

Molecular Basis of Inter-Individual Variability in Cytochrome P450

2D6-Mediated Drug Metabolism

BY

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THESIS

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CONTRIBUTION OF AUTHORS

Chapter 1 is a literature review that summarizes the previously published researches in the field and provides the context of this thesis research. Part of this chapter represents a published manuscript, in which I, Miaoran Ning, my research advisor, Dr. Hyunyoung Jeong, and a previous member of the laboratory, Dr. Xian Pan, contributed to the writing of the manuscript.

Chapter 2 represents an unpublished work in which I was the main investigator. This work is anticipated to be published as a manuscript with me as the first-author. Dr. Julio Duarte contributed to analysis for high-throughput DNA sequencing data. Dr. Nina Isoherranen's lab performed hepatic all trans retinoic acid measurement (i.e. Figure 10). Dr. Leah Robin contributed to statistical analysis (i.e. adjusted regression analysis). My research advisor Dr. Hyunyoung Jeong contributed to writing of the chapter.

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Chapter 4 represents a published manuscript (include complete citation) in which I was the first author. Dr. Kwi Hye Koh, a previous member of the laboratory, performed the experiments shown in Figure 15, and co-performed experiments shown in Figure 18 with me. Dr. Xian Pan provided the samples used in the experiments shown in Figure 16. My research advisor Dr. Hyunyoung Jeong contributed to the writing of the manuscript.

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LIST OF ABBREVIATIONS OR NOMENCLATURE

AS	Activity score
atRA	All trans retinoic acid
AUC	Area under the curve
BA	Bile acid
C/EBP α	CCAAT/enhancer-binding protein α
CA	Cholic acid, 5 β -cholanic acid-3 α ,7 α ,12 α -triol
CAR	Constitutive androstane receptor
CD	Collision energy
CDCA	Chenodeoxycholic acid, 5 β -cholanic acid-3 α ,7 α -diol
ChIP	Chromatin immunoprecipitation
C _{max}	Maximal plasma concentration
CNV	Copy number variation
CYP2D6	Cytochrome P450 2D6
CYP3A4	Cytochrome P450 3A4
CYP3A4	Cytochrome P450 3A4
CYP7A1	Cytochrome P450 7A1
CYP7A1	Cytochrome P450 7A1
CYP8B1	Cytochrome P450 8B1
CYP8B1	Cytochrome P450 8B1
DCA	Deoxycholic acid, 5 β -cholanic acid-3 α ,12 α -diol
DME	Drug metabolizing enzyme
DP	Declustering potential

DX	Dextrorphan
EM	Extensive metabolizer
EMSA	Electrophoretic mobility shift assay
ESC	Embryonic stem cell
FXR	Farnesoid X receptor
GCA	Glycocholic acid, 5 β -cholanolic acid-3 α ,7 α ,12 α -triol -N-(carboxymethyl)-amide
GCDCA	Glycochenodeoxycholic acid, 5 β -cholanolic acid-3 α ,7 α -diol-N-(carboxymethyl)-amide
GDCA	Glycodeoxycholic acid, 5 β -cholanolic acid-3 α ,12 α -diol-N-(carboxymethyl)-amide
GDHCA	Glycodehydrocholic acid, 5 β -colanic acid-3,7,12-trione-N-(carboxymethyl)-amide
GHCA	Glycohyocholic acid, 5 β -cholanolic acid-3 α ,6 α ,7 α -triol-N-(carboxymethyl)-amide
GHDCa	Glycohyodeoxycholic acid, 5 β -cholanolic acid-3 α ,6 α -diol-N-(carboxymethyl)-amide
GLCA	Glycolithocholic acid, 5 β -cholanolic acid-3 α -ol-N-(carboxymethyl)-amide
GS1	Gas 1, TurbolonSpray nebulizer
GS2	Gas 2, Heater gas
GUDCA	Glycoursodeoxycholic acid, 5 β -cholanolic acid-3 α ,7 β -diol-N-(carboxymethyl)-amide

GW4064	Synthetic farnesoid X receptor agonist
GW4064	3-[2-[2-Chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid
HCA	hyocholic acid, 5 β -cholanolic acid-3 α ,6 α ,7 α -triol
HDCA	Hyodeoxycholic acid, 5 β -cholanolic acid-3 α ,6 α -diol
HEK293T	Human embryonic kidney cell line 293T
HFD	High fat diet
HFD	High fat diet
HNF4 α	Hepatocyte nuclear factor 4 α , NR2A1
IM	Intermediate metabolizer
IS	Internal standard
KLF9	Krüppel-like Factor 9
LC-MS/MS	Liquid chromatography–mass spectrometry
LCA	Lithocholic acid, 5 β -cholanolic acid-3 α -ol
LRH-1	Liver receptor homolog-1, NR5A2
LTCDS	Liver tissue cell distribution system
MR	Metabolic ratio
MRM	Multiple reaction monitor
muroCA	Murocholic acid, 5 β -cholanolic acid-3 α , 6 β -diol
P0	Pre-pregnancy in gestational time point in mice
P17	17 days of pregnancy, term pregnancy in mice
PM	Poor metabolizer
PP7	Postpartum day 7 in mice

PXR	Pregnane X receptor
QC	Quality control
qRT-PCR	Quantitative real time- Polymerase chain reaction
RA	Retinoic acid
RE	Response element, DNA binding site for transcription factor
S.D.	Standard deviation
SHP	Small heterodimer partner, NR0B2
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
TCA	Taurocholic acid, 5 β -cholanolic acid-3 α ,7 α ,12 α -triol-N-(2-sulphoethyl)-amide
TCDCA	Taurochenodeoxyxholic acid, 5 β -cholanolic acid-3 α ,7 α -diol-N-(2-sulphoethyl)-amide
TDCA	Taurodeoxyxholic acid, 5 β -cholanolic acid-3 α ,12 α -diol-N-(2-sulphoethyl)-amide
TDHCA	Taurodehydrocholic acid, 5 β -colanic acid-3,7,12-trione-N-(2-sulphoethyl)-amide
tg-CYP2D6	CYP2D6 humanized mouse
THCA	Taurohyocholic acid, 5 β -cholanolic acid-3 α ,6 α ,7 α -triol-N-(2-sulphoethyl)-amide
THDCA	Taurohyodeoxycholi acid, 5 β -cholanolic acid-3 α ,6 α -diol-N-(2-sulphoethyl)-amide
TLCA	Taurolithocholic acid, 5 β -cholanolic acid-3 α -ol-N-(2-sulphoethyl)-

