difference spectra between 450 and 490 nm of dithionite-reduced samples. The CYP reductase activity was determined as the reduction of cytochrome C at 550 nm and 30ºC, in 0.33 M potassium phosphate buffer. The rate of NADPH oxidation was determined by continuously recording the absorbance at 340nm (loss of reduced NADPH) at 37˚C, in a cuvette with microsomes. As reference, a sample without added NADPH was used.

In paper III, *S*-mephenytoin hydroxylation was measured using the same method as in paper I, with the exceptions that the incubation times were 0-20 minutes, the final methanol concentration was 1% and the reactions were stopped by the addition of one volume of acetonitrile. *R*-omeprazole was used as an additional probe substrate for CYP2C19 activity in paper III. The rate of hydroxylation of *R*-omeprazole was measured using HPLC.

Table 2. In vitro techniques used for catalytic activity measurements in paper I (shortened from paper I).

Activity measured Commonly used as probe for (human CYP)	Initial probe conc. (μM)	Microsomal protein conc. (mg/ml)	Reaction time (min)	Final solvent conc.	Termination/stop solution (ml)	Analytical method			
						Column	Mobile phase	Flow rate (ml/min)	Detection System
Phenacetin O-deethylation (CYP1A2)	1	0.50	5	---	ACN	Symmetry C_{18} 50x2.1 mm, 3.5 μm	Gradient 2-95% ACN in 0.1% HAc	0.4	LC/MS
Coumarin 7-hydroxylation (CYP2A6)	4	0.25	10	---	Fluorescence recorded in real time	---	---	---	Ex./Em.: 390/460 nm
7-Ethoxy-4-triflouromethyl-coumarin deethylation (CYP2B6)	10	0.20	15	0.2% ACN	Fluorescence recorded in real time	----	---	---	Ex./Em.: 405/535 nm
Diclofenac 4' -hydroxylation (CYP2C9)	5	0.25	10	---	ACN: Glacial acetic acid (47:3)	Symmetry C_8 50x2.1 mm, 3.5 μm	Gradient 2-95% ACN in 0.1% HAc	0.4	LC/MS
Bufuralol 1´-hydroxylation (CYP2D6)	5	0.25	10	---	ACN	Symmetry C_{18} 50x2.1 mm, 3.5 μm	Gradient 2-95% ACN in 0.1% HAc	0.4	LC/MS
Dextromethorphan O-demethylation (CYP2D6)	5	0.50	15	---	70% perchloric acid	Symmetry C_8	30 mM ammonium acetate: ACN 75:25	1.0	Fluoresc.Ex./Em.: 270/312 nm
Chlorzoxazone 6-hydroxylation (CYP2E1)	40	0.50	15	0.6mM KOH	70% perchloric acid	LiChroCART 250-4	Gradient 30-60% ethanol	0.8	UV Absorbance: 280 nm
p-Nitrophenol O-hydroxylation (CYP2E1)	100	0.20	40	---	0.6M perchloric acid	---	---	---	VIS-photometry Abs.: 546 nm
Testosterone 6-β-hydroxylation (CYP3A4)	80	0.50	30	0.4% DMSO	ACN	LiChroCART 250-4	Gradient 30-60% ethanol	0.8	UV Abs.: 254 nm
Lauric acid 12-hydroxylation (CYP4A)	50	0.50	30	---	ACN: 1M H_3PO_4 (1:1)	AquaSil C18 30x2.1mm, 3μm	Gradient 2-80% ACN in 0.1% HAc	0.3	LC/MS
Amiodarone disappearance	1	0.50	10	1% ACN	ACN	Atlantis 30x2.1mm, 3μm	0.1% formic acid	0.35	LC/MS/MS
Paclitaxel disappearance	10	0.50	10	0.1% Ethanol	ACN	Atlantis 30x2.1mm, 3μm	0.1% formic acid	0.35	LC/MS/MS
S-Mephenytoin disappearance and 4-hydroxylation (CYP2C19)	10	0.50	40	0.1% Methanol	ACN	Symmetry C_8 50x2.1 mm, 3.5 μm	Gradient 2-80% ACN in 0.1% HAc	0.4	LC/MS

Clinical pathology (Paper II)

In paper II, multiple clinical pathology parameters (both haematology and blood chemistry) were investigated, see Table 3. In general, haematology parameters were analyzed using standard methodologies and commercially available kits were used when analyzing blood chemistry parameters. Bone marrow differentials were determined by flow cytometry.

Table 3. Parameters measured in clinical pathology

Haematology	**Blood chemistry**
Basophils	Albumin
Erythrocytes	Albumin/globulin ratio
Eosinophils	Alkaline aminotransferase
Hematocrit	Alkaline phosphatase
Haemoglobin	Aspartate aminotransferase
Large unstained cells	Bilirubin (total)
Leucocytes	Calcium
Lymphocytes	Cholesterol
Mean corpuscular haemoglobin	Creatinine
Mean corpuscular haemoglobin concentration	Glucose
Mean red cell volume	Potassium
Monocytes	Sodium
Neutrophils	Total protein
Platelets	Triglycerides
Red cell distribution width	Urea
Reticulocytes	

Anatomical pathology (Paper II)

Most tissue specimens were fixed in 10% buffered formalin; while testicles and epididymides were fixed in Bouin's solution and eyes were fixed in MFAA (methanol, formalin, acetic acid). All preserved tissues (Table 4) were embedded in paraffin according to standard methods, stained with haematoxylin and eosin (HE) and evaluated histologically.

