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EPIGENETIC REGULATION AND POSTTRANSLATIONAL MODIFICATION OF HUMAN CYTOCHROME P450s: FOCUS ON CYP2W1, CYP1A2 AND CYP2C18

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I want to know God's thoughts;
the rest are details.
-Albert Einstein

ABSTRACT

The cytochrome P450 superfamily represents a very important group of enzymes that are responsible for the metabolism of drugs as well as of endogenous compounds. Many P450s are genetically polymorphic causing important interindividual variability in P450 expression and activity whereas in some cases the basis for interindividual variation has still not been resolved. In this thesis, epigenetic and posttranslational aspects of the regulation of P450s are discussed, with special emphasis on CYP2W1, CYP1A2 and CYP2C18.

CYP2W1 was identified through search in the dbEST and Celera sequence databases and cloned. *CYP2W1* is a 5.5 kb long gene located on chromosome 7p22.3, was found to be expressed in HepG2 cells, and later in Caco2TC7 cells. Transient cDNA-based expression in HEK293 cells yielded a protein that is properly folded, as evidenced by its typical P450 spectrum and was active in the catalysis of arachidonic acid metabolism. Gene analysis revealed a high density of CG dinucleotides and the presence of CpG islands in promoter and exon 1-intron 1 region. Experiments using 5-Aza-2′-deoxycytidine treatment of HepG2 and B16A2 cells suggested involvement of DNA methylation in the regulation of CYP2W1 expression. Interestingly, the CYP2W1 enzyme was found to be expressed almost entirely in fetal colon (rat) and in human colorectal cancers. In these tumors there was a reverse correlation of DNA methylation and CYP2W1 expression, which further proved the involvement of DNA methylation in CYP2W1 regulation. The colorectal cancer-specific expression of CYP2W1 offers an attractive means of developing an anticancer chemotherapeutic strategy by prodrug activation. We found that the enzyme is localized at the cell surface, thus raising the possibility for cancer antibody therapy. It is posttranslationally modified by N-linked glycosylation on Asn177, which is the first time glycosylation is described for P450s in families 1-3. The functional role of glycosylation in CYP2W1 is yet to be determined.

Although the extensive variability in CYP1A2 expression and activity is well documented, the main genetic basis for this is poorly understood. We studied DNA methylation in the *CYP1A2* gene in relation to interindividual differences in hepatic expression as revealed from a human liver bank. The DNA methylation level of the CpG island was shown to be inversely correlated with the CYP1A2 mRNA levels. When evaluated against allele-specific expression, however, no correlation was found with DNA methylation. Interestingly, site-specific changes in DNA methylation correlated with allele-specific expression, suggesting the possible role of transcription regulators that bind to specific sites and are influenced by methylation changes. In addition, evidence is provided for the microRNA silencing of CYP2C18 explaining its lack of expression at the protein level.

In conclusion a new colorectal cancer specific form of P450 (CYP2W1) has been cloned and characterized and suggested to constitute a possible drug target. Epigenetic mechanisms that control certain P450 expression have been identified and could potentially provide additional understanding to the important interindividual differences in expression of these pharmacologically and physiologically important genes.

LIST OF PUBLICATIONS

I. Karlgren M, **Gomez A**, Stark K, Svärd J, Rodriguez-Antona C, Oliw E, Bernal ML, Roman y Cajal S, Johansson I, Ingelman-Sundberg M. Tumor-specific expression of the novel cytochrome P450 enzyme, CYP2W1. *Biochem Biophys Res Commun*. 2006, **341**: 451-458.

II. **Gomez A***, Karlgren M*, Edler D, Bernal ML, Mkrtchian S, Ingelman-Sundberg M. Expression of CYP2W1 in colon tumors: regulation by gene methylation. *Pharmacogenomics*. 2007, **8**:1315-25.
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III. **Gomez A**, Edler D, Wigzell H, Mkrtchian S, Ingelman-Sundberg, M. Glycosylation and cell surface localization of the colon cancer cell-specific cytochrome P450 2W1 (CYP2W1). *Manuscript*.

IV. Ghotbi R, **Gomez A**, Milani L, Syvänen A-C, Bertilsson L, Ingelman-Sundberg M, Aklillu E. Allele-specific expression and gene methylation in the control of CYP1A2 mRNA level in human livers. *Pharmacogenomics J. Provisionally Accepted*.

The following review articles are included in the discussion:

V. Ingelman-Sundberg M, Sim SC, **Gomez** A, Rodriguez-Antona C. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacol Ther*. 2007, **116**:496-526.

VI. **Gomez A**, Ingelman-Sundberg M. Pharmacoepigenetic Aspects of Gene Polymorphism on Drug Therapies: Effects of DNA Methylation on Drug Response. *Expert Rev Clin Pharmacol*. 2008, *In Press.*

VII. **Gomez A**, Ingelman-Sundberg M. Pharmacoepigenetics: Role in Interindividual Differences in Drug Response. *Clin Pharmacol Ther*. *Manuscript*. *Invited review*.

VIII. Rodriguez-Antona C, **Gomez A**, Ingelman-Sundberg M. Molecular genetics of the cytochrome P450 gene family and its relevance for cancer treatment. *Hum Genet*. *Manuscript. Invited review.*

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LIST OF ABBREVIATIONS

Ah	aryl hydrocarbon
AhR	aryl hydrocarbon receptor
AI	allelic imbalance
AR	androgen receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
ASE	allele-specific expression
ATCC	American Type Culture Collection
ATRA	all-*trans*-retinoic acid
AzaC	5-Aza-2'-deoxycytidine
B16A2	a human hepatoma cell line
CAR	constitutive androstane receptor
CMP	cytidine monophosphate
CNV	copy number variation
COS-7	African Green Monkey SV40-transf'd kidney fibroblast cell line
CpG	CG dinucleotide motif
CYP	cytochrome P450 (also P450)
DHT	dihydrotestosterone
DNMT	DNA methyltransferase (also MTase)
dNTP	deoxynucleotide triphosphates
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FXR	farsenoid X receptor
gDNA	genomic DNA
GDP	guanosine diphosphate
GR	glucocorticoid receptor
HEK293	human embryonic kidney fibroblast- 293
HDAC	histone deacetylase
HepG2	a human hepatocellular liver carcinoma cell line
HPLC	high performance liquid chromatography
LXR	liver X receptor
MBD	methylated cytosine binding domain
miRNA	microRNA
mRNA	messenger ribonucleic acid
MTase	methyltransferase
P450	cytochrome P450 (also CYP)
PAH	polyaromatic hydrocarbons
PPA	protease protection assay
PPAR	peroxisome proliferator-activated receptor
PPi	pyrophosphate
PTM	postranslational modification
PXR	pregnane X receptor
qPCR	quantitative real-time polymerase chain reaction
RISC	RNA-induced silencing complex
RXR	retinoid X-receptor

SNP	single nucleotide polymorphism
SUMO	small ubiquitin-like modifier
TRDMT1	tRNA aspartic acid methyltransferase 1
tRNA	transfer RNA
UDP	uridine diphosphate
UTR	untranslated region

1 THE CYTOCHROME P450 SUPERFAMILY OF ENZYMES

Often abbreviated as CYP or P450, the cytochrome P450 family of enzymes is a very large and diverse group of proteins present in various species and spanning the different domains of the biological taxonomy, namely Eukarya, Bacteria and Archaea (Danielson 2002; Ingelman-Sundberg 2005; Nelson et al. 1996). The CYPs metabolize a plethora of substrates with a wide range of chemical structures that originate both endogenously in the body and exogenously from the environment (Goodsell 2001; Guengerich 1994; Slaughter and Edwards 1995). They are responsible for majority of Phase I dependent metabolism of clinically used drugs (Ingelman-Sundberg and Evans 2001). Metabolism of these substrates most commonly occur via a monooxygenase reaction or the addition of oxygen to the substrate: $RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$ (Chang and Kam 1999). Aside from monooxygenase reactions, some CYPs can also catalyze other reactions, i.e. epoxidation, N-dealkylation, O-dealkylation, and S-oxidation (Chang and Yeung 2001; Kashiyama et al. 1997; Sanga et al. 2006; Ward et al. 2004).

The name cytochrome P450 originated from the term “pigment at 450 nm” when early scientists realized the importance of cytochromes in metabolism and needed a means of identifying them (Omura 1999). A solution of microsomal fraction absorbs light at a wavelength of 450 nm, the characteristic soret peak, when complexed to carbon monoxide and when the heme iron is reduced (Schenkman and Jansson 2006). At present, 57 CYP genes are documented and 58 are CYP pseudogenes. They are grouped based on their amino acid sequence similarity: CYPs belonging to the same family (e.g. CYP1, CYP2, etc.) are 40% identical and those belonging to the same subfamily (e.g. CYP2A, CYP2B, CYP2C, etc) are 55% identical (Nelson 2006).

1.1 Interindividual Variability in Drug Metabolism

Genetic variations cause variability in drug metabolism. Polymorphic CYPs can cause abolished, decreased or enhanced drug metabolism (Ingelman-Sundberg and Rodriguez-Antona 2005). Ultrarapid metabolism of drugs due to multiple genes or by induction of gene expression decrease drug concentration thus decreasing drug’s effect.

On the other hand, presence of defective alleles may allow prolonged presence of drug which may promote adverse reactions.

The growing list of CYP genetic polymorphisms provides drug targets that could influence drug response (www.cypalleles.ki.se). As all the major human drug-metabolizing P450 enzymes have been identified and cloned, and the major gene variants that cause interindividual variability in drug response and are related to adverse drug reactions have been identified, these information now provide the basis for the use of predictive pharmacogenetics to yield drug therapies that are more efficient and safer. We have also come to an understanding of which drugs necessitate dosing based on pharmacogenetics to improve drug treatment (Evans and Relling 1999; Ingelman-Sundberg 2004; Peter Guengerich et al. 2003; Shimada et al. 1994) and which different forms of cytochrome P450 are the most important for drug metabolism (Ingelman-Sundberg 2001).

Individual variability in drug metabolism among most of the relevant CYPs has been shown to be of genetic cause. CYP2D6 gene duplication has been found to be the major cause of increased activity of this gene (Ingelman-Sundberg 1999). Other genetic factors, like SNPs, have also been characterized to cause variation in CYP2D6 activity. Polymorphism in CYP2C19 (CYP2C19*2) and CYP2C9 (CYP2C9*3) have been thoroughly studied and have been found to be of high clinical significance (Rodriguez-Antona and Ingelman-Sundberg 2006).

Some members of the CYP superfamily display extensive interindividual variability in expression levels and activity although the genetic bases for these are not yet conclusive. One classis example is CYP1A2, which is a liver-specific member of the CYP superfamily that metabolizes many endogenous as well as exogenous substrates (Gunes and Dahl 2008) and there is a considerable interindividual variation in CYP1A2 mRNA, protein expression and activity (Eaton et al. 1995; Kalow and Tang 1991a, b; Rodriguez-Antona et al. 2001; Schweikl et al. 1993). The genetic reason for the interindividual variations, however, has not yet been pinpointed although a few studies have characterized genetic polymorphisms in the gene and how they relate to the activity of the enzyme (Aklillu et al. 2003; Djordjevic et al. 2008; Ghotbi et al. 2007; Jiang et al. 2006). Environmental factors like smoking, dietary intake that includes cruciferous vegetables and charcoal-grilled meat and caffeine also induce the

CYP1A enzymes and these complicate the understanding of the genetic bases of variation in CYP1A2 activity (Gunes and Dahl 2008). The same is true for CYP3A4, one of the most important members of the CYP superfamily and is involved in the metabolism of the largest range of substrates of all the CYPs. CYP3A4 likewise exhibits extensive interindividual variation but is not reflected genetically (Lamba et al. 2002). The genetic bases for interindividual variation in CYP1A2 and CYP3A4 expression and activity are currently not established. The role of epigenetics in the regulation of these genes, which is discussed in this thesis, is still barely analyzed but could potentially provide the answer to the cause of interindividual variation.