	amide
TUDCA	Tauroursodeoxycholic acid, 5 β -cholanolic acid-3 α ,7 β -diol-N-(2-sulphoethyl)-amide
T α MCA	Tauro- α -muricholic acid, 5 β -cholanolic acid-3 α ,6 β ,7 α -triol-N-(2-sulphoethyl)-amide
T β MCA	Tauro- β -muricholic acid, 5 β -cholanolic acid-3 α ,6 β ,7 β -triol-N-(2-sulphoethyl)-amide
UDCA	Ursodeoxycholic acid, 5 β -cholanolic acid-3 α , 7 β -diol
UM	Ultra-rapid metabolizer
UTR	Untranslated region
WT	Wild type
α MCA	α -muricholic acid, 5 β -cholanolic acid-3 α ,6 β ,7 α -triol
β MCA	β -muricholic acid, 5 β -cholanolic acid-3 α ,6 β ,7 β -triol
ω MCA	ω -muricholic acid, 5 β -cholanolic acid-3 α ,6 α ,7 β -triol

SUMMARY

Cytochrome P450 (CYP) 2D6 is a major drug metabolizing enzyme, metabolizing over 20% of clinically used drugs. CYP2D6-mediated drug metabolism exhibits large inter-individual variability. This large variability in CYP2D6 activity can lead to significant alternations in drug exposure, subsequently therapeutic responses or adverse effects after standard doses of CYP2D6 substrates. Genetic polymorphisms of CYP2D6 are known to contribute to the variability. Especially, individuals carrying two non-function CYP2D6 alleles exhibit poor metabolizer (PM) phenotype. However, PMs only account for 5-10% of the population and CYP2D6 genotype-phenotype association is weak in non-PM population. Previous studies suggest good correlation between CYP2D6 expression and activity levels. However, the extent of variability in CYP2D6 activity levels that can be explained by CYP2D6 genotypes and differential CYP2D6 expression has not been determined.

In Chapter 2, we determined the portion of variability in CYP2D6 activity explained by different parameters in a systematic manner by examining CYP2D6 genotypes, mRNA expression, protein expression and activity levels in 115 healthy human liver tissues. Our results showed that, CYP2D6 activity score (AS), a semi-quantitative collective representation of CYP2D6 genotypes, explained 23% of the variability in CYP2D6 activity in the human liver tissues; especially, in samples carrying one to two functional CYP2D6 genes, AS only explained 7.5% of the variability. On the other hand, 59% of the variability in CYP2D6 activity is explained by CYP2D6 protein levels. This indicates that CYP2D6 activity is governed by its protein expression. CYP2D6 protein and mRNA levels were also significantly correlated; however, the overall r^2 was 0.11, likely reflecting the dynamics gene regulation. The CYP2D6 protein-mRNA correlation was higher ($r^2 = 0.17$) in samples that do not carry CYP2D6 variants

that were previously known to affect protein translation. We also explored whether differential expression and/or activity of CYP2D6 transcriptional regulators play a role in explaining variability in CYP2D6 mRNA expression. Results from a series of adjusted analysis and multivariate regression showed that CYP8B1 expression (that share the same transcriptional regulatory pathway as CYP2D6) was the best predictor of CYP2D6 mRNA levels. In summary, our data from human liver tissues indicate that CYP2D6 genotypes only partially explain the variability in CYP2D6 activity and its role in non-PM population was limited. CYP2D6 activity level in the liver is governed predominantly by its protein amount highlighting the importance of CYP2D6 regulation in explaining the inter-individual variability of CYP2D6-mediated drug metabolism.

On the other hand, the significant correlation between CYP2D6 protein and mRNA expression observed in this study and previous studies underscores the need to understand CYP2D6 transcriptional regulation and its modulators. However, factors governing transcription regulation of CYP2D6 are not well understood. It's only known that hepatocyte nuclear factor (HNF) 4 α binds to CYP2D6 promoter and transactivates CYP2D6 expression, while small heterodimer partner (SHP) interacts with HNF4 α and subsequently represses CYP2D6 expression. Previous studies have shown that high fat diet (HFD) feeding and subsequent obesity in mice lead to altered expression of HNF4 α and SHP. Medical conditions accompanying obesity often require drug therapy, but whether and how obesity alters the expression of CYP2D6 is not known.

In Chapter 3, we examined whether diet-induced obesity alters CYP2D6 expression by modulating HNF4 α and SHP expression in CYP2D6-humanized (tg-CYP2D6) mice. Male Tg-CYP2D6 mice were fed with HFD or matching control diet for 18 weeks. Hepatic mRNA

expression of CYP2D6 decreased to a small extent in HFD group (by 31%), but the differences in CYP2D6 protein and activity levels in hepatic S9 fractions were found insignificant between the groups. While hepatic SHP expression did not differ between the groups, HNF4 α mRNA and protein levels decreased by ~30% in HFD group. Among major mouse endogenous Cyp genes, Cyp1a2 and Cyp2c37 showed significant decreases in HFD group while Cyp2e1 expression did not differ between groups. Cyp2b10 and Cyp3a11 expression was higher in HFD group, with corresponding 2.9-fold increases in hepatic CYP3A activities in HFD-fed mice. Together, these results suggest that obesity has minimal effects on CYP2D6-mediated drug metabolism while it modulates the expression of mouse endogenous CYPs in a gene-specific manner.