Table 4. Tissues sampled at necropsy

Adrenal glands	Kidneys	Salivary gland (parotid, submaxillary and lingual)
Aorta (thoracic)	Liver with gallbladder	
Sternum	Lungs	Seminal vesicles
Brain	Lymph node (mandibular and mesenteric)	Skeletal muscle
Brown fat deposit[s]		Skin
Cervix		Spleen
Epididymides	Nerves (sciatic and optic)	Spinal cord (lumbar and cervical)
Epididymal fat deposit		Stomach
Esophagus	Ovaries	Testes
Eyes	Pancreas	Thymus
Femur/femoro-tibial joint	Parathyroid glands	Thyroid glands
Harderian gland	Pituitary gland	Tongue
Heart	Prostate gland-ventral	Trachea
Intestines (duodenum jejunum, ileum, colon, caecum, rectum)	Retriperitoneal fat deposit	Urinary bladder
		Uterus
		Vagina

Fluorescent in Situ Hybridisation (Paper III)

In situ hybridization (Figure 6) was performed to detect the localization of the *CYP2C18/19* BAC segment used for producing the transgenic mice used in papers II-IV. Primary mouse fibroblasts were obtained from 13 days old mouse embryos. After culturing of the cells, chromosomes were obtained according to standard cytogenetic techniques. DNA from the BAC clone was labelled in parallel with biotin-dUTP or digoxin-dUTP. Labelled BAC DNA was applied to chromosomes fixed to a slide and DNA and chromosomes were denaturated together. Hybridization was performed overnight at 37°C. For probe detection, antidigoxigenin antibodies coupled with rhodamin/avidin coupled with a fluorescent dye (fluorescein isothiocyanate) were added to the slide. Slides were stained with DAPI (4', 6-Diamidino-2-Phenylindole) and the hybridization signals were analyzed.

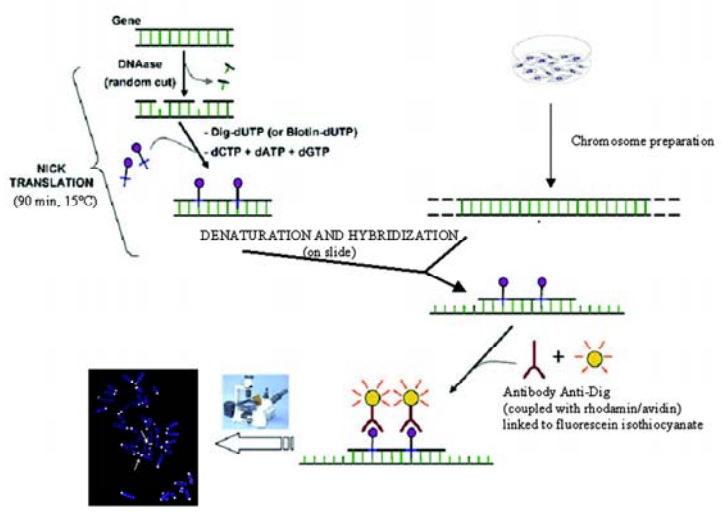

Figure 6. In situ hybridization. Picture modified from http://en.wikipedia.org/wiki/Fluorescent_in_situ_hybridization.

Electrophoretic Mobility Shift Assay (Paper IV)

In paper IV, nuclear extracts prepared from mouse livers were used in Electrophoretic Mobility Shift Assays (EMSAs) to find the regulatory elements for the sexually dimorphic expression of *CYP2C18* and *CYP2C19* in the humanized mouse model used. Wildtype and mutated STAT5b oligonucleotides were labelled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P] ATP. Binding reactions were carried out in a HEPES buffer with glycerol, Poly (dI-dC)-Poly (dI-dC), the double stranded radiolabeled probes and mouse nuclear protein. For supershift assays, liver nuclear extracts were first incubated with STAT5b antibodies on ice for 45 minutes. Competition experiments were carried out with 100-fold excess of unlabelled double stranded oligonucleotides. DNA was resolved on 4% non-denaturating polyacrylamide gels, which were dried and scanned using a phosphoimager.

Statistical Analysis (Paper I-IV)

In paper I, the statistical calculations were performed with SigmaStat software. Spearman's rank correlation coefficients were calculated for the relationship between activities and western blot densities, as well as for co-correlation analyses between activity-activity and western blot-western blot pairs.

In addition to the statistical analyses performed in Sigma Stat, some arbitrary cut offs were added. When comparing the average activity/protein expression for one strain with the pooled average activity/expression for the other mouse strains, a strain was considered to be different from the others when the average was less than half, or more than twice that of the average of the pool. Similarly, when all mouse strains investigated were pooled and compared with literature data, the results were considered to differ from literature data if the average of the pool was less than half, or more than twice that, of the value obtained from the literature.

In paper II, all comparisons between wildtype and transgenic mice were conducted with a *t*-test.

S-Mephenytoin and *R*-omeprazole metabolism between microsomes from mice with different genotypes (wildtype and transgenic) were compared using the Mann-Whitney test in paper III.

In paper IV, all genotype, treatment and gender comparisons of mRNA levels were performed with a *t*-test.

Results and Discussion

Mouse strain and gender differences in cytochrome P450 activities and protein levels (Paper I)

The first paper in the thesis focuses on differences in CYP metabolism between strains commonly used in different stages of medical research and drug development. The strains investigated were CBA, C57BL/6, 129/SvJ. NMRI, CD1 and in addition a transgenic mouse strain (Muta™Mouse) was included. The CBA strain is used in transgenic mouse development as a breeding strain, usually crossbred with C57BL. Both the CBA and the C57BL strains have relatively good reproductive performances (Les, 1980), and the C57BL/CBA crossbreed give large litter-sizes which is an advantage when aiming for offspring with altered genome. The larger the littersizes, the larger is the probability for obtaining pups with an altered genome. The C57BL strain is probably the most widely used mouse strain in research (http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml), both in industry and in academia. The 129 strain is the easiest strain from which embryonic stem cells, used for development of knock-out mice, can be obtained (Seong *et al.*, 2004). The NMRI strain is often used in efficacy tests (Kreppel *et al.*, 1995; Tingstedt *et al.*, 2007; Lamberty *et al.*, 2002), while the CD1 strain often is used for drug safety and toxicity tests (D'Cruz *et al.*, 2004; Stern *et al.*, 2005; Satoh *et al.*, 2006). Muta™Mouse is a transgenic mouse model used for detecting mutations *in vivo* (Szegedi *et al.*, 2000; Myhr, 1991).