1.2 Transcriptional Regulation of CYPs

Studies on regulation of CYP have been focused primarily at the transcriptional level through the involvement of nuclear receptors and transcription factors (Sonoda et al. 2003). Xenobiotics mediate transcriptional activation of CYPs and the xenobiotics are metabolized in return. Many studies have focused on the involvement of nuclear receptors (Waxman 1999). Roles of the constitutive androstane receptor (CAR), pregnane X receptor (PXR), and the peroxisome proliferator-activated receptor (PPAR), in respectively mediating the induction of hepatic P450s belonging to families CYP2, CYP3, and CYP4 in response to the prototypical inducers phenobarbital (CAR), pregnenolone 16alpha-carbonitrile and rifampicin (PXR), and clofibric acid (PPAR) have now been established (Sparfel et al. 2003; Yamamoto et al. 2003). The liver X receptor (LXR) and the farsenoid X receptor (FXR), belonging to the nuclear receptor gene family (family NR1), also play a role in liver P450 expression by dimerization with the retinoid X-receptor (RXR) and cross-talk with other nuclear receptors and a broad range of intracellular signaling pathways (Cai et al. 2002; Waxman 1999). The role of the aryl hydrocarbon receptor (Ah receptor or AhR) in CYP1A1 expression (Delescluse et al. 2000; Nebert et al. 2000; Whitlock 1999) has also been established. PXR regulates the expression of CYP3A4 by binding to two ER_6 motifs in the proximal promoter region and three at the distal enhancer region. CAR also binds to the ER_6 motif and enhances expression of CYP3A4. CYP2C9 has been shown to be up-regulated by glucocorticoid receptor (GR) as well as CAR both at the proximal and distal regions of the promoter (Ferguson et al. 2002; Gerbal-Chaloin et al. 2002) while CYP2C19 has been recently shown to be transactivated by CAR, PXR and GR (Chen et al. 2003) but the GATA transcription factor is a promising candidate for the

activation of CYP2C19 (Jessica Mwinyi, personal communication). CYP1A2 can be regulated by an AhR-dependent (Schmidt et al. 1996; Tukey and Nebert 1984) pathway. Some studies also show AhR-independent (Quattrochi et al. 1998; Ryu et al. 1996; Zaher et al. 1998) pathways can regulate CYP1A2.

1.3 CYPs in Cancer

Information about the CYP expression profile in cancer is of tremendous benefit in the therapeutic management as CYPs can either activate or inactivate anticancer drugs. Several studies have reported the array of CYPs expressed in different kinds of cancers and such knowledge should offer guidance in making clinical decisions to treat the disease. A review by Oyama, *et al*. (2004) provides a list of CYPs expressed in various cancers detected using different techniques and a comparative expression pattern between tumor and normal tissues was also presented (Oyama et al. 2004). This also gives idea on possible toxicity patterns that could arise after drug metabolism. Since the role of some CYPs in carcinogenesis has been presented, this could also impart further knowledge on cancer management (Scripture et al. 2005; van Schaik 2005). Recent discoveries of tumor-specific or the elevated expression of some CYPs provide a more cancer-specific treatment approach (Jiang et al. 2005; McFadyen et al. 2001a; McFadyen et al. 2001b; Murray et al. 1997; Rieger et al. 2004).

1.4 CYPs as Targets in Cancer Therapy

The ability of P450s to metabolize a wide array of substances and the characteristic expression pattern of some of the members provide the potential to use them as therapeutic targets in cancer therapy. CYPs can be classified as those with specific roles in the metabolism of endogenous molecules such as hormones, and those that non-specifically process exogenous molecules (drugs, chemicals, natural products, etc.). Although CYP expressions are mainly liver specific, they are extrahepatically expressed as well (Karlgren et al. 2005; Saarikoski et al. 2005; Swanson 2004).

The development of therapeutic strategies using the CYPs as targets has been initiated with the utilization of CYP19, an aromatase enzyme, to treat breast cancer. The standard pharmacological protocol on breast cancer treatment is to block the binding of estrogen to the estrogen receptor (ER), which then instigates molecular events that lead

to the transcription of target genes, with the anti-estrogen tamoxifen. Tamoxifen, however, can be an ER agonist which serves as a drawback. The alternative approach is to prevent the production of estrogen by inhibiting CYP19 since this enzyme is responsible for the conversion (aromatization) of androgens to estrogens (Bruno and Njar 2007; Bulun et al. 2003; Sebastian et al. 2002). In a similar manner as aromatase inhibition approach in breast cancer treatment, CYP17 has likewise been targeted for prostate cancer chemotherapy (Moreira et al. 2008; Yap et al. 2008). CYP17 is involved in the production of testosterone, an androgen, which, in concert with another androgen dihydrotestosterone (DHT), binds to the androgen receptor (AR) and initiates transcription of genes involved in cell proliferation and survival (Wadelius et al. 1999).

CYP family members that metabolize exogenous compounds have also been deemed attractive as targets for cancer therapy. Members of the CYP1 family (CYP1A1, CYP1A2 and CYP1B1) are under the transcriptional control of the aryl hydrocarbon receptor (AhR) and activate the pro-carcinogens polycyclic aromatic hydrocarbons (PAH) (Trombino et al. 2000). All CYP1 enzymes are expressed extrahepatically and CYP1B1 has been shown to be overexpressed in many tumor types relative to normal tissues (McFadyen et al. 2001a; Murray et al. 1997). Its ability to metabolize estradiol presents a role of CYP1B1 in estrogen-related tumorigenesis. Therapeutic tactics that target CYP1 members include inhibiting agonist binding to AhR as well as agonist induced AhR/ARNT/DNA complex formation and induction of CYP1 expression. Agents that directly inhibit activity of CYP1 enzymes are also being developed for clinical purposes. Members of the CYP2 family have recently been gaining attention for cancer therapy. They have been shown to metabolize both exogenous and endogenous substrates (i.e. CYP2S1 metabolizes ATRA and CYP2R1 metabolizes vitamin D) (Shinkyo et al. 2004; Smith et al. 2003).

Development of therapeutic approaches that utilize members of the CYP family as targets for cancer treatment has achieved but only a small success. There is still a big niche to be occupied and the potential seems enormous.

2 EPIGENETICS

It was in 1942 when the word ‘epigenetics’ was coined by Conrad H. Waddington to define the processes that dictate the phenotype that arise from ones genotype (Ubeda and Wilkins 2008). The mechanisms involved regarding this idea remained unknown for a few more decades and the definition of the word epigenetics metamorphosed. In 1994 Robin Holliday redefined epigenetics as “nuclear inheritance which is not based on differences in DNA sequence (Holliday 1994)." It can then be reduced to defining epigenetics as any process that is involved in the development of an organism that does not involve any change in DNA sequence (Bird 2007; Jaenisch and Bird 2003).

The expression of a gene is a product of several mechanisms in concert with each other. Several requirements must have to be met which includes the following: a stimulus that signals the need for a particular gene product, the presence of the transcriptional machinery and other proteins that facilitate transcription (i.e. transcription factors) and the degree of the openness of the gene locus from which to read the message (i.e. chromatin state). The latter is the domain of epigenetics.

The phenotype of an organism is dependent on the transcription of its genes and this is further dependent in part on the chromatin, the complex containing the DNA and various proteins (Elgin 1990, 1996). Several layers of regulatory mechanisms affect the chromatin state and hence the expression of a gene (Gelato and Fischle 2008; Matouk and Marsden 2008).

2.1 Posttranslational Modification of Histones

The main group of proteins that are involved in the control of chromatin structure is the histones (Jenuwein and Allis 2001; Strahl and Allis 2000). These much conserved proteins compact the 2 meter DNA in a mammalian cell nucleus and play very important roles in gene expression. The DNA is wrapped around the core histone (consisting of 2 molecules each of H2A, H2B, H3 and H4 histones and which are collectively known as the histone octamer) in a left-handed fashion 1.64 turns (146 bp of dsDNA) and this structure is called nucleosome (Felsenfeld and Groudine 2003). One of the characteristic of the histones is the presence of long N-terminal tails which

contribute to the stability of the nucleosome (Strahl and Allis 2000). This is made possible by the various posttranslational modifications in several positions of the histone tail and combinations of these (Figure 1). The histone code refers to the combination of modification in the histone tails that affect its binding with the DNA and depending on these combinations, the chromatin can be either open (relaxed) or closed (compact) (Gelato and Fischle 2008; Jenuwein and Allis 2001; Strahl and Allis 2000).

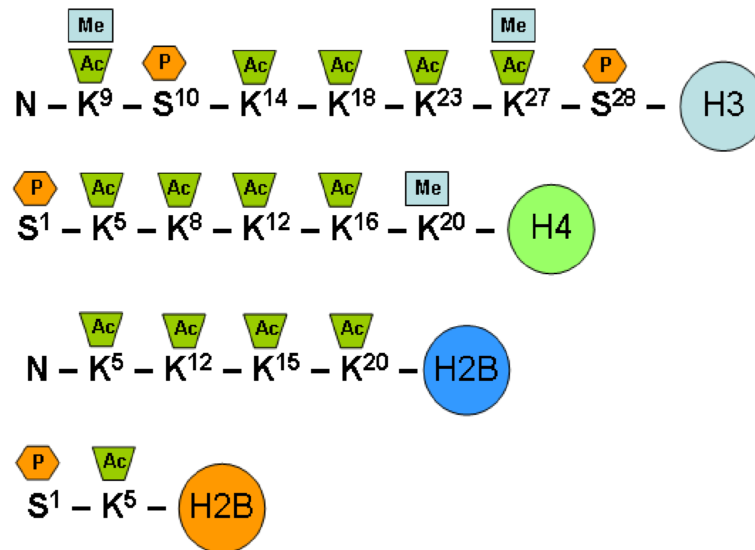

Figure 1: A map of the histone "tails" showing modification sites. The histone tails extend from the compact histone multimer to provide a platform for various posttranslational modifications. These modifications affect the histones' ability to bind DNA and each other, which in turn affects gene expression [Adapted from (Strahl and Allis 2000)].

2.2 DNA Methylation

The covalent attachment of methyl moieties to the DNA is termed DNA methylation. DNA methylation occurs predominantly at CpG sites in the mammalian genome (Bestor 2000; Bird and Wolffe 1999; Jones and Takai 2001; Robertson and Wolffe 2000). Of the four bases, only cytosine has a physiologically modified analogue, 5-methylcytosine, which is methylated at position C5 of the pyrimidine ring (Shiraishi et al. 2002a; Shiraishi et al. 2002b). 3-4% of all cytosines are methylated and the resulting 5-methylcytosines make up 0.75-1% of all nucleotide bases in the DNA of normal human tissue (Esteller 2003a, b). High concentrations of these CpG dinucleotides, called CpG islands (Gardiner-Garden and Frommer 1987), can be found most often at

promoter regions and first exons but may also occur in other regions of the gene (Bird 1980; Jones and Takai 2001; Larsen et al. 1992; Rountree et al. 2001).

Methylation of DNA is made possible by DNA methyltransferases (DNMTs or DNA MTase) (Bestor 2000; Goll and Bestor 2005; Rountree et al. 2001). Four active DNMTs have been characterized so far: DNMT1, DNMT2, DNMT3A and DNMT3B (Goll et al. 2006; Robertson et al. 1999). A fifth one has also been described, DNMT3L, but it is inactive although it is structurally related to DNMT3A and DNMT3B (Aapola et al. 2000; Chedin et al. 2002; Hata et al. 2002). DNMT1 is the most abundant of the DNMTs and it is mainly responsible for maintenance methylation by methylating hemimethylated DNAs, although it also has *de novo* methylation capacity which could even be greater than the other DNMTs. DNMT1 is therefore responsible for copying patterns of DNA methylation during DNA replication when new DNA strands are synthesized (Pradhan and Kim 2002). DNMT3A and DNMT3B have been suggested to be responsible for *de novo* methylation (Hata et al. 2002) although it also has the same capacity to methylate hemimethylated DNA as well (Fatemi et al. 2002). DNMT2 does not methylate DNA but methylates cytosine -38 in the anticodon loop of aspartic acid tRNA instead, hence being named tRNA aspartic acid methyltransferase 1 (TRDMT1) (Goll et al. 2006).

Reprogramming of DNA methylation occurs in the mouse germ cells as depicted in upper panel in Figure 2. The normal patterns of imprinting and high methylation levels observed in primordial germ cells in the mouse undergo a genome-wide demethylation. At the time that the genomes of the male and female primordial germ cells are demethylated, mitotic (male) and meiotic (female) cycles are halted. Remethylation in the prospermatogonia begins on embryonic day 16 followed by reentry of the cells into mitosis and then into meiosis. Remethylation of the female germ line takes place after birth during the growth of the oocyte. Reprogramming of the DNA methylation landscape in the germ lines has been suggested to have a role in resetting of imprints, a genetic phenomenon by which certain genes are expressed in a parent-of-origin-specific manner due to epigenetic mechanisms. Another likely purpose is to remove acquired epigenetic modifications due to environmental factors.