Clinical evidences suggest that CYP2D6-mediated drug metabolism is enhanced during pregnancy. Our laboratory recently recapitulated this phenomenon (i.e. enhanced CYP2D6 expression and activity) in pregnant tg-CYP2D6. This is caused by enhanced transactivation of CYP2D6 promoter by HNF4 α during pregnancy, and this is triggered in part by altered SHP and Krüppel-like factor 9 (KLF9). The objective of Chapter 4 is to determine whether this is conserved for mouse endogenous Cyp2d gene(s). Among the eight Cyp2d homologs of mouse we examined, only Cyp2d40 expression was found induced (by 6-fold) at term pregnancy as compared to pre-pregnancy level. In mice where hepatic Hnf4 α was knocked-down, the pregnancy-mediated increase in Cyp2d40 expression was abrogated. Results from transient transfection, promoter reporter assays, and electrophoretic mobility shift assays indicated that HNF4 α transactivates Cyp2d40 promoter via direct binding to -117/-105 of the gene. Chromatin immunoprecipitation assay showed a 2.3-fold increase in HNF4 α recruitment to Cyp2d40 promoter during pregnancy. Results from mice treated with an SHP inducer (i.e., GW4064) and HepG2 cells co-transfected with KLF9 suggest that neither SHP nor KLF9 is involved in the

increased HNF4 α transactivation of Cyp2d40 promoter during pregnancy. Together, our results indicate that while the underlying molecular mechanism is different from that for CYP2D6, Cyp2d40 is induced during pregnancy through enhanced transactivation by HNF4 α .

In conclusion, we determined the major sources of variability in CYP2D6 activity in human liver tissues and examined effect of physiological factors, i.e. obesity and pregnancy, on CYP2D6 or Cyp2d40 expression. These results highlighted the importance of CYP2D6 gene regulation for its expression at basal level and under different physiological states.

CHAPTER 1.OVERVIEW OF INTER-INDIVIDUAL VARIABILITY AND TRANSCRIPTIONAL REGULATION OF CYTOCHROME P450 2D6 ENZYME

Part of this chapter was previously published as Pan X, Ning M, Jeong H. (2017) Transcriptional Regulation of CYP2D6 Expression. Drug Metab Dispos. 45(1), 42-48.

Cytochrome P450 (P450) 2D6 (CYP2D6) is a major drug-metabolizing enzyme responsible for the clearance of over 20% of marketed drugs (Zhou 2009). Substrates of CYP2D6 include tricyclic antidepressants (e.g. desipramine), selective serotonin receptor inhibitors (e.g. paroxetine), β -blockers (e.g. metoprolol), antiarrhythmics (e.g. sparteine), opioid analgesics (e.g. codeine) and anti-cancer agents (e.g. tamoxifen) (He et al. 2015). CYP2D6 is expressed in the liver and in other organs such as brain and intestine (Miksys et al. 2002, Paine et al. 2006, Shimada et al. 1994). CYP2D6 protein accounts for ~1.5% of total P450 abundance in the liver (Shimada et al. 1994). Average values of CYP2D6 absolute protein expression in human liver microsomes range from 5 to 30 pmol/mg microsomal protein in different reports (Kawakami et al. 2011, Groer et al. 2014, Shimada et al. 1994, Langenfeld et al. 2009).

1.1. Inter-individual variability in CYP2D6-mediated drug metabolism

CYP2D6-mediated drug metabolism exhibits large inter-individual variability. For example, urinary metabolic ratio (MR, the ratio between metabolite and parent drug) of dextromethorphan, primarily metabolized by CYP2D6 into dextrorphan, ranged over 5 orders of magnitude in 660 US subjects (Gaedigk et al. 2008). MR of debrisoquine, another sensitive substrate of CYP2D6, also ranged for ~4 orders of magnitude in 695 Chinese and 1101 Swedish subjects (Bertilsson et al. 1992). Large variability in CYP2D6 activity is also observed in human liver microsomes in vitro. Dextromethorphan O-demethylation rate, representing CYP2D6 activity, ranged from no activity to over 1000 pmol/min/mg microsomal protein in individual

human liver microsomes (Yang et al. 2010, Temesvari et al. 2012, Hart et al. 2008). Based on the CYP2D6 phenotyping results, an individual can be classified as ultra-rapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM) and poor metabolizer (PM) with CYP2D6 activity from high to low (Gaedigk et al. 2008). The frequencies of the four phenotypes are 0.6, 75, 19, and 6%, respectively, in 660 subjects in the United States (Gaedigk et al. 2008). Of note, CYP2D6 phenotype frequencies vary in different ethnic groups (Llerena et al. 2014, Gaedigk et al. 2017).

This large variability in CYP2D6-mediated drug metabolism can lead to significant alternations in drug exposure, subsequently therapeutic responses or adverse effects after standard doses of CYP2D6 substrates. For instance, codeine is metabolized by CYP2D6 to pharmacologically active morphine. UM individual can have abnormally high concentration of morphine, subsequently intoxication, after a regular dose of codeine (Gasche et al. 2004). For another instance, nortriptyline is a tricyclic antidepressant with narrow therapeutic index cleared by CYP2D6. Regular doses of nortriptyline (i.e., 75 mg/day) can lead to high plasma drug concentration and cause adverse effect in a PM patient, while 300-500 mg daily dose was needed to achieve therapeutic plasma concentration in a UM patient (Bertilsson et al. 2002). Precision dosing based on CYP2D6 activity level is desirable for CYP2D6 substrates, especially for ones with narrow therapeutic index, to achieve optimum drug responses and to avoid adverse effects.

1.2. CYP2D6 genetic polymorphisms

Historically, inter-individual variability in debrisoquine and sparteine metabolism (later proved to be primarily mediated by CYP2D6) was observed in parallel and the deficiency in metabolizing these two drugs was associated with a recessive gene in the late 1970s (Eichelbaum

et al. 1979, Mahgoub et al. 1977). CYP2D6 cDNA was cloned and characterized in the 1980s (Gonzalez et al. 1988). The study also demonstrated that some debrisoquine poor metabolizer carry the mutant gene which produces negligible amount of the enzyme due to incorrect mRNA splicing. Since the discovery that genetic variations in CYP2D6 account for poor metabolizer phenotype, CYP2D6 genetic polymorphisms have been studied extensively. Up to date, over 100 CYP2D6 alleles have been identified (<http://www.cypalleles.ki.se/cyp2d6.htm>).