Although different mouse strains are used in pharmaceutical development, little consideration has been given to the metabolism differences that may occur between the strains and cause differences in test

results. The aim of the first paper was to investigate if such differences occurred and evaluate the importance of these, if any. In addition a literature search was made to compare mouse metabolism with human metabolism for the same substrates.

Differences in both protein expression and activities for the substrates used were shown, but the exact identity of the proteins detected and the metabolism they perform was sometimes difficult to assess. It was difficult to positively identify proteins detected in Western blots, since no mouse specific cytochrome P450 antibodies were available. Also, no mouse specific cytochrome P450 substrates to use in activity measurements were found.

In four of the five strains examined, male mice metabolized phenacetin (a human CYP1A2 substrate) faster than females from the same strain and this correlated with the protein expression detected with rat CYP1A2 antibodies. Coumarin (a human CYP2A6 substrate) was most efficiently metabolized in the 129/SvJ strain. Diclofenac (a human CYP2C9 substrate) was generally metabolized to diclofenac 4'-hydroxylase faster in female than in male mice. The rate of paclitaxel disappearance (paclitaxel 6α-hydroxylation is a marker activity for human CYP2C8) was higher in males than in females, except for the 129/SvJ strain where the rate of metabolism was the opposite, with females having the higher metabolism. Two human CYP2D6 marker substrates, i.e. bufuralol 1'-hydoxylation and dextromethorphan *O*-demethylation, were used in activity measurements and female mice metabolized both substrates faster than male mice. Two marker activities for human CYP2E1 were used in the activity studies, i.e. chlorzoxazone 6-hydroxylation and *p*-nitrophenol *O*-hydroxylation. Chlorzoxazone was hydroxylated, at position 6, twice as fast in CBA females as in CBA males, but no other evident gender differences were observed in the other strains investigated. Both the chlorzoxazone and *p*-nitrophenol activities correlated well with the protein detected by rat CYP2E1 antibodies, and it is likely that mouse Cyp2e1 is responsible for the two activities measured. Testosterone 6β-hydroxylation was faster in females than in males for all strains studied, except for C57BL6 where the activities were approximately the same in both genders. The testosterone 6β-hydroxylation also correlated well with one of the bands detected with antibodies against rat-CYP3A. Cyp3a11 has previously been shown to catalyze testosterone 6β-hydroxylation (Yanagimoto *et al.*, 1992), and the mRNA expression of Cyp3a11 is independent of sex in C57BL mice (Jarukamjorn *et al.*, 2006; Down *et al.*, 2007), while the information from other strains is scarce.

Human CYP3A4 (Diczfalusy *et al.*, 2008; Hunt *et al.*, 1992; Wolbold *et al.*, 2003), is expressed in a sex-dependent manner, with women having higher average enzymatic activity than men. The 7-ethoxy-4-triflouromethyl-coumarin deethylation (7-EFC, human CYP2B6 substrate), amiodarone disappearance (where the amiodarone *N*-demethylation is a marker for human CYP2C8 metabolism), *S*-mephenytoin disappearance (where *S*-mephenytoin 4-hydroxylation is a marker for human CYP2C19) and lauric acid 12-hydoxylation (marker for human CYP4A) did not show any consistent differences between strains or genders.

The results from the first paper clearly show large differences in CYP protein levels as well as in CYP-dependent metabolism. Although the proteins detected and the CYP enzymes metabolizing the different substrates could not be positively identified, it was clear that the differences could cause differences in test results. In addition to the strain differences identified, quite a few enzymes showed sex-dependent expression and several activities indicated sex-dependent metabolism. These results are interesting since many early studies in pharmaceutical development are only conducted in males.

Differences between mouse and human cytochrome P450 activities (Paper I)

Mouse liver microsomes had higher metabolic rates for bufuralol 1'-hydroxylation and dextromethorphan *O*-demethylation (both human CYP2D6 marker substrates) than human liver microsomes (literature data). Human liver microsomes on the other hand had higher metabolic rates for coumarin 7-hydroxylation (marker for human CYP2A6), diclofenac 4'-hydroxylation (marker for human CYP2C9) and testosterone 6β-hydroxylation (marker for human CYP3A4) than mouse liver microsomes. Interestingly, more than 50% of the drugs on the market today are metabolized by members from these four subfamilies (Williams *et al.*, 2004; Wienkers & Heath, 2005) and drugs have been developed in animal models where these relatively large differences in drug metabolism may exist between humans and the model used.

In vivo characterization of a heterozygous CYP2C18 and CYP2C19 gene-addition mouse (Paper II)

Better animal models and better prediction of human toxicity are always desired and new models are continuously being developed. Once such models are developed, it is important to find out if the animals are healthy or if the phenotypes are connected to adverse effects that may shorten the lifespan, affect the health of the animals or in any other way make the animal models unsuitable.

When a phenotypic characterization was performed on the heterozygous *CYP2C18* and *CYP2C19* gene-addition mouse, no pathological changes were observed. Despite the fact that many clinical- and anatomical pathology parameters were measured, only a few differences between wildtype and heterozygous mice were observed.

The average brain was shown to have somewhat reduced weight in the humanized mouse as compared to wildtype littermates in both genders. The adrenal weight and brown fat deposits were reduced in transgenic males compared to controls, while the heart weight was increased. The spleens of female transgenic mice weighed less than spleens from controls. When analyzing clinical pathology parameters, transgenic males were found to have higher blood glucose levels and female transgenes had lower levels of blood triglycerides than wildtype littermates. The reasons for these organ weight and blood chemistry alterations were not further investigated, but some of the differences could possibly be attributed to the role of members in the cytochrome P450 2c subfamily in arachidonic acid (Wang *et al.*, 2004; Alkayed *et al.*, 1996) and lipid metabolism (Stewart & Strother, 1999).