Methylation reprogramming occurs likewise in early embryos (Figure 2, lower panel). The rapid demethylation in the paternal genome suggests active demethylation, the

mechanism of which is still largely unknown. The maternal genome, on the other hand, suggests passive demethylation that is dependent on DNA replication. Postzygotic demethylation and remethylation are likely to have roles in the removal of epigenetic modifications in the same manner as has been suggested in germ line reprogramming. A reexamination of the original epigenetic scheme that links methylation and differentiation is also another of postzygotic reprogramming that leads to first lineage decisions during mammalian development.

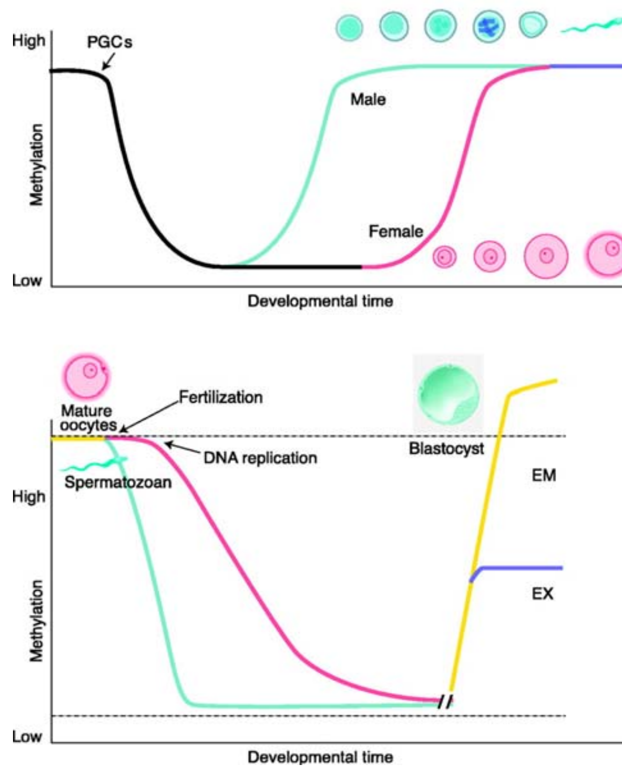

Figure 2. Reprogramming of DNA methylation in germ cells (upper diagram) and in the zygote (lower diagram) (Reik et al. 2001) [With permission from Science].

2.3 DNA Methylation in Gene Expression

Methylation of DNA is regarded as a means of regulating gene expression through two general mechanisms. First, DNA methylation of gene promoters may reject the binding of some transcription factors to their DNA binding sites (Rountree et al. 2001; Tate and Bird 1993). Second, the transcriptional silencing capability of DNA methylation may

occur via complex indirect mechanisms involving changes in chromatin formation (Caiafa and Zampieri 2005; Keshet et al. 1986; Nan et al. 1998a; Nan et al. 1998b; Padjen et al. 2005; Stirzaker et al. 2004). The interaction of methylated DNA to proteins that detect methylated DNA and other chromatin remodeling proteins render an altered chromatin configuration that prevents the expression of a gene (Rountree et al. 2001). In connection with its role in gene expression, DNA methylation has been reported to be involved in a phenomenon called allele specific expression (ASE) or allelic imbalance (AI). Although ASE has been classically associated with the epigenetic phenomena of X-chromosome inactivation, the process by which one of the two copies of the X chromosome in female mammals is inactivated, and genomic imprinting (Knight 2004) several recent studies have shown that the extent by which gene expression varies within and between populations can be explained by allele-specific expression that is relatively common among non-imprinted autosomal genes that are also potentially heritable.

2.4 DNA Methylation and Cancer

It has been common to speculate that cancer is due to changes in the sequence of a gene or genes that are involved in maintaining the integrity of the physiological mechanisms inside the cell or in protecting the genomic material from external insults. These changes can be as minute as a single nucleotide change or it can also involve a longer sequence of DNA. However, mutations in the DNA or cytogenetic abnormalities may not be as common as alterations in DNA methylation.

Dramatic genomic hypomethylation (loss of DNA methylation) may invoke carcinogenesis through three mechanisms: chromosomal instability, reactivation of transposable elements and loss of imprinting (Esteller 2005). Undermethylation of DNA might favor mitotic recombination that leads to loss of heterozygosity as well as promoting karyotypically detectable rearrangements. The possibility of reactivating intragenomic parasitic DNA and other previously silent transposons may now be transcribed and even ‘jump’ to other parts of the genome which then can disrupt normal genes. Hypomethylation can likewise affect imprinted genes (genes whose expression is determined by the parent that contributed them) which have been shown to contribute to carcinogenesis. It has also been observed that genomic DNA hypomethylation

increases through all the tumorigenic steps (Abe, *et al*., 2005), from the benign proliferations to the invasive cancers (Esteller 2005).

Hypermethylation of CpG islands located in the promoter region of tumor suppressor genes can lead to their silencing (Feinberg 2004; Feinberg and Tycko 2004; Feinberg and Vogelstein 1983a, b; Szyf et al. 2004; Teodoridis et al. 2005; Tsujimoto et al. 2002). Specificity of methylated genes to type of cancer has been observed. A list of these genes and tumor profile is listed in a review article by Esteller (Esteller 2005). CpG island methylation in human cancer has been shown to affect various cellular pathways and have relevant consequences. These pathways include cell cycle, the p53 network, APC/β-catenin/E-cadherin pathways, DNA repair, hormonal response, cytokine signaling and other imaginable molecular routes whose alteration promotes transformation of the cell.

2.5 DNA Methylation and the CYPs

A few studies on the role of DNA methylation on the expression of CYPs in different species have been reported. In humans, the role of several putative regulatory elements in determining inter-individual variability in CYP1A2 expression has not been conclusive. However, differential methylation of a specific CCGG site (-2759 nt) located adjacent to an AP-1 site in the 5'-flanking region of the *CYP1A2* gene has been shown by Hammons, *et al*. (2001) to be correlated with expression of CYP1A2 (n=55, $p<0.05$), with hypermethylation reducing expression (Hammons et al. 2001). For CYP2E1, the difference in the expression pattern in human fetal and adult liver samples has been shown to be due to differences in methylation patterns between fetal and adult in the 5' region of the gene (Jones et al. 1992). Overexpression of CYP1B1 in various human malignancies has also been shown to involve DNA methylation. Hypomethylation at multiple CpG sites at the promoter region of *CYP1B1* was suggested to be involved in the overexpression of the gene in prostate cancer (Tokizane et al. 2005) which may alter accessibility of DNA binding sites for proteins involved in AhR-mediated regulation and may also alter estrogen-mediated regulation of CYP1B1 (Sissung et al. 2006). Promoter methylation of the *CYP1B1* gene, encoding a tamoxifen- and estradiol-metabolizing CYP, predicted response differentially in tamoxifen-treated and non-tamoxifen-treated patients (Widschwendter et al. 2004).

In the mouse, a conserved regulatory element between nt -93 and -100 of Cyp2d9 promoter is differentially methylated between males and females at CpG/-97 (Yokomori et al. 1995a; Yokomori et al. 1995b). The male-preferential demethylation at this site allows the binding of the heteromeric transcription factor GABP and promotes its transcription. The methylation of this site in the female mouse prevents the binding of the methylation-sensitive GABP and prevents it transcription. CYP2A4 differential expression between the sexes operate in the same way as *Cyp2d9* but the female-preferential demethylation at CpG/-50 of the *Cyp2a4* allows its transcription while in males, it is methylated and transcription of the gene is inhibited (Yokomori et al. 1995a).

A developmental and tissue-specific expression of CYP1A2 involves a domain-specific hypomethylation in the CYP1A2 promoter in the liver where CYP1A2 is constitutively expressed whereas high methylation profiles were seen in lung and kidney tissues (Jin et al. 2004). This pattern was also observed in the developing embryo. The developmental involvement of DNA methylation has also been observed in rats (Umeno et al. 1988) wherein the transcriptional activation of the gene during early development coincided with specific demethylation only at the 5' end of the *CYP2E1* gene.

A cell type-specific transcription of the rabbit CYP1A1 has been suggested to be dictated by DNA methylation (Takahashi et al. 1998). Hypermethylation of CpG dinucleotides within the xenobiotic-response elements (XRE) prevented the binding of the AhR/Arnt complex. Methylation status at these sites also explained the differential response of different tissues to 3-methylcolanthrene (MC). In the rabbit kidney RK13 cells, CYP1A1 was expressed after MC treatment due to its unmethylated XREs while in the rabbit lung R9ab cells, MC was not able to induce CYP1A1 expression due the hypermethylation status of the XREs in these cells.

2.6 MicroRNAs

MicroRNAs (miRNAs) are a family of non-coding RNAs that base-pair to target mRNAs and typically inhibit their expression (Massirer and Pasquinelli 2006). MiRNAs are involved in the regulation of genes involved in various cell processes like proliferation, morphogenesis, apoptosis and differentiation.

In animals, miRNAs are processed from primary transcripts known as pri-miRNAs or pri-miR. These are then processed into a ~60 bp hairpin precursor step called pre-miRNAs or pre-miR which are further processed into the mature forms that are ~22 bp in length. This sequential cutting is done by two RNase III enzymes, Drosha and Dicer. miRNAs are found in plants and animals but not in fungi (Berezikov et al. 2006a; Berezikov et al. 2006b; Carthew 2006). They control the expression of a gene by targeting the 3'UTR of mRNAs and results ultimately in the degradation of the mRNA. This occurs through the formation of a ribonucleoprotein complex called RISC (RNA-induced silencing complex) that guides the recognition and translational repression or degradation of target mRNAs. In many cases, target mRNA levels diminish but do not disappear (Farh et al. 2005; Lim et al. 2005).

At present, about 0.5-1.5% of the total genes in sequenced animal species are known miRNA genes (Carthew 2006). Through genomic and bioinformatic approaches, the number of miRNAs being characterized is increasing rapidly and it is foreseen that many target genes will be described in the future.

2.7 MiRNAs and the CYPs

A recent work by Tsuchiya, *et al*. has revealed the involvement of miRNA in the regulation of a member of the CYP family, CYP1B1 (Tsuchiya et al. 2006). Previous studies have shown greater expression levels of CYP1B1 protein in various types of malignant cancers compared with normal tissues, and thus become associated with cancer. The CYP1B1 transcript has an especially long 3' UTR with regions of extreme inter-species conservation, which were suggestive of potential miRNA regulation. The authors discovered a region in the 3'-UTR with near-perfect complementarity to miR-27b which they then validated through several complimentary routes of experimentation. This forerunning study of miRNA regulation of CYP1B1 strongly supports a potential and significant role for miRNAs in the regulation of drug metabolizing enzymes. Furthermore, this additional layer of post-transcriptional regulation could be responsible for a portion of the significant unexplained interindividual variability in enzyme expression and activity.

Regulation of CYP regulators can potentially present a source of variation in CYP levels. This was suggested by a study on the regulation of the PXR by miRNAs. Takagi et al. (2008) described has shown that miR-148a could recognize the miR-148a recognition element of PXR mRNA and PXR protein level was decreased by the overexpression of miR-148a, whereas it was increased by inhibition of miR-148a, suggesting the involvement of this miRNA in PXR regulation (Takagi et al. 2008). This study has likewise shown the PXR protein level was significantly correlated with the CYP3A4 mRNA (n=25, $p < 0.05$) and protein levels (n=25, $p < 0.001$).

3 POST-TRANSLATIONAL MODIFICATION OF PROTEINS

The released polypeptide from the translational machinery in ribosomes is not the last chemical step in the formation of the protein. A diverse sort of covalent modifications or other physical alterations in the peptide occur in many of these translational products. These may happen during (co-translational) or after (post-translational) the assembly of the polypeptide chain. These, however, are collectively known as posttranslational modification (PTM) of proteins. PTM can be grouped into various types depending on how they institute change in the polypeptide chain. They can involve the addition of functional groups (i.e. phosphorylation, glycosylation, methylation, acetylation, etc.), addition of other proteins or peptides (i.e., ubiquitination, SUMOylation), change in the chemical nature of amino acids (i.e., citrullination or deimination: conversion of arginine to citrulline; deamidation: conversion of glutamine to glutamic acid or asparagine to aspartic acid) and change in the structure of the protein (i.e, formation of disulfide bridges between two cysteine amino acids; proteolytic cleavage at a peptide bond). Many of the properties of the protein can be described by knowledge of these modifications. Some of these can be alteration of the physical and chemical properties of the protein, folding, conformational distribution and activity. PTM of proteins have also been shown to have roles in the health of the individual, as it is particularly important for the study of heart disease, cancer, neurodegenerative diseases and diabetes.