CYP2D6 genetic polymorphisms are complex. CYP2D6 locates on the negative strand of Chr 22q13.1. The whole CYP2D6 locus spans about 45 kb, containing CYP2D6 and two pseudogenes, namely CYP2D7 and CYP2D8. CYP2D7 is highly homologous with CYP2D6, sequence identical or near-identical over large regions (Gaedigk 2013). A common CYP2D6 sequence (GeneBank entry M33388), published by Kimura et al. (Kimura et al. 1989), was designated as reference and named as CYP2D6*1. For CYP2D6 nomenclature, nucleotide positions are assigned based on M33388, despite three errors found in the sequence when compared with the nowadays gold-standard sequence AY545216. Of note, the CYP2D6 reference sequence on human genome assembly hg19 is not that of CYP2D6*1 allele, rather the sequence of CYP2D6*2, another common CYP2D6 allele. In addition to the complexity of different reference sequences and coordinates, the types of CYP2D6 genetic variations are also complex. The most common type of CYP2D6 genetic variations contains single nucleotide variations (SNVs) that include single nucleotide polymorphisms (SNPs) and insertions or deletions of one or a small number of nucleotides. Other types are characterized by gene rearrangements, including gene deletions, duplication/multiplication or CYP2D6-CYP2D7 gene hybrids (Gaedigk 2013).

Different CYP2D6 variant alleles can be associated with increased, normal, decreased or no activity of the enzyme. Due to the complexity and large number of CYP2D6 genetic variants (some are rare variants), a CYP2D6 activity score (AS) system was proposed to simplify CYP2D6 genotype interpretation for predicting CYP2D6 phenotype with genotype information (Gaedigk et al. 2008). CYP2D6 AS assignment to each individual follows the these rules: (1) each CYP2D6 allele is assigned with functional gene dose based on the activity that the allele associated with (i.e. 0 for non-functional, 0.5 for decreased-functional, 1 for normal functional and 2 for increased-functional alleles due to functional gene duplication/multiplication); (2) each individual has two alleles of CYP2D6 from maternal and paternal heritages respectively; (3) AS is the sum of the functional gene doses of maternal and paternal alleles (Gaedigk et al. 2008, Gaedigk et al. 2017). Of note, activity score classification, as inferred by the name, is based on the association between allelic variants and CYP2D6 activity levels. The molecular mechanisms by which these variants alter CYP2D6 activity can be different for alleles with the same functional gene dose. For example, CYP2D6 gene deletion (i.e. CYP2D6*5 allele) and multiplication (e.g. CYP2D6*1×N allele) lead to no expression or increased expression of CYP2D6 mRNA, respectively, and subsequently alter the activity levels of CYP2D6. On the other hand, mutations in alleles that lead to frameshifts (e.g. CYP2D6*4) (Hanioka et al. 1990) or producing increased level of nonfunctional mRNA splice variant (e.g. CYP2D6*41) (Toscano et al. 2006) affect CYP2D6 activity by altering protein expression. Some alleles with point mutations that lead to amino acid changes (e.g. CYP2D6*10 and *12) can affect CYP2D6 enzymatic activity directly (Niwa, Murayama, and Yamazaki 2011, Tsuzuki et al. 2001, Matsunaga et al. 2009).

1.3. CYP2D6 genotype-phenotype association

In the effort to explain inter-individual variability in CYP2D6-mediated drug metabolism, CYP2D6 genotypes are most intensively studied. In general, there was a good agreement between CYP2D6 genotype and phenotype for PMs in the way that individuals carrying two non-functional CYP2D6 alleles (AS of 0) will exhibit PM phenotype. Two independent reviews across different ethnicities showed that frequencies of phenotypic PMs are similar to the frequencies predicted by CYP2D6 genotype (Llerena et al. 2014, Gaedigk et al. 2017). Notably, observed frequencies of PMs tend to be higher than the predicted PM frequencies in all the ethnicities. For non-PM subjects, the genotype-phenotype association remains significant that median CYP2D6 activity increases with the number of functional CYP2D6 alleles or AS in the carriers. However, CYP2D6 activity levels (i.e. dextromethorphan metabolic ratio) in carriers with one to two functional CYP2D6 alleles (AS 1 to 2) are largely overlapping (Gaedigk et al. 2008). Specifically, CYP2D6 activity within the same AS or even with the same genotype still ranges for ~100 folds, comparing with ~3 fold change in medians of AS 1 and 2 groups (Gaedigk et al. 2008).

Similar conclusion can be drawn from the results of a twin study of metoprolol pharmacokinetics (Matthaei et al. 2015). Metoprolol is also a CYP2D6 substrate, with CYP2D6-mediated metabolism accounting for ~80% of its clearance (Ito et al. 2005). Results from 88 monozygotic and 24 dizygotic twins showed that CYP2D6 genotype only explained 39% of the variability in metoprolol AUC, reflecting CYP2D6 activity in the subjects (Matthaei et al. 2015). Subjects with semiquantitative gene dose (the same as AS) of 0 and 0.5 had the highest metoprolol AUC, while subjects with gene dose above 1 had overlapping metoprolol AUC range. Together, these evidences suggests that CYP2D6 genotype as a contributor to inter-individual variability in CYP2D6 activity accounts for PM phenotype; however, it does not play a major

role in explaining variability in CYP2D6 activity in non-PM population (~90% of the population).

1.4. Association between CYP2D6 activity and expression

CYP2D6 activity is highly correlated with its protein abundance in human liver microsomes (Langenfeld et al. 2009, Lee et al. 2009, Ohtsuki et al. 2012, Achour et al. 2014). Three independent studies measured CYP2D6 absolute protein levels in individual human liver microsomes (n=17-23), and showed that CYP2D6 activity measured by dextromethorphan O-demethylation significantly correlated with CYP2D6 protein levels with Spearman coefficient of 0.65, 0.8 and least-square regression r^2 of 0.78 (Langenfeld et al. 2009, Ohtsuki et al. 2012, Achour et al. 2014). Lee et al. measured CYP2D6 protein expression in human liver microsomes from 49 Korean donors by western blotting and showed high correlation (Spearman $r^2=0.70$) between CYP2D6 protein and activity levels (Lee et al. 2009).