Despite the few alterations observed between wildtype and *CYP2C18* and *CYP2C19* gene-addition mice, the heterozygous transgenic mice were considered to be viable and healthy animals. Although the mice were healthy, it remains to be evaluated if this animal model could be considered a better model than currently available mice for examination of metabolism of drugs that are mainly CYP2C19 substrates.

Chromosomal integration of human CYP2C18 and CYP2C19 (Paper III)

The human *CYP2C18* and *CYP2C19* genes were shown to be inserted into region C1 of murine chromosome 2 in the mouse genome. The number of incorporated segments was determined using a real time PCR approach based on existing methodologies (De Preter *et al.*, 2002) utilizing quantification of the human and murine IL-2 genes as standards, (Hoebeeck *et al.*, 2007), and the number of tandem copies was determined to be approximately 12. An example (using simple whole numbers) of how to estimate the copy number is given in Figure 7.

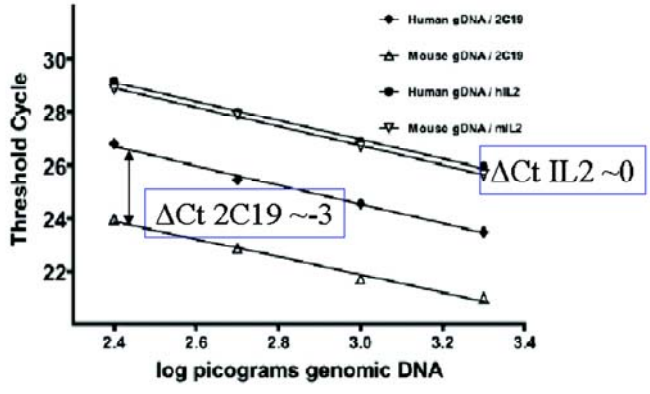

Figure 7. The mouse CYP2C19 copy numbers were calculated relative to human gDNA samples, using the following equation: $(1+E)^{-\Delta Ct\ 2C19}/(1+E)^{-\Delta Ct\ IL2}$, where E is the efficiency of the PCR reaction (in this example set to 100%), ΔCt 2C19 and ΔCt IL-2 are the differences in mean CYP2C19/IL-2 threshold cycles between mouse and human gDNA samples (in this example set to –3 and 0 respectively). In this example, the mouse CYP2C19 gene copy number would be: $2^3/2^0=8$. Since the human sample contains two copies of the gene, while a heterozygous mouse only a single copy, the total number per mouse haploid gene would be 2x8=16.

It is not unusual to have a high number of transgene copies incorporated (Xing *et al.*, 2007) when using the pronuclear injection technique. In the CYP2D6 transgene (Corchero *et al.*, 2001), 5 ± 1 copies per haploid genome were found and approximately 5 gene copies were found in the homozygous CYP3A4 model (Granvil *et al.*, 2003). Two copies have been found in two humanized CYP2E1 founders and 20 copies were found in another CYP2E1 founder (Morgan *et al.*, 2002).

Sexually dimorphic expression of CYP2C18 and CYP2C19 (Paper III and IV)

Differences in CYP2C19 expression between the sexes at the mRNA (liver, kidney, heart), protein (liver) and activity (liver) levels are shown in papers III and IV. Differences in CYP2C18 mRNA expression are also evident between the sexes in liver and kidney. No specific antibodies could be found for western blot analysis, while the primers used for mRNA analyses were considered to be specific and gave distinct differences between the genotypes. When trying to determine CYP2C19 protein levels from western blots (no CYP2C18 protein could be detected), the antibody used cross-reacted with murine Cyp2c70. Similarly, when activities for *R*-omeprazole and *S*-mephenytoin were measured, the metabolism was carried out by more than one enzyme, as shown by the fact that metabolites were also detected in wildtype littermates. Despite this cross reactivity, an increase in the total level of immunoreactive protein (CYP2C19/Cyp2c70) was evident (42% increase) in transgenic male mice as compared to wildtype controls. Similarly, an increased hydroxylation of omeprazole and mephenytoin was observed in transgenic males compared to wildtype littermates. The increase in metabolism and protein expression observed in the transgenic mice compared to wildtype control mice was nevertheless considered to be low, bearing in mind the high number of *CYP2C19* copies inserted (~12) in the genome. A low rate of metabolism performed by inserted cytochrome P450 segments in transgenic CYP mice has also been described for other models (Gonzalez, 2007; Felmlee *et al.*, 2008), and compensatory downregulation of the murine Cyp enzymes has been suggested. No statistically significant increase in the metabolism of *R*-omeprazole or *S*-mephenytoin substrates could be detected in female CYP2C18/CYP2C19 humanized mice compared to their wildtype littermates, explained by the absence of expression of these genes in female liver (see below).

Sex differences in the expression of CYP2C family members have previously been shown in the rat where the male-specific CYP2C11, and the female-specific CYP2C12, are the major isoenzymes. Expression of CYP2C11 can be down-regulated by continuous administration of GH, while the same gene can be up-regulated in response to intermittently administered GH (Mode *et al.*, 1998). In hamsters, Cyp2c27 has been shown to be male predominant in the liver (Sakuma *et al.*, 1994). In paper IV we show that the mouse *Cyp2c37, Cyp2c38, Cyp2c39 and Cyp2c40* genes also show a sexually dimorphic mRNA expression with all four genes being

female predominant. Despite the fact that clear sex differences in CYP2C isoenzyme expression have been shown in rat, mouse and hamster, no corresponding unambiguous dimorphic expression of CYP2C enzymes has been observed in human livers.