3.1 Glycosylation

The enzymatic incorporation of sugar moieties (saccharides) to a protein or peptide, giving rise to a proteoglycan, is called glycosylation. Majority of the proteins that are synthesized in the rough ER undergo glycosylation, which is a process that is site-specific and enzyme-directed.

The necessary saccharide components employed in protein glycosylation come from dietary sources or from recycling and salvage processes from glycoproteins synthesized earlier. The initial step involves the conversion of monosaccharides into nucleotide-sugar donors that first entails the phosphorylation of one or more of the hydroxyl

groups of the monosaccharides. Phosphorylated monosaccharides then undergo stereochemical configurations and when this is achieved, they are converted to UDP, GDP or CMP analog, depending on the sugar, to create a nucleotide-sugar donor. This facilitates the assembly of the monosaccharides into complex carbohydrates by the stepwise action of a group of enzymes, known as glycosyltransferases, which mostly reside in the Golgi apparatus. Glycosyltransferases that are involved in the assembly of complex carbohydrates have subtly different substrate specificities and, depending in part on the route of transit of the growing oligosaccharide through the secretory pathway, the exact composition of the final product is determined as it gets in contact with the various glycosyltransferases present along the way. This also indicates that glycosylation of a protein can be synthesized differently from another copy of the same protein (Ruddock and Molinari 2006).

Various types of glycosylation have been described and the most common of these are *N*-linked and *O*-linked glycosylation. *N*-linked glycosylation involves the addition of oligosaccharides to the Asparagine (Asn or N) residue, the sequon, in the context of the amino acid pattern N--Asn-Xxx-Ser/Thr—C where Xxx can be any amino acid followed by either Serine (Ser or S) or Threonine (Thr or T). A sequon, however, cannot be glycosylated if it contains or if it is followed by a Proline (Pro or P) (Gavel and von Heijne 1990), and glycosylation may be inhibited by certain combinations of N-X-S or when followed by certain amino acids (Kasturi et al. 1995). Upon entering the lumen of the ER, the nascent protein chain is scanned by the luminal complex called oligosaccharyltransferase (OST) for the N-X-S/T motif, which is then modified covalently by the addition of the pre-assembled, tri-antennary core glycan composed of *N*-acetylglucosamine, nine mannose and three glucose residues (Figure 3) (Ruddock and Molinari 2006). After *N*-glycosylation of the nascent protein, a glycoprotein-dedicated chaperone system, consisting of calnexin and calreticulin, comes into action where two of the outermost glucose residues are sequentially cleaved by glycanases GI and GII. Removal of the third glucose dissociates the substrate from calnexin and is required for release of native polypeptides from the ER and transport to their final destination. Folding defective proteins undergo cycles of dissociation/reassociation with calnexin but terminally misfolded proteins are retrotranslocated into the cytosol and degraded through the proteasome-operated disposal, a process known as ER-associated degradation (ERAD).

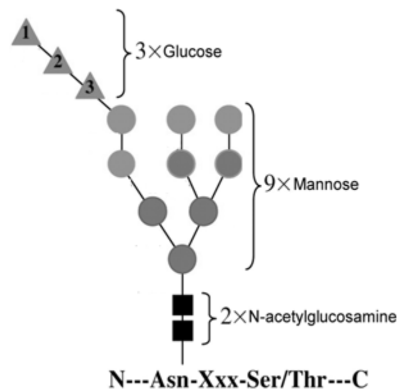

Figure 3: The complete oligosaccharide is composed of *N*-acetylglucosamine residues, nine mannose residues and three glucose residues employed in N-linked glycosylation. [Adapted from (Ruddock and Molinari 2006)].

O-linked glycosylation involves the addition of glycans to Serine or Threonine residues of the peptide and does not require a consensus sequence. No oligosaccharide precursor is required for protein transfer in O-linked glycosylation. O-linked glycans are heterogeneous and are classified based on their core structure and includes glucosamine, xylose, galactose, fucose, or mannose as the initial sugar bound to the Ser/Thr residues.

3.2 Glycosylation in CYPs

Posttranslational modifications occur in CYP enzymes and these are presented in a few review articles (Aguiar et al. 2005; Neve and Ingelman-Sundberg 2008; Oesch-Bartlomowicz and Oesch 2005). Only a few of them, however, focus on glycosylation, and the aromatases (CYP19) are the best known members of the CYP superfamily to be modified by N-linked glycosylation (Jo Corbin et al. 2003; Shimozawa et al. 1993). Although other studies on the glycosylation of CYPs have basically looked into its role in protein localization (Loeper et al. 1998; Szczesna-Skorupa and Kemper 1989, 1993), the task of this modification in CYPs is still largely unknown.

4 AIMS

The general aim of this thesis is to study the mechanisms of regulation of members of the CYP superfamily. It is also of interest to consider these as a source of interindividual variability in the CYPs' expression and activity.

The specific aims are as follows:

• to assess the role of DNA methylation in the expression of CYP2W1 in cancer, particularly in colorectal tumors, and in the interindividual variation in CYP1A2 expression in the livers
• to investigate and identify miRNAs that regulate CYP2C18 post-transcriptionally
• to characterize posttranslational modification in CYP2W1 protein and how it affects the protein's function
• to determine the catalytic activity of the CYP2W1 protein and the functional significant of genetics polymorphisms (SNPs) in the coding region
• to establish the idea of pharmacoepigenetics—the interplay of factors originating from the environment and how these influence genomic expression signatures that ultimately affect how an individual responds to drug therapy

5 MATERIALS AND METHODS

5.1 CELL LINES

The following cell lines were used in this thesis: the human hepatoma HepG2 cells, the human embryonic kidney HEK 293 cells and the human cervix cancer HeLa cells and the human hepatoma B16A2 cells. HepG2, HEK 293 and HeLa cells were propagated based on the ATCC procedures while B16A2, which was obtained from Drs. Laurent Corcos and Andre Guillouzo of the Faculte des Sciences Pharmaceutiques et Biologiques, Universite de Rennes I, Rennes, France, was cultured as previously described.

5.2 TISSUE SAMPLES

Tumor tissue samples for the CYP2W1 project were obtained from a couple of collaborating hospitals. Tumor tissues and the corresponding normal tissue sample for some were obtained from the Hospital Clinico Universitario 'Lozano Blesa' in Zaragoza, Spain and from the Karolinska University Hospital in Solna, Sweden. Although they have been examined by a pathologist, samples may have been handled differently which have to be taken into account during the study. For the CYP1A2 project, liver tissues were obtained from our biobank established at the Division of Clinical Pharmacology, Karolinska University Hospital-Huddinge, Sweden. Approval by the ethics committee from both Karolinska Institutet and Universidad de Zaragoza were obtained prior to utilization of the samples for experimental purposes.

5.3 MOLECULAR CLONING AND EXPRESSION

The CYP2W1 cDNA was obtained from HepG2 total RNA extract after reverse transcription. Expression of the gene after insertion to an expression vector was carried on in various systems that include mammalian cells (i.e. HEK293), yeast (*Saccharomyces cerevisiae*) that overexpressed the yeast or human P450 reductase, common fruit fly (*Drosophila melanogaster*), and bacteria (*Escherichia coli*). Although the advantage of using a mammalian cell system is apparent in that it contains the necessary factors for posttranslational modifications or protein folding, we observed expression to occur best in the bacterial system (Wu et al. 2006).

5.4 ANALYSIS OF PROTEIN STABILITY

Protein stability studies was performed by treating cells with cycloheximide, an antibiotic that inhibits protein synthesis in eukaryotes, for various time points. Lysates were used for immunoblotting experiments and a non-specific band that did not change intensity during treatments was used to normalize CYP2W1.

5.5 ANALYSIS OF PROTEIN TOPOLOGY

The sidedness of the CYP2W1 protein in a closed membrane was determined by the protease protection assay (PPA) (Wu et al. 2003). PPA involves the complete digestion of exposed domains or proteins on the outside of the sealed compartment in the presence or absence of detergents. This is followed by the detection of the protected domains or proteins by western immunoblotting.

5.6 SUBCELLULAR FRACTIONATION

In order to detect the localization of proteins of interest, sucrose gradient centrifugation is a very useful method. Since the different organelles have their characteristic densities, passing them through a sucrose gradient, which may consist of layers extending from 70% sucrose to 20% sucrose either in a continuous or non-continuous manner, at a centrifugal force in excess of 150,000 x g would allow them to travel through the gradient until they reach the point in the gradient where their density matches the surrounding sucrose. The layers of sucrose are then obtained and aliquots are used for western immunoblotting to reveal the location of a protein. This is confirmed further by comparing them with proteins that are known to be localized in particular organelles.

5.7 IMMUNOLOGICAL TECHNIQUES

5.7.1 Immunoblotting

The expression level of a protein is routinely detected by western immunoblotting techniques using the appropriate primary and secondary antibodies after protein separation via polyacrylamide gel (Burnette 1981; Laemmli 1970).

5.7.2 Immunocytochemistry

The visual localization of proteins is well detected by immunofluorescence techniques that involves fixing cells, grown on cover slips, using para-formaldehyde,

permeabilization (which can be disregarded when looking for cell surface localization), and detection of proteins by indirect means, i.e. treatment first with a primary antibody that binds to the antigen or protein of interest and then treatment with a secondary antibody, which is conjugated with a fluorescent dye, that binds to the primary antibody. Detection is done using a fluorescent microscope. Confocal microscopy was also used to create a three-dimensional picture of the cell and to better understand localization.

5.8 REAL-TIME PCR FOR QUANTIFICATION OF mRNA

Also called quantitative real time polymerase chain reaction (qPCR) or kinetic polymerase chain reaction, real-time PCR is a powerful method to amplify as well as quantify the expression level of genes at the mRNA level. Prior to the PCR step, reverse transcription was performed to obtain the first strand DNA. The SYBR Green real-time PCR procedure was then used in the experiments. SYBR Green is a fluorescent dye that normally exhibits low fluorescence levels. It binds to double-stranded DNA and the resulting DNA-dye complex absorbs blue light (λ_{max} = 488 nm) and emits green light (λ_{max} = 522 nm) which is then detected (Zipper et al. 2004). All double-stranded DNA produced during the amplification steps are potential binding sites for SYBR Green and because of this situation, non-specific PCR products can interfere with the signal arising from the wanted PCR product. Stringency of PCR reaction is confirmed by melting curve analysis, an assessment of the dissociation characteristics of double-stranded DNA during heating. The relative concentrations of DNA were determined by plotting the fluorescence against the number of cycles on a logarithmic scale. A threshold for detection is set and the cycle at which the fluorescence crosses the threshold, called the cycle threshold or C_t value, is employed for quantification using the ΔΔCt method. Various other genes were used to normalize the readings obtained for the genes of interest.

5.9 ANALYSIS OF DNA METHYLATION

5.9.1 Enzymatic digestion to detect variation in DNA methylation status

The initial detection of variation in the methylation status of the CYP2W1 CpG island was performed by the use of the isoschizomers *MspI* and *HpaII*. Isoschizomers are pairs of restriction enzymes that recognize the same DNA sequence and cut the same location. *MspI* and *HpaII* both recognize the CCGG site (note the presence of the CpG

dinucleotide) which is present in the CYP2W1 CpG island. *MspI*, however, can only cut the DNA when the cytosine is unmethylated. On the other hand, *HpaII* is not methylation sensitive. Subsequent to digestion, amplification of the DNA region by PCR was performed to determine its methylation status.

5.9.2 Bisulfite PCR and sequencing

Site specific analysis of DNA methylation pattern is currently best done by initially treating the DNA with bisulfite. Generally, bisulfite treatment converts unmethylated cytosines to uracil but methylated cytosines or 5-methylcytosines remain recalcitrant. After treatment, the methylation status of individual cytosines are reflected on the sequence of the DNA, i.e. unmethylated cytosines become thymines while unmethylated cytosines remain cytosines after either conventional sequencing or pyrosequencing. This is shown in Figure 4.