In addition, studies have shown that CYP2D6 activities positively correlate with its mRNA expression levels (Yang et al. 2010, Temesvari et al. 2012, Rodriguez-Antona et al. 2001, Zanger et al. 2001). For example, in 398 human liver tissues, CYP2D6 mRNA expression and activity levels were correlated with a correlation coefficient (r) value of 0.53 (Yang et al. 2010). In another study, CYP2D6 mRNA expression and activity levels exhibited stronger correlation ($r = 0.95$) in 164 human liver tissues (Temesvari et al. 2012). Interestingly, the extent of correlation was similar to that of CYP3A4 ($r = 0.55$ and 0.90 in the former and latter study, respectively). CYP3A4 is a drug-metabolizing enzyme whose expression and activity is governed at the level of gene transcription via actions of multiple transcription factors (Goodwin, Redinbo, and Kliewer 2002, Goodwin et al. 2002). The fact that both CYP2D6 and CYP3A4 exhibit similar extent of correlation between mRNA and activity levels (in human liver tissues) raises the

possibility that CYP2D6 expression and activity may also be determined at the level of CYP2D6 transcription, and differential transcriptional regulation of CYP2D6 may contribute to large inter-individual variability in CYP2D6-mediated drug metabolism.

1.5. Transcription factors of CYP2D6

Historically, CYP2D6 was not considered to be inducible, due to the fact that prototypical inducers of drug-metabolizing enzymes (e.g., rifampin or phenobarbital) do not induce CYP2D6 expression. Despite good correlation between CYP2D6 activity and expression, transcriptional regulation of CYP2D6 is poorly understood. To date, only a small number of transcription factors have been identified to be involved in the transcriptional regulation of CYP2D6. These transcription factors include hepatocyte nuclear factor (HNF) 4 α , small heterodimer partner (SHP) and CCAAT/enhancer-binding protein (C/EBP) α .

HNF4 α is a member of nuclear receptor family and a global regulator of genes involved in liver development or liver-specific functions (Gonzalez 2008). Whole body deletion of HNF4 α results in embryonic lethality in mice (Chen et al. 1994). On the other hand, mice absent hepatic HNF4 α (as a result of organ-specific, conditional targeted gene deletion) are viable but exhibit disrupted lipid homeostasis (Hayhurst et al. 2001), demonstrating a key role of HNF4 α in maintenance of normal hepatic function. Also, HNF4 α is known to regulate expression of drug-metabolizing enzymes including CYP2D6. A systematic study using over 400 human liver tissues showed that HNF4 α mRNA expression significantly correlated with microsomal CYP2D6 enzyme activity (Yang et al. 2010). In HepG2 cells, ectopically expressed HNF4 α enhanced promoter activity of CYP2D6 by binding to a response element (i.e., -53/-41) in CYP2D6 promoter (Cairns et al. 1996). HNF4 α knockdown (by using antisense targeting) led to significant decreases in CYP2D6 expression in human hepatocytes, indicating that HNF4 α is

required for the constitutive expression of CYP2D6 (Jover et al. 2001). Similarly, liver-specific deletion of HNF4 α led to (greater than 50%) decreases in CYP2D6 expression and activity in mice (Corchero et al. 2001). Together, these results indicate key roles of HNF4 α in the regulation of constitutive CYP2D6 expression in liver.

Numerous genetic polymorphisms have been reported for HNF4 α , and some have shown to modulate CYP2D6 expression. G60D polymorphism confers lower DNA binding activity of HNF4 α , and is associated with decreased hepatic CYP2D6 expression (Lee et al. 2008). Human pharmacokinetic study of tolterodine (a CYP2D6 substrate) demonstrated that HNF4 α G60D and CYP2D6 genotypes together account for a significant proportion of the variation in tolterodine area under the curve ($AUC_{0-\infty}$) and maximal plasma concentration after an oral dose (C_{max}) ($r^2 = 0.81$ and 0.59 , respectively; $p < 0.01$), and the relative contribution by HNF4 α genotype to tolterodine pharmacokinetic variability is approximately 25% of that by CYP2D6 genotypes (Jiang et al. 2013). HNF4 α G60D allele frequency is very low (at most 1.3%, observed in Korean population; almost nonexistent in Caucasians and African Americans (Lee et al., 2008)), and thus its overall contribution to CYP2D6 variability in non-Asian populations may be low. Other rare missense SNPs in HNF4 α have been reported that leads to maturity-onset diabetes of the young (MODY) or hyperinsulinemic hypoglycemia (reviewed in (Colclough et al. 2013)). Structural studies and *in vitro* experiments have shown that some of these disease-linked mutations are linked to decreased HNF4 α binding to DNA and subsequently reduced HNF4 α transactivation of target gene promoters (Lu et al. 2008, Chandra et al. 2013). Whether these HNF4 α polymorphisms lead to altered HNF4 α transactivation of CYP2D6 promoter remains unclear.

Studies on functional SNPs in HNF4 α regulatory regions (i.e., upstream, 5'-, or 3'-untranslated region) have been sporadic. rs11574744 is a SNP in 3'-untranslated region of

HNF4 α (minor allele frequency of 3.4%) and located in putative binding sites of multiple microRNAs (Ramamoorthy et al. 2012, Wirsing et al. 2011). Subjects carrying rs11574744 (both homozygous and heterozygous) exhibited lower CYP2D6 metabolic ratio (of dextromethorphan) than those with wild-type HNF4 α , but the difference did not reach statistical significance ($p = 0.16$) (Ramamoorthy et al. 2012), suggesting that its clinical implication may be minor.

In the investigation of the molecular mechanism underlying altered CYP2D6-mediated drug metabolism during pregnancy, our laboratory identified SHP as a novel repressor of CYP2D6 expression. SHP is a nuclear receptor that does not have a DNA-binding domain, and behaves as a co-repressor by physically interacting with various transcription factors (including HNF4 α) and inhibiting their activities (Zhou et al. 2010). In HepG2 cells, SHP overexpression suppressed HNF4 α transactivation of CYP2D6 promoter; in tg-CYP2D6 mice, SHP knockdown (using small interfering RNA) led to a significant increase in CYP2D6 expression (Koh, Pan, Shen, et al. 2014). These studies in *in vitro* and *in vivo* systems demonstrated a role of SHP as a repressor of CYP2D6 expression.