Both GH (Waxman & O'Connor, 2006) and gonadal hormones (MacGeoch *et al.*, 1985; Janeczko *et al.,* 1990; van den Berg *et al.*, 1996) have been shown to be essential in the differentiation and expression of many sexually dimorphic CYPs. "Feminization" of male mice was accomplished using two different methods in paper IV. The first method was to castrate male mice prior to puberty and the second was to administer GH continuously (using a subcutaneously implanted micro-osmotic pump) for 4 or 7 days. Wildtype males release GH in a pulsatile manner while females have a more constant release (Kato *et al.*, 1986; Waxman & O'Connor, 2006). Both the performed attempts to feminize male mice, i.e. castration and continuous GH administration, were successful in the sense that castration and GH treatment feminized in part or completely the CYP2C18 and CYP2C19 expression in liver and kidney (an example of test results from GH administration is given in Figure 8.

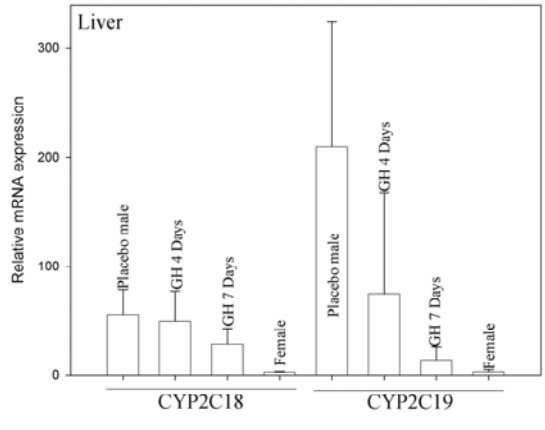

Figure 8. Effect on hepatic CYP2C18 and CYP2C19 mRNA expression by continuous administration of GH to heterozygous CYP2C18/CYP2C19 male mice. As control groups, placebo treated heterozygous CYP2C18/CYP2C19 males and untreated heterozygous CYP2C18/CYP2C19 females were used.

In addition, female mice were ”masculinized” by administrating testosterone (using subcutaneously implanted 60-day release pellets) in a constant manner. The testosterone administration to female mice had the most prominent “masculinizing” effect on CYP2C19 in the kidney.

Neither CYP2C18 nor CYP2C19 showed sexually dimorphic expression in brain, or in small intestine and no response to the hormonal treatments was observed in these organs. It is quite interesting though that the mRNA expression of both CYP2C18 and CYP2C19 is relatively high in the small intestine compared with in the liver (paper III), especially for CYP2C19 where the expression in the small intestine is higher than in liver for female heterozygous mice.

Genetic regulation of CYP2C18 and CYP2C19 (Paper IV)

The regulatory mechanisms for the sexually dimorphic expression of the inserted human *CYP2C18* and *CYP2C19* genes are investigated in paper IV. Pulsatile GH secretion is known to stimulate translation of STAT5b and it has been suggested that STAT5b is the responsible transcription factor, regulating the sexually dimorphic expression of CYP2C11 and CYP2C12 in rats (Gebert *et al.*, 1997; Kaufhold *et al.*, 2002). There is also a close relationship between GH and other steroids by feedback mechanisms within the hypothalamic-pituitary-target organ axis. Gonadal steroid imprinting during the neonatal period influence the sex-dependent GH profiles in plasma that emerge at puberty (Waxman *et al.*, 1985; Jansson *et al.*, 1985). Although a STAT5b binding site was identified in the promotor region of *CYP2C19*, no binding to that region could be identified in EMSA studies (Figure 9). However, other proteins may interfere with binding of STAT5b to the identified promotor region and other promotor regions could also be responsible for the sexually dimorphic expression of the inserted genes.

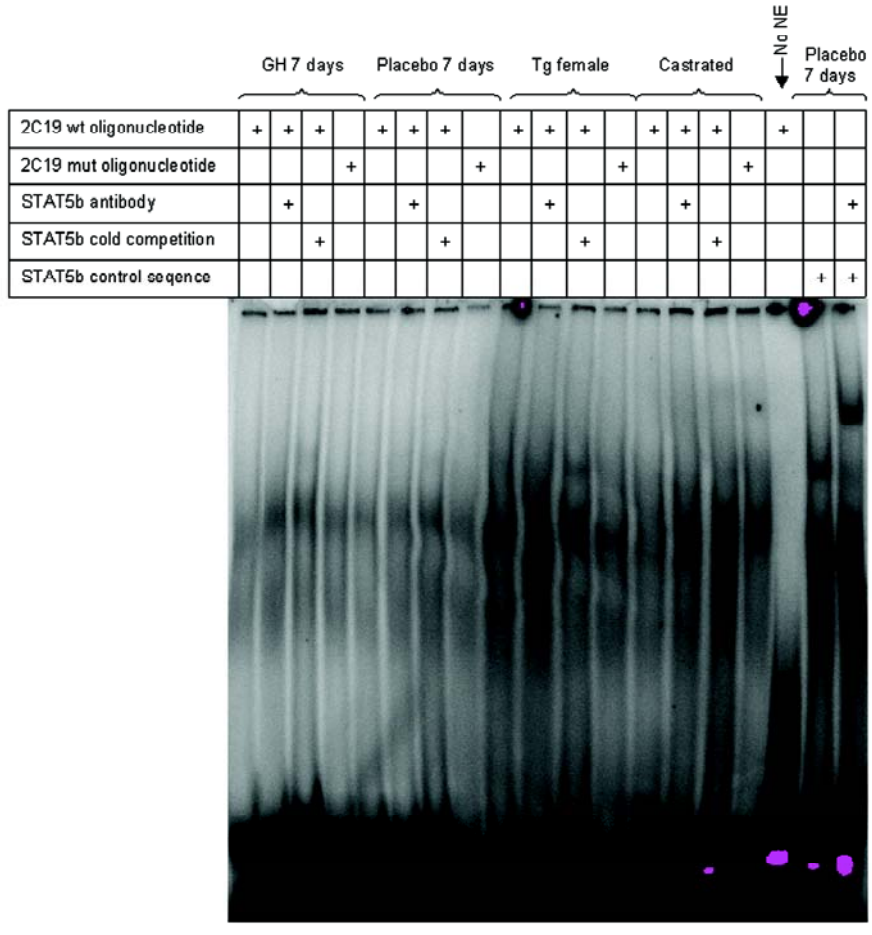

Figure 9. Electrophoretic mobility shift assay. Oligonucleotides for a putative wildtype (wt) and mutated (mut) STAT 5b as well as a control STAT5b sequence were labelled with [γ-^{32}P] ATP. Nonradiolabelled control competitor (100x) and STAT5b antibodies were incubated with nuclear extract at 4°C for 45 minutes (cold competition), followed by a 15 minutes preincubation at 37°, whereafter radioactively labelled oligonucleotides were added and incubated for another 15 minutes at 37°C. NE: Nuclear Extract.