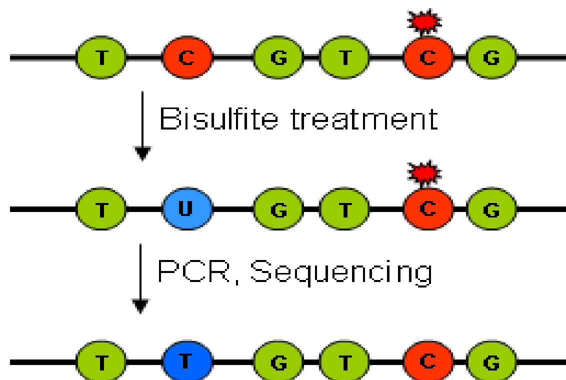

Figure 4. Bisulfite treatment of DNA distinguishes methylated and non-methylated cytosines.

Chemically, bisulfite treatment of DNA occurs in the Figure 5.

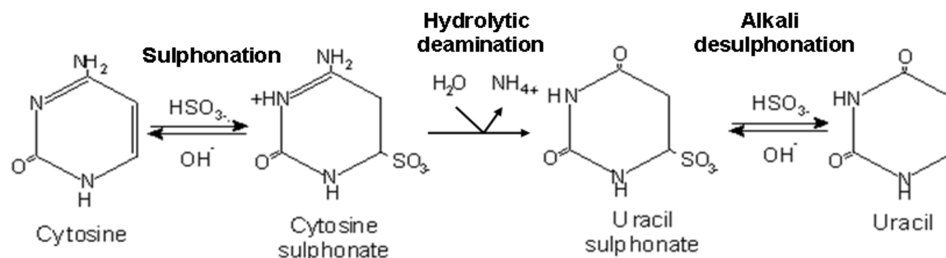

Figure 5. Chemical pathway in bisulfite treatment of DNA shows modification from cytosine to uracil.

5.9.3 Pyrosequencing

Based on the principle of 'sequencing by synthesis,' pyrosequencing involves taking a template DNA and enzymatic synthesizing its complementary strand. This technique detects the activity of the DNA polymerase enzyme by the use of a chemiluminescent enzyme. The template DNA, i.e. a PCR product with one strand being biotinylated, is first allowed to bind to sepharose beads. The bound double stranded template DNA is then made single stranded by treatment with NaOH and the bound DNA is washed. A sequencing primer is then allowed to anneal to the bound DNA. The synthesis of the complementary strand is performed by the sequential addition of deoxynucleotide triphosphates (dNTP) based on the template DNA (dispensation order). DNA polymerase catalyzes the addition of a dNTP if it is complementary to the template DNA. Incorporation of the dNTP is accompanied by the release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. The PPi produced during this process in quantitatively converted to ATP by ATP sulfurylase in the presence of adenosine 5' phosphosulfate. The ATP then drives the luciferase-mediated conversion of luciferin to oxyluciferin, in a manner that is proportional to the amount of ATP that generates visible light which is then detected by a charge couple device (CCD) camera and is shown as a peak in a pyrogram. Unincorporated dNTPs, i.e. excess dNTPs or when there is no complementarity and excess ATPs are actively and continuously degraded by the enzyme apyrase, a nucleotide degrading enzyme. When degradation is complete, another dNTP is introduced into the system following the dispensation order previously designed. Incorporation of dNTPs are done one at a time and as the sequencing process progresses, the complementary DNA is built up and the nucleotide sequence is determined from the signal peak in the pyrogram.

5.10 DETECTION OF DIFFERENTIAL ALLELIC EXPRESSION BY TAG ARRAY MINISEQUENCING

5.10.1 Assay design

Several SNPs in the *CYP1A2* gene from the Hapmap project (www.hapmap.org) were considered as a candidate for the present study to use as a marker for the AI assay. A non-functional SNP (rs2470890) located in exon 7 of the *CYP1A2* gene was selected as marker. PCR primers and a minisequencing primer with 5′ Tag sequences covering the SNP site were designed using the Autoprimer (http://www.autoprimer.com) (Beckman

Coulter) software. The primers were obtained from Integrated DNA Technologies (IDT Inc., Coralville, IA, USA).

5.10.2 PCR minisequencing and hybridization

Both Genomic DNA and cDNA were genotyped for the marker SNP using 1.5 µl DNA (0.5 ng/µl) by SNaPshot primer extension analysis as described previously (Lindroos et al. 2002; Milani et al. 2007). In brief, a 164 bp PCR fragment spanning the marker SNP site was amplified by PCR from both genomic DNA and cDNA. The oligonucleotides complementary to the tag sequence attached to the 5´of the minisequencing primer were immobilizated on the CodeLink Activated Slides (GE Healthcare, Uppsala, Sweden) in duplicate spots in each subarray. Cyclic minisequencing of the PCR products from both genomic DNA and cDNA, hybridisation of the minisequencing reaction products to the captured oligonucleotides (cTags) on the slides and washing of the slides were performed as described previously (Lovmar et al. 2003). Each sample (DNA and the corresponding cDNA) was run in triplicates on the same slide.

5.10.3 Signal detection and data analysis

Fluorescence signals from the microarrays were measured using a ScanArray® Express instrument (Perkin Elmer Life Sciences, Boston, MA, USA). The laser power was kept constant at 80% and the photo multiplier tube gain adjusted to obtain equal signal levels from the reaction control spots in all four-laser channels. The excitation lasers were Blue Argon 488 nm for R110; Green HeNe 543.8 nm for Tamra; yellow HeNe 594 nm for Texas Red and Red HeNe 632.8 nm for Cy5. The signal intensities were determined with the QuantArray® analysis 3.1 software (Perkin-Elmer Life Sciences, Boston, Ma, USA). The SNP genotype were assigned using the SNPsnapper software v3.0.0.191 (http://www.bioinfo.helsinki.fi/SNPsnapper/) based on scatter plots with the logarithm of the sum of both fluorescence signals ($S_{Allele1}+S_{Allele2}$) on the vertical axis and the fluorescence signal fractions [$S_{Allele1}/(S_{Allele1}+S_{Allele2})$] on the horizontal axis. AI for each heterozygous individual was determined by calculating the fluorescence signal ratio between the two alleles [$S_{Allele1}/(S_{Allele1}+S_{Allele2})$] in both cDNA and the respective genomic DNA as a reference for each heterozygous liver. The mean signal intensity of the duplicate spots run in triplicates on one sub-array was considered as one replicate assay.

6 RESULTS

6.1 CHARACTERIZATION OF CYP2W1 (PAPERS I, II AND III)

6.1.1 The discovery (Paper I)

The complete sequence of the CYP2W1 cDNA was established in our group. In a search using the EST database, a sequence corresponding to a part of CYP2W1 was found. Alignment of this sequence with the sequences in the Celera database which contained genomic clones, the *CYP2W1* gene sequence was obtained. The whole *CYP2W1* gene spans a length of 5.5 kb and is located on chromosome 7p22.3. Its open reading frame is 1473 bp. In comparison to other CYP genes, CYP2W1 has the highest identity with CYP2D6 (42%) and CYP2S1 (40%). CYP2W1 is also found in other species.

6.1.2 Distribution (Papers I and II)

CYP2W1 expression has been determined both at the mRNA level and the protein level. The initial search for the expression of CYP2W1 at the mRNA level was done using different mRNA blots, i.e. Multiple Tissue Expression (MTE) array, Multiple Tissue Northern (MTN) blot and Northern blotting using various cell lines. There was no signal observed when using the MTE array and the MTN blot; however, CYP2W1 was detected in HepG2 cell line but not in the other cell lines used (I.e., HeLa, B16A2, HEK 293). It was found later that CYP2W1 is also expressed the in epithelial colorectal adenocarcinoma cell line CaCo2-TC7.

Using real-time PCR as a more sensitive method to detect gene expression, several human tumors were examined for CYP2W1 expression. It was found in highest levels in colon tumors although there was also moderate expression in adrenal tumors. The expression of CYP2W1 in different tissues was also checked during development by using rat tissues as the model. We have shown that there is a very significantly high expression of CYP2W1 in the rat colon during fetal life that gradually decreased after birth. Some expression was also detected in the brain and lung but was almost at background level.

Since our earlier studies suggested high expression of CYP2W1 in the colon, we embarked further on investigating this phenomenon by examining more colon samples. We found high expression of CYP2W1 in colon tumor samples. Interestingly, the expression of CYP2W1 in the tumor samples were significantly higher than in normal colon tissues obtained from the same patient in most cases.

At the protein level, CYP2W1 is consistent with the expression at the colon tumor tissues. Although CYP2W1 mRNA was detected in adrenal tumor tissues, the CYP2W1 protein was not detected. On the other hand, there was a detectable CYP2W1 protein in breast and lung tumors.

6.1.3 Expression (Paper I)

In order to further characterize the CYP2W1 protein, it was necessary to produce the protein in high amounts. We expressed CYP2W1 in various expression systems. In the mammalian cell line HEK293, expressed CYP2W1 gave a peak at 450 nm in the reduced CO-bound form indicating the presence of correctly folded CYP enzyme. A mock transfected control did not demonstrate the same result suggesting the absence of CYP in HEK293 cells. The advantage of using a mammalian expression system for CYPs is that it can be assumed that the necessary factors for expression and folding of the protein are already present.

As the amount of CYP2W1 protein in HEK293 cells was not high, we tried to express CYP2W1 in other expression systems. Using yeast, which is the typical expression system for most CYPs, we did not get good spectra. We then finally used a bacterial system which expressed the CYP2W1 protein that properly folded as shown by the P450 spectrum.

6.1.4 Genetic polymorphism

Genetic polymorphism in the form of single nucleotide polymorphisms (SNPs) and copy number variation (CNV) are the most commonly examined variations in different population for pharmacogenetic purposes. Although a number of SNPs have been reported for the CYP2W1 gene (HapMap), only two SNPs have the ability to change the amino acid sequence of the CYP2W1 protein: rs3735684 (A181T) and rs3808348 (P488L). With regard to potential change in the protein structure and activity, these non-synonymous SNPs are of the most interesting. Frequency of the rs3735684 SNP

has been reported previously in databases (HapMap) but not rs3808348. The frequency of this SNP was investigated in Spanish, Swedish, Tanzanian and Chinese populations (Figure 6).

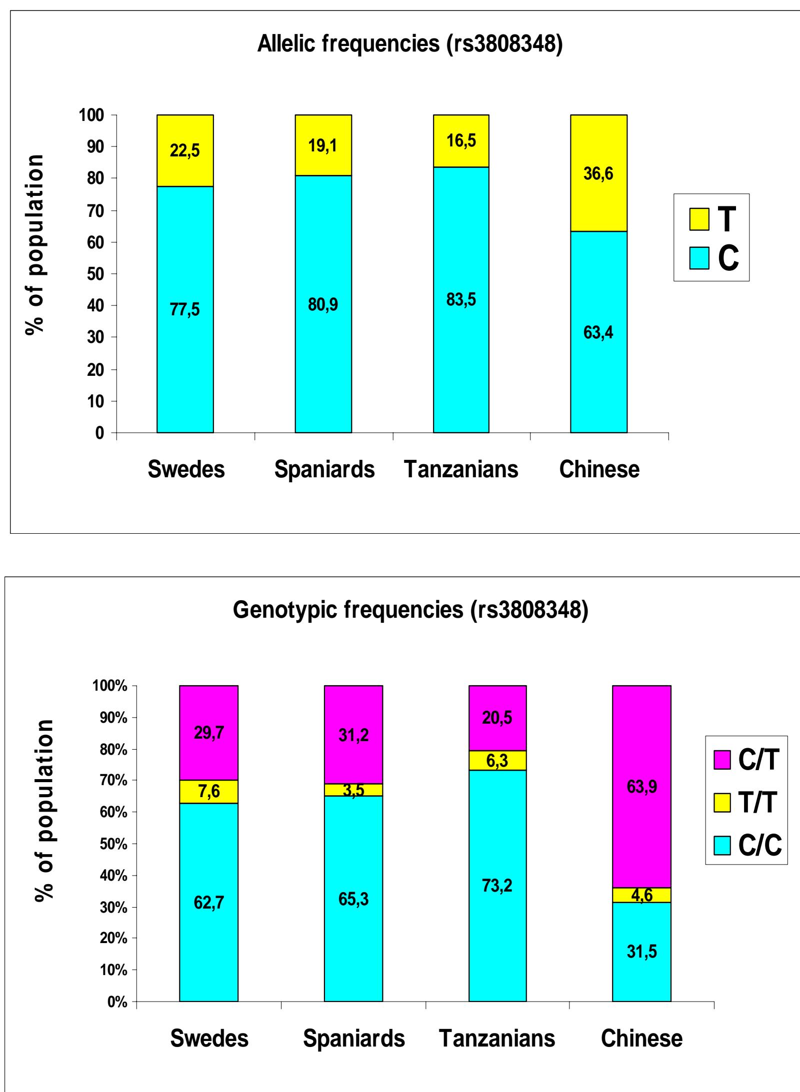

Figure 6. Allelic and genotypic frequencies of rs3808348 in CYP2W1.