C/EBP α is a transcription factor expressed in many different tissues including placenta, liver, and small intestine (Schrem, Klempnauer, and Borlak 2004). Biological functions of C/EBP α are diverse and include nutrient metabolism, cell cycle control, and liver regeneration (Schrem, Klempnauer, and Borlak 2004). C/EBP α is expressed at later stages of liver development, and overexpression of C/EBP α in HepG2 cells increased the mRNA levels of CYP2D6 as well as other drug-metabolizing enzymes such as CYP2B6 and CYP2C9 (Jover et al. 1998). On the other hand, knockdown of C/EBP α expression (by using small interfering RNA) led to decreased expression of CYP2D6 in HepG2 cells (Matsunaga et al. 2012), suggesting that C/EBP α is a transcriptional regulator involved in basal CYP2D6 expression. C/EBP α binding

site was identified within -1231/-1220 of CYP2D6 via *in silico* promoter analysis, promoter reporter assays, and chromatin immunoprecipitation assays in HepG2 cells (Matsunaga et al. 2012). These results indicate that C/EBP α is a transcriptional activator of CYP2D6 expression. Its regulatory role in CYP2D6 expression in *in vivo* systems or in human liver tissues remains to be verified.

1.6. Genetic polymorphisms of CYP2D6 that modulate CYP2D6 expression

While a majority of genetic polymorphisms identified to date are located in the exonic or intronic region of CYP2D6 gene, there have been sporadic reports for the presence of single nucleotide polymorphisms (SNPs) within regulatory regions of CYP2D6 and their roles in differential expression of CYP2D6. For example, carriers of -1584C>G (rs1080985) polymorphism (first discovered in the carriers of CYP2D6*2) exhibited increased metabolism of CYP2D6 substrates (i.e., dextromethorphan and thioridazine) (Dorado et al. 2009, Llerena et al. 2013). CYP2D6 protein levels in the liver tissues of -1584 C/C carriers were ~50% of those of either -1584 C/G or G/G carriers (Dorado et al. 2009, Llerena et al. 2013, Zanger et al. 2001). The underlying molecular mechanisms for the finding and the role of -1584C>G in transcriptional regulation of CYP2D6 remain unclear.

A systematic approach to identify SNPs associated with altered P450 expression and/or activity (using 398 human liver tissues and SNP arrays) revealed that three long-range polymorphisms of CYP2D6, namely rs8138080 (126 kb downstream of CYP2D6), rs17478227, and rs5751247 (~127 and ~106 kb upstream of CYP2D6, respectively) are associated with lower CYP2D6 activity as well as the mRNA levels (Yang et al. 2010). It is unknown whether the long-range polymorphisms are functional; however, they are in strong linkage disequilibrium with CYP2D6*4 (rs3892097, rs1065852) and CYP2D6*10 (rs1065852) that result in

nonfunctional or decreased-function enzymes, respectively (Yang et al. 2010), raising the possibility that the decreased CYP2D6 activity of CYP2D6*4 and *10 alleles may be in part responsible for the lower CYP2D6 activity in the liver tissues carrying the long-range polymorphisms.

Most recently, studies have found another SNP rs5758550 (A>G) that is associated with enhanced CYP2D6 expression (Wang, Papp, and Sun 2014, Wang et al. 2014). SNP rs5758550 is located at ~115 kb downstream of CYP2D6 gene, and is in linkage with CYP2D6*2. Results from *in vitro* promoter reporter assays demonstrated that rs5758550G is associated with (2-fold) higher CYP2D6 promoter activity as compared to rs5758550A (Wang, Papp, and Sun 2014). In HepG2 cells, deletion of the region surrounding rs5758550 (using CRISPR-Cas9 technology) led to >2-fold decreases in CYP2D6 mRNA level (Wang, Papp, and Sun 2014), suggesting an important regulatory role of rs5758550 in basal CYP2D6 expression. Together, these results suggest that SNPs in upstream or downstream regulatory regions of CYP2D6 gene may alter CYP2D6 expression and thus its activity. Systematic approaches appear necessary to identify other promoter or enhancer SNPs that may play a role in the transcriptional regulation of CYP2D6 expression and to elucidate their roles in inter-individual variability in CYP2D6-mediated drug metabolism.

1.7. Physiological factors affecting CYP2D6 expression

Prescription and over the counter drug use during pregnancy are common (Glover et al. 2003, Andrade et al. 2004). Accumulating clinical data indicate that CYP2D6-mediated drug metabolism is enhanced during pregnancy, presenting a rare case of CYP2D6 induction. The apparent induction in CYP2D6 activity during pregnancy was first observed in 1983; the peak plasma concentrations obtained after an oral dose of metoprolol in pregnant women were only

20-40% of that after delivery (Hogstedt, Lindberg, and Rane 1983). A subsequent study showed that systemic clearance of metoprolol estimated after an intravenous dose was 26–97% higher at term pregnancy as compared to after delivery (Hogstedt et al. 1985). Later studies using other CYP2D6 substrates [e.g., dextromethorphan (Wadelius et al. 1997, Tracy et al. 2005), clonidine (Buchanan et al. 2009, Claessens et al. 2010), and paroxetine (Ververs et al. 2009)] showed similar increases in CYP2D6-mediated drug metabolism during pregnancy. For example, plasma metabolic ratio of dextromethorphan (i.e., dextromethorphan/dextrorphan) at 2-hour after an oral dosing was decreased (by 53%) during pregnancy (Wadelius et al. 1997), and urinary metabolic ratio of dextromethorphan in a cumulative 24-hour urine sample was decreased by 26-48% (Tracy et al. 2005). Together, these studies demonstrated significant increases in CYP2D6-mediated drug metabolism in pregnant women.