Use of humanized CYP2C mice as drug safety models (general comments)

Quite a few humanized models have been generated and characterized today, i.e. mice expressing CYP1A1/CYP1A2 (Cheung *et al.*, 2005a), CYP2C18/19 (Löfgren *et al.*, 2008), CYP2E1 (Cheung *et al.*, 2005b), CYP2D6 (Corchero *et al.*, 2001), CYP3A4 (Granvil *et al.*, 2003; van Herwaarden *et al.*, 2005) and CYP3A7 (Li *et al.*, 1996). Although all these models contain human *CYP* genes, many of them suffer from drawbacks as safety and toxicity models. Compensatory mechanisms in the mouse *Cyp* expression may occur, and although parts of the human promotor regions are incorporated, the inserted genes are shown to be under mouse hormonal control. The inserted genes may show species specific expression, for example the CYP2C18/CYP2C19 model studied in this thesis has a clearly sexually dimorphic expression although there is no proof for CYP2C18 and CYP2C19 having sex-dependent expression in humans. These facts raise the question whether these models are more appropriate as safety models than conventional wildtype mice. Maybe they are more suitable for studying genetic regulation and expression of the human genes and increase our understanding of species specific (hormonal) regulation. To avoid a compensatory mechanism performed by murine subfamily members, knock-out models and gene-swap models (i.e. human genes replacing the murine subfamily counterparts) could be a way forward.

General conclusions

- Sex and strain differences in cytochrome P450 mRNA levels, protein expression and metabolism are common for murine enzyme families involved in drug metabolism (CYP1, CYP2 and CYP3).

- The humanized *CYP2C18/CYP2C19* gene addition mice investigated are considered to be healthy, viable animals that could possibly be used in drug safety testing and investigation of the hormonal regulation of *CYP2C18* and *CYP2C19*.

- The humanized *CYP2C18/CYP2C19* gene addition mouse investigated shows a sexually dimorphic expression of the inserted genes. CYP2C18 and CYP2C19 mRNA levels and *S*-mephenytoin metabolism are much higher in liver from male transgenic mice than in female transgenic mice.

- The mRNA expression of CYP2C18 and CYP2C19 was decreased in livers from castrated male transgenic mice and male transgenic mice treated with continuous exogenous growth hormone as compared to untreated transgenic male mice. The mRNA expression of CYP2C18 and CYP2C19 was increased in female transgenic mice treated with testosterone compared to untreated transgenic female mice.

Future research

- **Investigate the regulatory mechanisms behind the sexually dimorphic expression of CYP2C18 and CYP2C19**

No STAT5b binding could be identified in the promotor region of *CYP2C19* in EMSA studies. It would be of interest to identify and investigate putative STAT5b binding sequences in the promotor region of *CYP2C18*. It would also be of interest to investigate other nuclear receptors in the promotor regions of *CYP2C18* and *CYP2C19* for possible gender differences. Possible nuclear receptors to examine in EMSA studies could be hepatocyte nuclear factors, which have been shown to be involved in the regulation of rat *CYP2C11* and *CYP2C12*. Other important nuclear receptors of interest are the constitutive androgen receptor, the pregnane X receptor and the glucocorticoid receptor, all of which have been shown to be involved in *CYP2C19* expression in humans.

- **Distribution of the inserted CYP2C18 and CYP2C19 genes**

Organ weight alterations between transgenic and wildtype mice were observed in the brain, heart, spleen, adrenal gland and brown fat deposits. No further investigations to elucidate these differences were performed. It would be of interest to study the mechanisms behind these alterations. Immunohistochemistry could show distributions of the inserted genes within these tissues. Is the expression more intense in some specific cell types? This could hopefully give some information when investigating the organ weight alterations.

- **Use of the humanized CYP2C18/19 transgenic mouse in safety assessment**

Would this mouse model be better than conventional wildtype mice for drug safety testing? It would be of great interest to evaluate this model as a safety model, by administering known human CYP2C18 and CYP2C19 substrates, for example omeprazole or diazepam. However, in order to be a useful model it is considered important to make a *Cyp2c* knockout model and crossbreed our transgene with these null mice in order to get a model that is reasonably well humanized with respect to the metabolism of human CYP2C19 substrates.

- **To further develop, and investigate, the CYP2C knock out model**

Within AstraZeneca's CYP2C disease model project, attempts were made to develop a knockout model completely devoid of the mouse *Cyp2c* cluster without success. It would be of major interest to investigate why no such model could be developed. Is the *Cyp2c* cluster too large/too important to be deleted? The embryonic stem cells are viable and chimeras survive but no germline transmission occurs. Why is that? At what stage do the embryos die *in utero*?

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Populärvetenskaplig sammanfattning

Läkemedel som finns tillgängliga utvecklas och förbättras kontinuerligt och numera kan vi behandla sjukdomar som förut var livshotande och obotliga. Idag är det också allt mer vanligt med läkemedel som är riktade mot olika organ och individanpassade. För att i möjligaste mån undvika biverkningar och sidoeffekter är det väsentligt att veta vad som händer med läkemedlet när det kommer in i kroppen. Bieffekter är inte alltid beroende av mängden läkemedel som tillförs, de kan också bero på patientens mat- och dryckesvanor, om patienten röker eller om patienten använder flera läkemedel.