6.1.5 Activity (Paper I)

The close relatedness of the CYP2W1 protein to other CYPs that metabolize arachidonic acid compelled us to test the ability of CYP2W1 to metabolize arachidonic acid. Microsomes obtained from transfected HEK 293 cells were incubated with arachidonic acid and other necessary factors for the enzyme's activity like NADPH, cytochrome b_5, and NADPH-cytochrome P450 reductase in HEPES buffer. Subsequent analysis of metabolic products revealed the presence of 14,15-DHET, 11,12-DHET and

8,9-DHET. These were confirmed by comparison with authentic reagents during LC/MS. Microsomes obtained from mock transfected HEK 293 cells showed significantly less activity. The results showed that recombinant CYP2W1 enzyme has the ability to metabolize arachidonic acid. Although it was not to a great extent as compared to other CYPs, it was nonetheless significant.

The metabolism of the substance AQ4N (banoxantrone) by members of the CYP superfamily has been reported earlier (McCarthy et al. 2003; Raleigh et al. 1998; Raleigh et al. 1999; Yakkundi et al. 2006). AQ4N is an inactive prodrug that is metabolized to its end product AQ4 via an intermediate AQ4M (Loadman et al. 2001; Patterson et al. 2000; Swaine et al. 2000) (Figure 7).

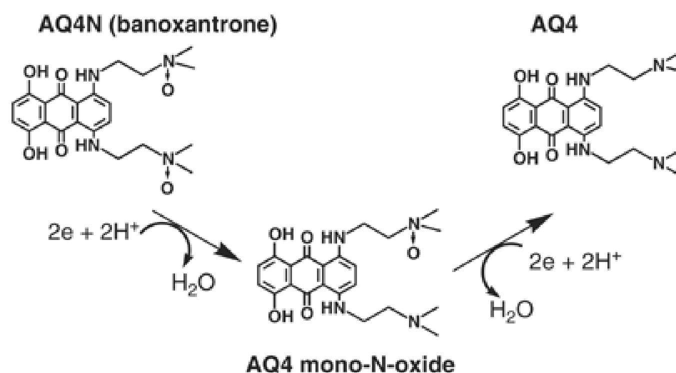

Figure 7. Metabolic pathway in the activation of AQ4N to the active product AQ4 via the intermediate AQ4 mono-N-oxide or AQ4M.

AQ4, the active product, is produced when the prodrug enters a hypoxic cell or environment (Atkinson et al. 2007; Lalani et al. 2007; Steward et al. 2007). The toxicity of AQ4 is demonstrated by its ability to inhibit topoisomerase, which leads to compromise in progression of the cell cycle that ultimately causes single strand breaks and causes DNA damage and cell death. It has been described that the cytotoxic effect of AQ4N treatment sensitizes tumors to radiotherapy (Atkinson et al. 2007; McKeown et al. 1996; McKeown et al. 1995).

The ability of CYP2W1 to metabolize AQ4N to its active product AQ4 was investigated by HPLC experiments (Swaine et al. 2000). Figure 8 shows metabolism studies using CYP2W1 enzyme that has been heterologously expressed in bacteria. The

activity of the enzyme was compared with background activity observed in null-transformed bacteria even in hypoxic conditions. By interrogating their metabolic capabilities with variations in the amount of CYP2W1 enzyme (50 µM AQ4N was incubated at 37°C for 30 minutes in hypoxic condition) and time of incubation (50 µM AQ4N was incubated at 37°C with 0.25 nmol/mL CYP2W1), AQ4 was observed to be produced more by the CYP2W1 obtained from transformed bacteria.

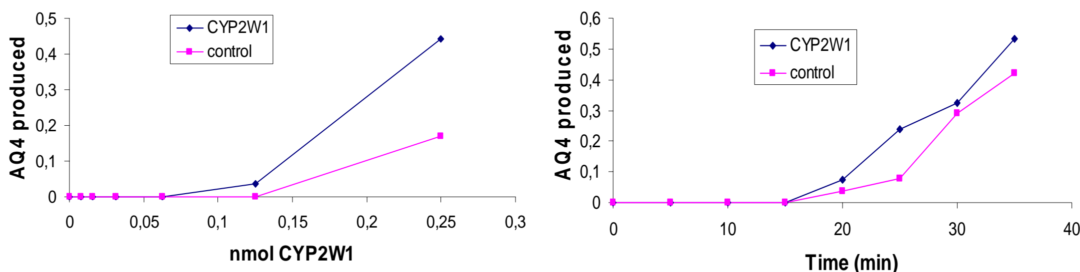

Figure 8. Metabolism of AQ4N by CYP2W1.

6.1.6 Regulation (Papers I and II)

Analysis of the promoter region of the *CYP2W1* gene until about 2000 bp upstream of the ATG revealed a high density of CpG dinucleotides. By utilizing the CpGPlot/CpGReport (http://www.ebi.ac.uk/emboss/cpgplot/) program, two CpG islands were detected: one at the 5' flanking region (-2032 to -1471 bp) and another at the first exon and first intron region (+55 to +305 bp). It has to be noted though that although no other CpG islands are detected elsewhere in the region, there is still a high number of CpG dinucleotides in the region.

The high density of CpG dinucleotides and the presence of CpG islands in the promoter of *CYP2W1* made us to look into the possible role of DNA methylation in the regulation of CYP2W1 expression. To answer this question, we compared the methylation status of the HepG2 and B16A2 cells, both being hepatoma cell lines but HepG2 expresses CYP2W1 while B16A2 does not. The isoschizomers *HpaII* and *MspI* restriction enzymes were used to investigate this difference. Following digestion of genomic DNA obtained from HepG2 and B16A2, amplification of the CpG island regions using appropriate oligonucleotide primers showed DNA methylation difference only in the CpG island at the exon 1- intron 1 region while there was no difference in

the upstream CpG island, i.e. both HepG2 and B16A2 are methylated at the distal CpG island region. DNA from HepG2 did not show any amplification product after *HpaII* digestion (methylation of CpG abrogates the ability of *HpaII* to digest DNA) which suggested that the exon 1- intron 1 region in HepG2 is completely demethylated. On the other hand, there was a band from the *HpaII* digested DNA obtained from B16A2 suggesting presence of DNA methylation in the region. The methylation status of the investigated region inversely corresponds to the expression of CYP2W1, i.e. in HepG2, CYP2W1 is expressed and it has demethylated DNA while B16A2 does not express CYP2W1 and has methylated DNA.

By treating B16A2 cells with the DNMT inhibitor AzaC, the level of DNA methylation at the exon 1- intron 1 CpG island was significantly reduced. We also examined the expression of CYP2W1 in the treated B16A2 and we showed that CYP2W1 can be re-expressed in B16A2 cells by demethylating it. This further suggests that DNA methylation is involved in the regulation of CYP2W1 expression.

To further investigate the role of DNA methylation in CYP2W1 expression, we probed clinical samples to find out if the hypothesis holds. By comparing the methylation levels of a number of colon tumor tissues compared to their normal colon tissue counterpart, we presented a significant inverse correlation between DNA methylation level and CYP2W1 expression. This result strengthened our hypothesis that DNA methylation is an important factor in affecting the expression of CYP2W1.

6.1.7 Posttranslational modification (Paper III)

Based on our earlier results showing multiple immunoreactive bands from HEK293 transfected with the CYP2W1 construct during western blotting experiments (52 and 54 kDa), we investigated the possible involvement of posttranslational modification in this phenomenon. By employing various programs that predict the presence of sites where posttranslational modifications may occur, N-glycosylation gave the plausible event. By employing glycosidases that cleave off sugar moieties from glycoproteins, we showed the disappearance of a CYP2W1-specific band in samples obtained from HEK293 cells transfected with the CYP2W1 construct. This was further proven by mutation of the glycosylation site Asn177 and mass spectrometry analysis.

By utilizing clinical samples, we have shown that glycosylation of the CYP2W1 protein also occurs *in vivo*. It is, however, interesting to note that in HepG2 and B16A2 cell lines where CYP2W1 is expressed, the CYP2W1 bands are recalcitrant to glycosidase treatment which implies the absence of the posttranslational modification in these samples.

6.1.8 Topological orientation (Paper III)

The topological orientation of CYP2W1 was monitored in both the microsomal and mitochondrial fractions by using the protease protection assay. In both organelles, it was shown that the CYP2W1 protein, both the glycosylated and the non-glycosylated forms, face the luminal space of the mitochondria and endoplasmic reticulum. This was further substantiated by employing control proteins whose orientations have already been established by other groups.

6.1.9 Protein Stability

By treating cells with the protein biosynthesis inhibitor cycloheximide, we were able to follow the levels of CYP2W1 protein at various time points in a western blotting experiment. By comparing the band intensity densitometrically, results suggest that there is faster turnover of the glycosylated species of CYP2W1 compared to the non-glycosylated species (Figure 9).

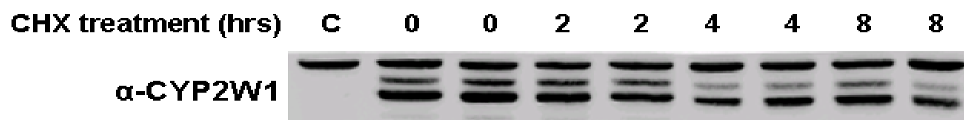

Figure 9. Degradation of the CYP2W1 protein is shown to be enhanced by N-linked glycosylation of the protein

6.2 CYP1A2, ALLELIC IMBALANCE AND DNA METHYLATION (PAPER IV)

6.2.1 Allelic imbalance

Owing to the fact that there is lack of information regarding the molecular basis for the interindividual variation in CYP1A2 expression, we hypothesized that allelic imbalance

is involved in this phenomenon, an idea that has not yet been tested previously in CYP1A2. Sixty-five liver samples were tested for heterozygosity at the marker SNP rs2470890. Twenty-three were found to be heterozygous and these samples were used subsequently to check for imbalance in allelic expression. Subsequent to PCR amplification of the region containing the SNP marker from both gDNA and mRNA (after obtaining the cDNA via reverse transcription), minisequencing analysis was performed for each allele. The averages of the signal ratio, expressed as $S_{Allele1}/(S_{Allele1}+S_{Allele2})$, from the cDNA were compared with the signal ratio from the respective reference gDNA. Eleven of the twenty-three samples (47.8%) were found to be expressed in an allelic fashion. No correlation, however, was observed between the total mRNA expression level and allelic expression.

6.2.2 DNA methylation and mRNA level

Following treatment of the hepatoma cell line B16A2 at various concentrations of the hypomethylating agent AzaC, CYP1A2 mRNA was determined using quantitative RT-PCR. The increase in CYP1A2 mRNA levels with the increase in AzaC concentration suggests the involvement of DNA methylation in the expression of *CYP1A2*.

Using the Methyl Primer Express® Software from Allpied Biosystems, a CpG island was detected at the transcription start site in exon 2. Variation in DNA methylation levels was then performed focusing on this CpG island by employing gDNA obtained from the twenty-three human liver samples. Ensuing bisulfite treatment and PCR amplification of the 309 bp region of the CpG island that included 17 CpG sites, methylation levels at each CpG site was examined by pyrosequencing. Analysis of methylation data revealed a significant negative correlation between the overall mean methylation frequency of the 17 CpG sites and the total CYP1A2 mRNA expression ($p=0.018$), i.e. a lower methylation level is associated with a higher CYP1A2 mRNA level.

Further analysis of the methylation data also suggested that there could be region-specific variation within the CpG island with regard to the distribution and level of CpG methylation, which we call here as domains: domain-1 included CpG sites 5, 6, 7, 13, 17, 18, and 19; domain-2 included CpG sites 8, 9, 10, 11, 12, 14, 15, 16, 17, and 21; Cpg site 17 as being common to both domains. The methylation level of domain-1 did not correlate with mRNA levels ($p=0.18$) while that of domain-2 showed significant

correlation (p=0.02). At the individual CpG methylation level, comparisons were also performed with the CYP1A2 mRNA levels and CpG sites 14 (p=0.006) and 16 (p=0.009) showed the highest negative correlation.