Notably, the increases in CYP2D6-mediated drug metabolism were not observed in CYP2D6 IM or PM subjects. For example, while steady-state plasma concentrations of paroxetine decreased in CYP2D6 EM subjects (indicative of CYP2D6 induction), such changes were not shown in IM or PM subjects (Ververs et al. 2009). Similarly, increases in dextromethorphan metabolism in pregnant women (as compared to postpartum women) were not observed in CYP2D6 PM subjects (Wadelius et al. 1997). One potential explanation of these findings is increased transcriptional activation of CYP2D6 during pregnancy.

Recently, our laboratory recapitulated CYP2D6 induction during pregnancy and studied the underlying mechanism in a CYP2D6-humanized (tg-CYP2D6) mouse model. Tg-CYP2D6 genome harbors the human CYP2D6 gene plus 2.5-kb of upstream regulatory region of CYP2D6 (Corchero et al. 2001, Hayhurst et al. 2001), thus enable us to study transcriptional regulation of human CYP2D6 gene in an *in vivo* system. In tg-CYP2D6 mice, pregnancy led to 2-3-fold

increases in CYP2D6 expression (at mRNA and protein levels) as well as in CYP2D6 activity as compared to pre-pregnancy or postpartum period (Koh, Pan, Shen, et al. 2014), presenting the first animal model for the study of CYP2D6 regulation during pregnancy. Previous studies have suggested potential roles of female hormones (i.e., estradiol and progesterone) in altered expression of P450 (such as CYP3A4) in pregnancy (Choi, Koh, and Jeong 2013). However, these hormones (at high concentrations attained at term pregnancy) did not affect CYP2D6 expression in human hepatocytes (Choi, Koh, and Jeong 2013). These results suggest insignificant roles of female hormones, if any, in CYP2D6 induction during pregnancy.

As the only well-established CYP2D6 transcriptional regulator at the time, HNF4 α expression and recruitment to CYP2D6 promoter were examined. Hepatic HNF4 α expression (at mRNA and protein levels) did not differ among different gestational time points in tg-CYP2D6 mice (Koh, Pan, Shen, et al. 2014). Interestingly, however, HNF4 α recruitment to CYP2D6 promoter exhibited significant increases at term pregnancy as compared to pre-pregnancy or postpartum period. The pivotal role of HNF4 α in CYP2D6 induction during pregnancy was further verified in mice where HNF4 α is conditionally knocked down in the liver; CYP2D6 induction during pregnancy was abrogated in these mice (Koh, Pan, Shen, et al. 2014). cDNA microarray using liver tissues of tg-CYP2D6 mice collected at different gestational time points revealed that SHP was down-regulated in the livers of pregnant mice (as compared to those of non-pregnant mice) (Koh, Pan, Shen, et al. 2014, Koh, Pan, Zhang, et al. 2014). The above-mentioned in vitro and in vivo results demonstrated role of SHP in regulating CYP2D6 expression during pregnancy.

Of note, Tg-CYP2D6 mice exhibit robust CYP2D6 expression in liver and intestine but not in the brain (Miksys et al. 2005). Identification and characterization of mouse Cyp2d

homologs whose expression or activities are co-regulated as CYP2D6, if any, may potentially enable establishing study models to explore the biological functions of CYP2D6 in *in vivo* systems. While human expresses only one functional CYP2D (i.e., CYP2D6), mouse genome harbors the nine Cyp2d homologs (i.e. *Cyp2d9*, *2d10*, *2d11*, *2d12*, *2d13*, *2d22*, *2d26*, *2d34*, and *2d40*). To explore whether and how mouse Cyp2d genes are regulated during pregnancy may shed light to conserved physiological/functional significance of CYP2D induction during pregnancy.

On another note, obesity is a prevalent medical condition that 35% of adults and 17% of children in the United States are overweight (Ogden et al. 2014). Obesity is associated with increased incidences of chronic comorbidities, such as diabetes, cardiovascular diseases, and non-alcoholic fatty liver diseases (Fujioka 2015), which often require drug treatment. Molecular changes accompanying obesity include altered expression and/or activity of different transcriptional regulators of drug-metabolizing enzymes (DMEs). Specifically, high fat diet (HFD) feeding in mice has been shown to alter expression levels of nuclear receptors that are involved in the regulation of major DME expression, including HNF4 α and SHP. In mice, HFD induces hepatic expression of microRNA (miR)-34a that targets HNF4 α and thus decreases HNF4 α expression (Xu et al. 2015). On the other hand, SHP protein levels were shown to be higher in the obese mice of genetic-based or diet-induced models, likely due to decreased proteasomal degradation of SHP (Miao et al. 2009). Despite the reports of altered expression of HNF4 α and SHP accompanying in obesity, whether the changes in the major regulators of CYP2D6 expression affect CYP2D6 expression remains unclear.

1.8. Modulators of CYP2D6 transcription

SHP is a representative target gene of a nuclear receptor farnesoid X receptor (FXR). FXR is a bile acid sensor and activated by endogenous bile acids. Upon activation, FXR transactivates SHP promoter by binding to FXR response elements in the proximal promoter region (Chanda, Park, and Choi 2008). SHP (as a co-repressor) in turn inhibits the activity of other transcription factors (such as HNF4 α) and modulates expression of genes involved in bile acid homeostasis (Li and Chiang 2014). SHP expression in *Fxr*-null mice was only ~20% of that in the wild-type mice, indicating that FXR is critical in governing basal SHP expression (Sinal et al. 2000). Synthetic FXR agonists (such as GW4064) or endogenous bile acids enhance SHP expression by activating FXR. In primary human hepatocytes, GW4064 treatment (for 48 hours) led to increased SHP and decreased CYP2D6 expression and activity (Pan, Lee, and Jeong 2015). A similar finding was observed in tg-CYP2D6 mice treated with GW4064 (10 mg/kg/day; i.p. for 5 days). CYP2D6 repression by GW4064 was abrogated when *Shp* was deleted in tg-CYP2D6 mice, indicating essential roles of SHP in CYP2D6 repression by GW4064 (Pan, Lee, and Jeong 2015). Importantly, these results provide proof-of-concept evidence for the roles of SHP modulators in altering CYP2D6 expression, and suggest the possibility that other modulators of SHP may also affect CYP2D6 expression.