I modern läkemedelsutveckling används djurmodeller för att undersöka hur läkemedel tas upp och utsöndras ur kroppen, och från dessa försök drar man slutsatser om förväntade effekter på människa. Tolkningen av resultaten från dessa studier kan dock vara svåra då det finns stora skillnader mellan djurmodeller och människa, men också mellan individer. Olika djurslag har olika nivåer av de ämnen som bryter ned läkemedel, s.k. enzymer. För vissa av enzymerna ses också skillnader mellan könen. Det är viktigt att känna till skillnader i enzymnivåer mellan arter, individer och kön för att kunna undvika oönskade effekter då läkemedlen ges till människa.

Under de senaste årtiondena har möss blivit allt vanligare som djurmodeller inom läkemedelsindustrin, dels som modell i säkerhetstestningar av läkemedel ämnade för människa, men också som modelldjur för olika sjukdomar. Till exempel finns det möss som har en ökad tendens att utveckla olika cancerformer, och på dessa djur kan man prova olika behandlingar för att se om antalet tumörer och/eller deras storlek minskar. Trots att möss används allt mer flitigt inom forskning och

utveckling har relativt få studier gjorts där man undersöker skillnader i enzymnivåer mellan möss och människor.

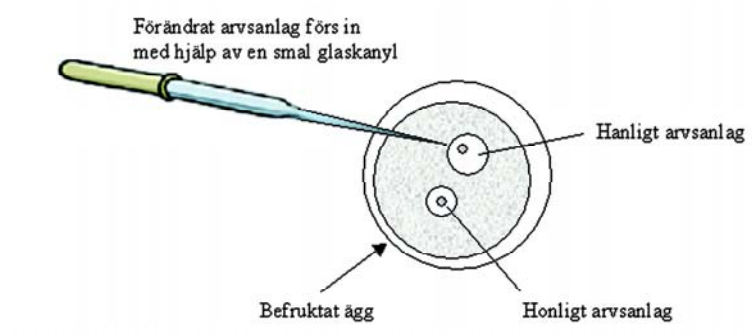
Läkemedel avlägsnas ifrån kroppen genom metabolism (ämnesomsättning/omvandling), vanligtvis till en mer vattenlöslig form som sedan kan lämna kroppen via urin eller galla. Hur snabbt läkemedlet avlägsnas från kroppen är starkt beroende av hur snabbt det omsätts. Metabolismen, tillsammans med hur snabbt läkemedlet tas upp ifrån tarmen, bestämmer vilken koncentration av läkemedlet man har i kroppens olika organ.

Omsättningen av läkemedel och andra ämnen som kommer in i kroppen delas traditionellt upp i två delsteg. Det första steget (Fas I) uträttas av de så kallade cytochrom P450 enzymerna, som exempelvis introducerar en syreatom i läkemedlet. Reaktionen gör vanligtvis läkemedlet mer vattenlösligt och dessutom kan läkemedlet lättare genomgå nästa steg (Fas II) i metabolismen. I Fas II binds en kroppsegen substans, som till exempel ett socker, till läkemedlet vilket gör det än mer vattenlösligt, och kan ännu lättare utsöndras via urin eller galla.

Kombinationen av Fas I och Fas II reaktioner anses generellt utgöra avgiftningsreaktioner, men det råder inget tvivel om att dessa processer ibland kan ge upphov till mellanprodukter som är giftigare eller mer cancerframkallande än ursprungsmolekylen. Ibland kan dessa reaktioner också ge upphov till mellanprodukter som är mer aktiva som läkemedel än det ursprungliga läkemedel som intagits.

Fas I steget utgörs som sagt av de reaktioner som uträttas av de så kallade cytokrom P450 enzymerna, som är en grupp bestående av flera hundra enzymer som skiljer sig åt både i uppbyggnad och genom vilken typ av läkemedel/substanser som de föredrar att omvandla. Den så kallade cytokrom P450 2C (CYP2C) subfamiljen av enzymer bidrar till omvandlingen av ungefär 30 % av de läkemedel som finns på marknaden idag. Hos människa består CYP2C subfamiljen av fyra medlemmar (CYP2C8, CYP2C9, CYP2C18 och CYP2C19) medan denna familj hos mus tillhör en av de mest komplexa, med så mycket som 15 medlemmar (Cyp2c29, Cyp2c37, Cyp2c38, Cyp2c39, Cyp2c40, Cyp2c44, Cyp2c50, Cyp2c54, Cyp2c55, Cyp2c65, Cyp2c66, Cyp2c67, Cyp2c68, Cyp2c69 och Cyp2c70).

Genetiskt modifierade möss blir också allt vanligare inom forskningen och det kan vara svårt att förstå att det bara är ca 35 år sedan som de första stegen togs för att utveckla transgena möss. Idag finns det två principiellt olika metoder för att genetiskt förändra möss. Den första metoden innebär att man för in det förändrade materialet i arvsanlaget som kommer från den manliga individen strax efter befruktning (pronukleär injektion, se bild nedan), medan man i den andra metoden för in arvsanlaget i några dagar gamla embryon genom användning av embryonala stamceller. Vid pronukleär injektion kan det tillförda genetiska materialet fastna var som helst i arvsanlaget, och ofta inkorporeras flera kopior av generna.