6.2.3 DNA methylation and Allelic Imbalance

The overall mean methylation of the CpG island did not correlate with the allele-specific expression (ASE) of CYP1A2 in the twenty-three samples used in the study (p=0.11). Analysis of the methylation levels of the domains likewise did not show any significant correlation with ASE of CYP1A2. However, further analysis of each individual CpG sites showed that CpG sites 9 (p=0.04) and 11 (p=0.006) are inversely correlated with the ASE, i.e. lower methylation frequency predicted a higher variation in the relative transcript level obtained from the two alleles.

6.3 THE ROLE OF MIRNA IN CYP2C18 POST-TRANSCRIPTIONAL REGULATION

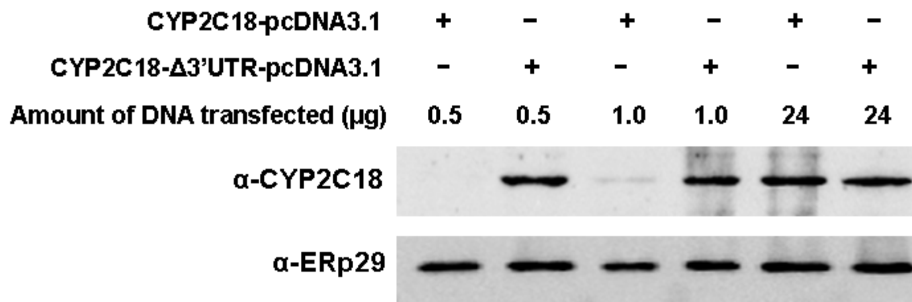

Figure 10. Post-transcriptional regulation of CYP2C18 is suggested by downregulation of transiently expressed CYP2C18 in the presence of its 3'-UTR.

CYP2C18 has been observed at the mRNA level but not at the protein level (Goldstein 2001; Lapple et al. 2003; Lofgren et al. 2008; Nishimura et al. 2003). This suggests a post-transcriptional control system that prevents the translation of the CYP2C18 mRNA to its protein product. We hypothesized that miRNAs are involved in the post-transcriptional regulation of CYP2C18. To find this out, two constructs of CYP2C18 were prepared: one with only the ORF (CYP2C18-Δ3'UTR-pcDNA3.1) and another that contains the 3'-UTR (CYP2C18- pcDNA3.1). These constructs were transfected to COS-7 cells at varying amounts. In Figure 10, we show that at lower amounts of transfected DNA, the difference in the protein product between the two constructs are

more apparent in lower amounts of transfected DNA (0.5 and 1.0 µg) than the one with the high amount of DNA (24 µg). This could be due to the fact that there is a strong promoter in the vector (pcDNA3.1+) and it's only at lower transfection levels that the difference can be observed. This result suggests that the 3'-UTR of the CYP2C18 mRNA is a regulatory site that happens post-transcriptionally.

6.4 PHARMACOEPIGENETICS: EPIGENETIC BASIS OF INTERINDIVIDUAL VARIATION IN DRUG METABOLISM (PAPERS V, VI, VII AND VIII)

Epigenetic modifications in the genome as a response to environmental cues can create discrepancies in response to drug therapy. This is the basic idea of pharmacoepigenetics. Many enzymes and proteins are involved in drug pharmacokinetics from the very introduction of a drug to the body, to its metabolism, until its excretion. These are gene products that can potentially be regulated by epigenetic mechanisms like DNA methylation, posttranslational modification of histones and non-coding regulatory RNA molecules. In the present thesis, this is exemplified by variation in DNA methylation in CYP2W1 and CYP1A2 genes and the result on their expression levels. Aberrant changes in DNA methylation in cancer is potentially the cause of variation in CYP2W1 gene expression while the cause of DNA methylation variation in CYP1A2, which can either be due to inherent individual differences or due to environmental factors, is currently unknown. In addition to gene products involved in drug metabolism and disposition, there is also a plethora of proteins that are used as drug targets (Paper VI). Epigenetic control of these genes can likewise result in differences in drug response.

The environmental aspect of pharmacoepigenetics cannot be over-emphasized. As individuals are exposed to fast changing habitats that include diet, pollution, therapeutic drug entities, and even lifestyle, changes in the gene expression pattern are expected creating a phenotypic spectrum in the population. It is therefore important to have a much fuller understanding of the epigenetic changes that occur upon exposure to environmental factors in order to have a better concept on changes in drug pharmacokinetics.

7 DISCUSSION

The cytochrome P450 superfamily is a very well studied group of enzymes that metabolize a wide variety of substrates ranging from exogenous chemicals, i.e. drugs, or environmental toxicants or pollutants to endogenous compounds. Interindividual variation in CYP metabolism due to genetic polymorphisms is another topic that has spurred the scientific community's efforts to better decisions in clinical drug therapy; hence, the rapid development of the field of pharmacogenetics.

7.1 CYP2W1— A POTENTIAL DRUG TARGET

CYP2W1 was discovered as a result of the sequencing of the human genome by employing the BLASTN and TBLASTN algorithms to probe the dbEST and Celera sequence databases for novel members of the CYP2 subfamily. The gene is expressed at the mRNA and protein level in two cell lines thus far, HepG2 and Caco2TC7. High mRNA levels, compared to HepG2 mRNA, were also observed in various tumor tissues like colon (being the tumor with the highest level of CYP2W1 mRNA expression) and adrenal gland (moderate mRNA level). Examination of the CYP2W1 protein level by western immunoblotting revealed high CYP2W1 protein in the colon tumor samples. The expression of CYP2W1 in development was also examined with the hypothesis that CYP2W1 is expressed highly in rapidly growing cells. By employing embryonic and adult rat tissues, CYP2W1 mRNA was detected at the highest level in the colon of the embryo peaking just before birth. A steady decrease was observed in the post-natal rat suggesting a possible function of the enzyme in development. These results further suggest the tumor-specific expression of CYP2W1 in the adult.

Although a few reports described substrates that are metabolized by CYP2W1, the optimal substrate has not yet been discovered. The tumor-specific expression of CYP2W1 presents itself as a very favorable target for drug therapy. Activation of an inactive prodrug by CYP2W1 that generates an active product would create a tumor-specific killing while leaving the surrounding normal, non-CYP2W1 expressing cells unscathed. AQ4N, an inactive substance, would be a very beneficial substrate for CYP2W1 for cancer chemotherapeutic purposes. The activation of this substrate by

other members of the CYP superfamily has been reported and has been exploited for therapeutic purposes in a strategy called gene-directed enzyme prodrug therapy (GDEPT). In GDEPT, CYP enzymes as well as the P450 reductase are inserted into the tumor cells by viral infection with the concomitant treatment with anticancer agent. Activation of the prodrug where the CYPs are expressed therefore promotes killing of the tumor cells. The disadvantage of this, however, is that most of the CYP enzymes are also expressed in other non-tumor tissues; hence, toxicological side effects of the drugs are not eliminated. The tumor-specific expression of CYP2W1 therefore creates a very ideal situation for cancer-specific therapy. Activation of AQ4N requires a hypoxic setting. The solid tumor tissues where CYP2W1 is expressed can offer this environment. AQ4, the active product after the metabolic reduction of AQ4N, is a topoisomerase II inhibitor and DNA intercalator. Its ability to damage the nucleic material ultimately promotes apoptotic cell death in these cells.

In the same fashion as the other CYPs, it is understandable that unwanted products could be produced by CYP2W1. The metabolic activation of procarcinogens has been reported for CYP members and even for CYP2W1. In this case, the expression of CYP2W1 in the tumor could even promote tumorigenicity depending on the habitat situation, i.e. individuals that are exposed to environmental pollutants/procarcinogens that are activated by CYP2W1 are more prone to the 'carcinogenic potential' of CYP2W1. Analysis of a number of colorectal tissues showed that the expression of CYP2W1 is higher in tumor tissues that are deemed of higher tumorigenic stage (Edler et al., manuscript). This could indeed indicate the tumorigenic role of CYP2W1.

A few genetic polymorphisms (i.e. SNPs) of the CYP2W1 gene have been reported and are already presented in the NCBI dbSNP and in the HapMap project database. Rs3735684 (exon 4) and rs3808348 (exon 9) are non-synonymous SNPs in the coding region of CYP2W1, i.e. rs3735684 corresponds to T181A while rs3808348 corresponds to L488P. Various possible consequences that could affect the protein's function include folding of the protein, splicing and mRNA stability, among others. What is quite interesting is that our initial results show that a CYP2W1 cDNA construct that bears the rs3808348 SNP and expressed in bacteria has lower expression level, when analyzed by immunoblotting, compared to the construct with the rs3735684 SNP (Masaya Tachibana, personal communication). This was also evident in their P450 spectra (Figure 11). Also, these SNPs are not located in the predicted substrate

recognition sites of CYP2W1, which leads to the assumption that if there is variation in the metabolic activity of the enzyme the genetic polymorphisms do not affect the binding of substrates per se but through other mechanisms. Whether they affect the stability of the mRNA or influence the structure of the protein remains to be further elucidated. It is also worthy to mention that the SNPs in the introns where regulatory sites reside may influence the expression of the gene. Analysis of data from the dbEST and preliminary experiments also revealed the presence of a splicing variant of CYP2W1. It is valuable to take into account that rs3808348 is located close to the translational termination site of the shorter variant. The role of this SNP in splicing is also worthy of recognition.

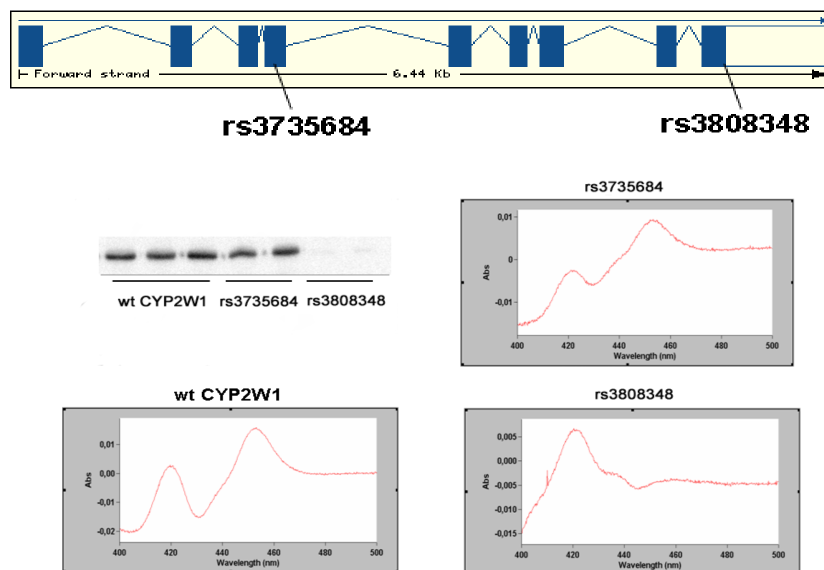

Figure 11. Bacterial expression of CYP2W1 and its genetic variants and characterization of their folding capabilities as evidenced by their signature P450 spectra.

7.2 REGULATION IN THE CYPs: DNA METHYLATION

7.2.1 CYP2W1

An important aspect of a gene’s role in the physiology of the cell is its capability to be expressed. The tumor-specific expression of CYP2W1 suggests that this could be an effect of mis-regulation that happened in the progenitor cancer cell that has been carried on as the cell grew, i.e. produced daughter cells. Aberration in DNA methylation status is a result of such mis-regulation in cancer. However, whether this occurs early or later

in the carcinogenesis process is a question that the scientific community still ponders on. Although it is generally accepted that DNA methylation changes in cancer involve the global decrease (hypomethylation) and loci-specific increase (hypermethylation events that occur in CpG islands at the promoter region of genes) in the methylation levels, some genes that have been found to be re-expressed in cancer are due to hypomethylation of their CpG islands, and CYP2W1 is one of them. In addition to the CYP2W1 CpG island in the exon 1- intron 1 region that was studied in detail with regard to its methylation level and how it is correlated with CYP2W1 expression, the promoter region of CYP2W1 is also abounding with CG dinucleotides, which are the targets for methylation by the DNMTs. To date, nothing is known regarding the transcription factors involved in the transcriptional regulation of CYP2W1 although preliminary results in our group suggested the role of AhR. The binding of AhR (or other transcription factors) in the promoter could be affected by the methylation status of the CpGs in the proximal promoter region even if there is no CpG island there. Another aspect of DNA methylation in individual CpG sites is their ability to attract proteins that contain the methyl-CpG binding domain. A group of proteins called the MBD family includes MeCP2, MBD1, MBD2, MBD3 and MBD4. They have the ability to recruit histone modifying and chromatin-remodeling complexes to methylated DNA sites like histone deacetylases (HDAC). When this happens, the configuration of the chromatin is changed into a more closed arrangement that prevents the transcriptional machinery and other important transcription factors to express the gene. Another CpG island that was detected at the 5' flanking region of CYP2W1 (-2032 to -1471 bp of the ATG) did not show variation in DNA methylation in HepG2 and B16A2 cell lines. These are the two cell lines that we used initially to study the role of DNA methylation in control of CYP2W1 expression: HepG2 expressed CYP2W1 while B16A2 does not. It is still important though to study it in more detail (by carrying out the bisulfite sequencing method), considering the fact that we employed a simple enzymatic digestion that detects methylated versus unmethylated CpG sites, i.e. *HpaII* and *MspI*. The role of the promoter even if they are kb away from ATG site cannot be ignored.

7.2.2 CYP1A2

CYP1A2 is one of the most important members of the CYP superfamily as it metabolizes many clinically important drugs. The high interindividual variability in the expression level and activity of this enzyme has not yet been correlated with any known genetic polymorphism of the gene. As it is also strongly affected by environmental factors like intake of caffeine and smoking, it can be difficult to pinpoint the exact genetic factor that plays a role in interindividual variation. We hypothesized that allelic imbalance (AI) or allele specific expression (ASE) is involved in the variation in mRNA expression of the gene and our results showed that 11 out of 23 samples tested displayed ASE. ASE can be caused by various mechanisms that include putative allele-specific *cis*-acting factors of either genetic or epigenetic origin. We followed the epigenetic route partly owing to the fact that an epigenetic mark in the *CYP1A2* distal promoter (-2759 bp) has been studied earlier by another group and has been correlated with the expression profile of the gene (Hammons et al. 2001). We, however, investigated the CpG island located in exon 2 of *CYP1A2*, which is the transcriptional start site. Although the methylation level of the whole CpG island was inversely correlated with the mRNA level, no statistically significant correlation was seen with ASE or AI. By close examination of the CpG island, there seems to be division in such a way that one part, which we call domain, is significantly correlated with the mRNA expression while the other domain is not. This probably suggests that there is a more important message in a CpG island than just the CpG density. For AI, only a few CpG dinucleotides were found to have correlation with AI. The same idea that was presented for CYP2W1 may also be helpful for understanding transcriptional regulation of CYP1A2: to identify the transcription factors or any regulatory proteins that bind to this region and how DNA methylation or other epigenetic factors affect their binding. To further scrutinize AI, it may also be important to examine the regions of each individual allele in the same individual and see how their chromatin configuration differs from each other. Although this would entail more technical work it would, nonetheless, show a better picture of the structure of each allelic environment (chromatin configuration) and may resolve the cause of ASE.

7.3 REGULATION IN THE CYPs: MiRNAs

CYP2C18 is not known as a protein because the high levels of CYP2C18 mRNA are not translated to the corresponding protein product. It is therefore hypothesized that a post-transcriptional regulatory mechanism prevents the subsequent translation of the mRNA. The recent characterization of non-coding regulatory RNAs has centered these molecules in the area of gene regulation and many genes have been described to be regulated by them, including one member of the CYP superfamily, which has been shown to be regulated by miRNA (Tsuchiya et al. 2006). In a similar manner, we want to know if miRNAs are involved in the regulation of CYP2C18. Our preliminary results show that the 3'-UTR of CYP2C18 is involved in its regulation because its presence abolishes the translation to its protein product, as shown in our western immunoblotting experiment. The identity of the miRNA/s involved is presently being examined but bioinformatic (miRBase) work suggests that the putative miRNAs are has-miR-623, has-miR-188, has-miR-539, has-miR-155 and has-miR-654 (Figure 12). The binding of miRNAs are also affected by changes in the binding site in the RNA (red letters in Figure 12). This could be due to changes in the secondary structure of the mRNA, thus preventing the binding of the miRNAs.

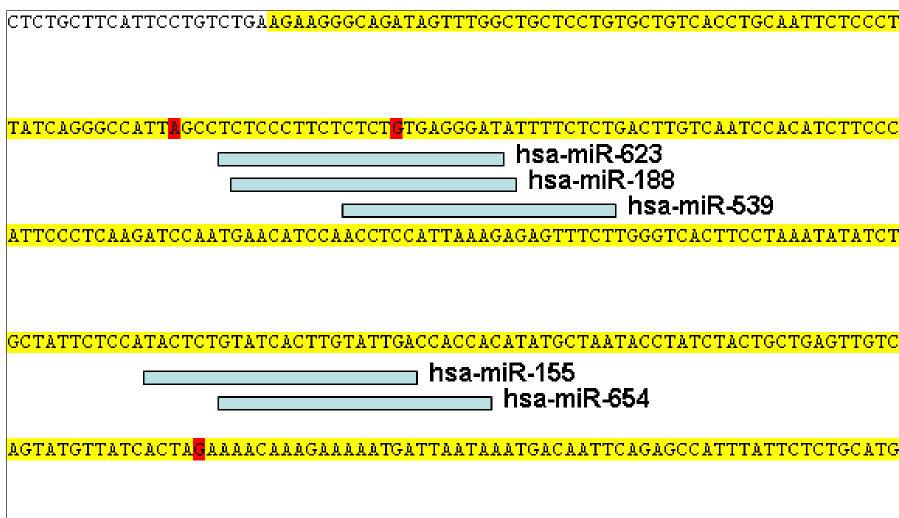

Figure 12. Putative miRNAs that bind to the 3'-UTR of the CYP2C18 mRNA and the SNPs that could potentially affect miRNA binding.

7.4 REGULATION IN THE CYPs: POSTTRANSLATIONAL MODIFICATION VIA GLYCOSYLATION

Glycosylation has been characterized in various proteins and has been found to play important roles in the protein's activity. The multiple bands observed in our western immunoblotting experiments lead us to look into posttranslational modification and bioinformatic and experimental work proved that *N*-linked glycosylation is involved in this phenomenon. The role of glycosylation includes protein folding (Loriol et al. 2007; Molinari 2007), protein targeting/trafficking and ligand recognition/binding (Ohtsubo and Marth 2006; Shi et al. 2005; Shi and Elliott 2004), biological activity, stability, regulation of protein half-life and immunogenicity (Walsh and Jefferis 2006). In CYP2W1, we looked into the various roles that glycosylation could potentially do to the protein. One aspect is intracellular localization. Although it has been detected along with the mitochondrial fractions, technical issues may complicate our observations. In order to do this, a more powerful technique such as electron microscopy is needed but a means of detecting the glycosylated species from the non-glycosylated ones must have to be established first. Our preliminary results also show that glycosylation is potentially involved in determining the half-life of the protein, as shown by cycloheximide treatments. However, a routine method, pulse-chase assay, to measure protein stability should be undertaken by using a more appropriate antibody. The surface localization of glycosylated CYP2W1 was also studied. This phenomenon would create a situation for using the protein as a target for antibody recognition which then would be tumor-cell specific and minimizes side-effects. The effect of glycosylation on substrate metabolism is another aspect that needs to be studied but at present the right substrate is not yet established.

7.5 PHARMACOEPIGENETICS—ENVIRONMENT, GENOME AND DRUG METABOLISM

Epigenetic mechanisms that include DNA methylation and modifications of the histone tails ultimately affect the structure of the chromatin. Although these have been established early in development, recent results show that the environment can shape them. Epigenetics potentially affects all genes in the genome that are involved in many cellular processes; therefore, every pathway can be under the regulatory control of epigenetics.

The epigenetic control of the expression of members of the CYP superfamily can potentially create obstacles in therapeutic strategies by changing the pharmacokinetics of drugs. In addition, drug transporters and other drug metabolizing enzymes that are also epigenetically regulated can intensify variety in drug pharmacokinetics. In addition to enzymes involved in drug pharmacokinetics, a number of other gene products are also utilized as drug targets. Examples of these are enzymes involved in the apoptotic pathway and the DNA damage response. The expression characteristics of these enzymes would be critical in the success of drug therapy. It is, therefore, vital to characterize the expression pattern of genes that are involved in drug pharmacokinetics and those that are utilized as drug targets in response to environmental cues in order to improve therapeutic strategies and to personalize medicine.

8 GENERAL CONCLUSION

The main results obtained from this thesis can be summarized as follows:

8.1 CYP2W1

- CYP2W1 is highly expressed in 50-60% of colon tumor tissues. The enzyme is also expressed in HepG2 and CaCo2TC7 cell lines.
- DNA methylation is involved in the regulation of CYP2W1 expression as shown in the hypomethylation of the CpG island of *CYP2W1* gene in the CYP2W1-expressing cell line HepG2 and hypermethylation in the non-CYP2W1-expressing cell line B16A2.
- A negative correlation between CYP2W1 expression and DNA methylation of the CpG island in the exon 1- intron 1 junction of *CYP2W1* was found in colon tumor tissues. Colon tumors over-expressing CYP2W1 have significantly lower methylation levels as compared to their corresponding normal tissues.
- Based on CYP2W1 over-expression, colon tumors can be divided into two phenotypes: high and low CYP2W1 expression, compared to their normal tissue counterpart.
- CYP2W1 can be modified posttranslationally via glycosylation at Asn177. This has been found to occur to transiently expressed CYP2W1 and in colon tumor and normal tissues.
- The CYP2W1 protein can be detected at the surface of the cell. This phenomenon can be exploited as target for anticancer antibody therapy.
- CYP2W1 can potentially metabolize AQ4N to its active metabolite AQ4. This offers a cancer-specific killing effect where production of AQ4 occurs only in CYP2W1-expressing cancer cells.

8.2 CYP1A2

- Allele-specific expression occurs in CYP1A2, which could describe variation in CYP1A2 expression among individuals.
- DNA methylation affects the mRNA expression of CYP1A2.

- Site-specific methylation variation affects imbalance in allelic expression.

8.3 CYP2C18

- Regulation of CYP2C18 expression happens post-transcriptionally.
- The CYP2C18 3'-UTR harbors the site/s for its post-transcriptional regulation, potentially by non-coding regulatory RNA molecules, i.e. miRNAs.

8.4 PHARMACOEPIGENETICS

- Interindividual variation in drug metabolism and disposition (pharmacokinetics) can be explained by discrepancies in the epigenetic status of the genes, in addition to genetic polymorphisms.
- Epigenetics offers the elucidation of the effects of environmental stimuli and disease states in changes in drug pharmacokinetics.

9 FUTURE PERSPECTIVE

Several other observations during the study merit further scrutiny. The main ideas are presented as follows:

- In order to fully utilize CYP2W1 for enzyme-based cancer chemotherapy, it is vital to describe the metabolic characteristics of CYP2W1. Although finding the optimal prodrug that can be activated into a cytotoxic agent that will promote cancer cell-specific killing is the aim, this goal can be facilitated by examining the structure of the enzyme and, through this, one can generate ideas on what kind of substrates CYP2W1 can metabolize. In addition to this, the development of anticancer agents by computational chemistry should then offer a more straightforward answer.
- The possibility of utilizing CYP2W1 as target for other types of cancers, aside from colorectal tumors, is apparent from studies that report the expression of CYP2W1 in other cancers (Aung et al. 2006). A few other tumor tissues also showed expression of CYP2W1 and this can be further studied by examining more tissues.
- Another way of utilizing CYP2W1 as an anticancer target is to take advantage of its cell surface localization by antibody recognition. The molecular basis for this localization is currently unknown and understanding its cause would be important as it relates to cellular transport, development of disease (i.e. colorectal cancer), and for biotechnological applications.
- The group of molecules non-coding regulatory RNAs could provide a more extensive as well as intensive understanding of regulation of CYP members. Gene regulation potentially provides answers to questions regarding expression patterns and even interindividual variations that cannot be offered by genetic reasons. At present, only CYP1B1 has been shown to be regulated directly by a miRNA. This is a promising area of research for the CYPs and for drug metabolism and disposition research as a whole.
- The interplay of the environment, genome and the resulting ability of an individual to respond to therapy can be conclusively included in a proposed idea called 'pharmacoepigenetics.' While the genomic sequence is rather stable and we are in constant relationship with a changing environment, epigenetic

mechanisms are the way individuals respond. This also potentially creates a phenotypic spectrum within the population. In order to create a personalized medicine strategy in therapeutics, an understanding of how our environment affects us is very vital.

10 ACKNOWLEDGEMENTS

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