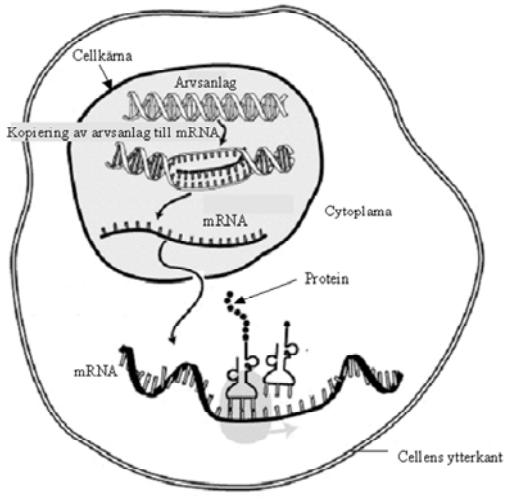
Detta arbete handlar i stora delar om en musmodell där man använt sig av pronukleär injektion för att få en mus som förutom sina egna CYP enzymer även uttrycker de mänskliga enzymerna CYP2C18 och CYP2C19.

I den första delstudien i avhandlingen undersöktes skillnader i nivåerna av musens egna CYP enzymer hos olika musstammar. I arbetet undersöktes också förekomsten av skillnader i enzymnivåer mellan könen. För vissa av enzymerna påvisades stora skillnader mellan de olika stammarna och hos vissa stammar tycktes en del CYP enzymer saknas helt. För en del enzymer sågs också stora könsskillnader, där vissa enzymer dominerar hos honor medan andra är specifika för hanar.

I delstudierna två och tre påvisades vad som kännetecknar en transgen musstam som innehåller de humana CYP2C18 och CYP2C19 enzymerna. Delstudie två fokuserade på musstammens egenskapers *in vivo*, dvs. på de biologiska processer som sker i celler och vävnader i den levande musen. Den tredje delstudien fokuserade istället på hur stammen fungerar *in vitro*, dvs. biokemiska processer som sker i förenklade system, till exempel isolerade celler i en artificiell miljö som exempelvis ett provrör.

Från *in vivo* studierna drogs slutsatsen att den transgena musstammen är sund och frisk utan några påtagliga skillnader jämfört med normala, icke genetiskt modifierade, möss. Några få skillnader i organvikter och halter av ämnen, såsom glukos och fett i blodet, kunde dock påvisas. Skillnaderna var dock små och bedömdes inte påverka musen.

Från studier av de biologiska processer som sker *in vitro* kunde det visas att mellan 11 och 13 kopior av det tillagda genetiska materialet hade inkorporerats i arvsanlaget. Det visades också att transgena hanar hade högre nivåer i lever och njure av så kallat mRNA för CYP2C18 och CYP2C19 än transgena honor. ”Messenger RNA” eller mRNA som det förkortas är den molekyl som överför genetisk information från arvsanlaget i cellkärnan till cytoplasman (se bild nedan), information som sedan används för att tillverka äggviteämnen (proteiner).



Vid analyser av proteinmängder och även enzymaktivitet blev tolkningen av resultaten något mer komplicerade. Eftersom många CYP enzymer är mycket lika i sin uppbyggnad reagerar de ofta lika på de substanser som används för att identifiera ämnen i molekylärbiologiska analyser. Detta gjorde det svårt att avgöra om proteinerna som påvisades och aktiviteterna som uppmättes härrörde från de tillförda mänskliga *CYP2C18* och *CYP2C19* generna, eller från något av musens egna enzymer. Dock kunde en ökning av både proteinnivåer och enzymaktivitet påvisas i transgena hanar jämfört med ej genetiskt modifierade möss.

I den fjärde och sista delstudien undersöktes könsskillnaderna som upptäcktes i delstudie tre djupare. Hanmöss gjordes mer feminina på två olika sätt, dels genom kastration innan de kommit upp i puberteten, dels genom att de tillfördes tillväxthormon (GH) kontinuerligt. På de flesta djurslag, men speciellt uttalat på gnagare, frisätts GH olika hos hanar och honor. Hanar utsöndrar GH i pulser med höga nivåer följt av perioder med nästan ingen utsöndring alls, honor däremot har en mer kontinuerlig utsöndring av GH med endast små fluktuationer i koncentration. Honmöss gjordes också mera maskulina genom att de tillfördes testosteron från tre veckors ålder (före pubertet). Samtliga möss avlivades vid ca 11 veckors ålder (de anses då vara fullvuxna) och mRNA-nivåer för dels CYP2C18 och CYP2C19 samt några av musens egna CYP2C enzymer undersöktes *in vitro*. Båda behandlingarna på transgena hanmöss (kastration samt GH behandling) gav ett mer feminint uttryck av båda *CYP2C18* och *CYP2C19* i lever och njure, där enzymnivåerna var lägre än i obehandlade möss. Testosteronbehandling av transgena honmöss gav inte lika stort utslag, men nivåerna av mRNA för både CYP2C18 och CYP2C19 i lever och njure, ökade hos dessa jämför med obehandlade honor. Nivåerna av musens Cyp2c37, Cyp2c38, Cyp2c39 och Cyp2c40 enzymer uppvisade också ett könsspecifikt uttrycksmönster, men här var nivåerna omvända mot de som observerades för CYP2C18 och CYP2C19, dvs. nivåerna var högre i honor än i hanar. Även musens egna Cyp enzymer svarade på de genomförda behandlingarna genom att kastrerade och GH behandlade hanar fick mer feminint uttryck och testosteronbehandlade honor fick mer maskulint uttryck.

Sammanfattningsvis konstaterades att det finns stora stam- och könsskillnader i uttryck av cytokrom P450 hos mus. I den transgena musmodell som undersöks i delstudierna två, tre och fyra, uppvisar de inkorporerade *CYP2C18* och *CYP2C19* segmenten ett könsspecifikt uttryck med högre nivåer av mRNA och protein i hanar än i honor trots att arvsanlaget är detsamma för båda könen. Detta könsspecifika uttryck kan påverkas genom kastration och GH behandling av hanar samt av testosteronbehandling av honor. Den transgena musen anses vara livskraftig och frisk och kan förhoppningsvis hjälpa till att förstå skillnader i läkemedelsomsättning mellan mus och människa. Den kan möjligtvis även anses vara en bättre mus att använda vid läkemedelstestning av substanser som omsätts av CYP2C18 och CYP2C19 än traditionella försöksdjur.