Estrogen is the major component in oral contraceptives and hormone replacement therapy. Estrogen regulates growth and differentiation as well as multiple physiological functions by activating estrogen receptor. While estrogen at physiological concentration does not seem to have significant effects on CYP2D6-mediated drug metabolism (Bock et al. 1994, Tamminga et al. 1999, Zanger et al. 2005), intrahepatic cholestasis that often accompanies the therapeutic use of estrogen may alter CYP2D6 expression. Intrahepatic cholestasis has been reported in women on oral contraceptives or (postmenopausal) hormone replacement therapy (Schreiber and Simon

1983), or in men taking estrogens for the treatment of prostate cancer (Kontturi and Sotaniemi 1969). Estrogen-induced cholestasis also occurs during pregnancy that intrahepatic cholestasis of pregnancy is the most common liver disease during pregnancy (Reyes 1997, Riely and Bacq 2004).

Considering that cholestasis leads to FXR activation and subsequent upregulation of SHP (Goodwin et al. 2000), the effects of estrogen-induced cholestasis on CYP2D6 expression were examined in tg-CYP2D6 mice. Mice administered with high-dose ethinylestradiol (10 mg/kg/day; s.c. for 5 days) exhibited increased SHP expression accompanied by 2-3-fold decreases in CYP2D6 expression and activity (Pan and Jeong 2015). In these mice, recruitments of SHP and HNF4 α to CYP2D6 promoter were increased and decreased, respectively, suggesting that enhanced SHP expression represses HNF4 α transactivation of CYP2D6 promoter in ethinylestradiol-induced cholestasis.

Bile acids are major products of cholesterol catabolism in liver and also major components of bile. Primary bile acids (cholic acid and chenodeoxycholic acid) are synthesized in human liver and mainly conjugated with taurine or glycine before excretion into bile canaliculi and then small intestine. Once in the intestine, the conjugated primary bile acids are deconjugated and converted into secondary bile acids (e.g., deoxycholic acid and lithocholic acid) by gut microbiota. Most bile acids are reabsorbed from intestine, conjugated in the liver, and re-excreted into bile (i.e., enterohepatic circulation). Human hepatic bile acids are mainly (>95%) composed of conjugated or unconjugated cholic acid, chenodeoxycholic acid, and deoxycholic acid (Garcia-Canaveras et al. 2012), the most potent bile acid species in activating FXR (Wang et al. 1999, Lew et al. 2004). In human liver tissues, concentrations of bile acids exhibit large inter-individual variations (i.e., coefficient of variation greater than 100% for major bile acids)

(Garcia-Canaveras et al. 2012). Potential roles of bile acids in the regulation of CYP2D6 expression and inter-individual variability in CYP2D6-mediated drug metabolism remain to be defined.

Retinoids are derivatives of vitamin A and play a key role in physiological processes including vision, epithelial differentiation, embryogenesis, and reproduction (D'Ambrosio, Clugston, and Blaner 2011, Napoli 2012). Liver is a major organ for retinoid storage and metabolism. Hepatic stellate cells are major sites of retinoid storage as retinol and retinyl ester, and retinoids are converted to retinoic acid (RA) in hepatocytes (Shirakami et al. 2012). Among three stereoisomeric forms of RA [*all trans* RA (atRA), 9-*cis* RA, and 13-*cis* RA], atRA is the major RA that exerts biological activities via binding to its cognate nuclear receptor (i.e., retinoic acid receptor). 9-*cis* RA and 13-*cis* RA can be converted to atRA in the body (Chen and Juchau 1998). RAs are also used as pharmacological agents for the treatment of cancers or skin diseases (Chen et al. 2013, Veal et al. 2013, Baldwin et al. 2013).

The mechanistic studies for CYP2D6 induction during pregnancy revealed that decreased SHP expression at term pregnancy is potentially responsible for the phenomenon (Koh, Pan, Shen, et al. 2014). A previous study reported that atRA induces SHP expression in HepG2 cells (Cai et al. 2010), and this is potentially responsible for hyperlipidemia often seen in patients treated with high-dose RA for cancer treatment. More importantly, this raises the possibility that altered retinoid homeostasis during pregnancy may underlie SHP repression. Indeed, in tg-CYP2D6 mice, hepatic atRA levels were significantly decreased at term pregnancy as compared to pre-pregnancy (Koh, Pan, Shen, et al. 2014). Also, in non-pregnant tg-CYP2D6 mice, atRA (5 mg/kg/day, intraperitoneally administered for 5 days) significantly increased hepatic SHP expression and decreased CYP2D6 mRNA and activity levels (Koh, Pan, Shen, et al. 2014),

suggesting that lower hepatic retinoids may be responsible for SHP down-regulation during pregnancy. Similar changes in SHP and CYP2D6 expression were observed in human hepatocytes upon atRA treatment (data not shown; a manuscript in preparation), indicating that CYP2D6 regulation by atRA is likely conserved in humans. The molecular mechanisms underlying how retinoids upregulate SHP expression are currently unclear. In healthy adults, hepatic levels of hepatic vitamin A exhibit ~300-fold inter-individual variability (Ukleja et al. 2002), and whether this plays a role in inter-individual variability in CYP2D6-mediated drug metabolism remains to be determined.

1.9. Epigenetic and post-translational regulation of CYP2D6

Studies of epigenetic and post-translational regulation of CYP2D6 are sporadic and their roles in CYP2D6 regulation still need to be defined. Epigenetic regulation of genes includes DNA methylation, histone modification and non-coding RNAs (i.e. miRNA) (Fisel, Schaeffeler, and Schwab 2016). CYP2D6 gene has two CpG islands that can be methylated (Zhang et al. 2016). In 20 human adult livers, CYP2D6 was found methylated at relatively variable statuses, and its methylation levels were inversely correlated with its mRNA expression (Habano et al. 2015). Park et al. compared CYP2D6 mRNA expression and epigenetics between embryonic stem cell (ESC)-derived hepatocytes and primary hepatocytes (Park et al. 2015). ESC-hepatocytes had ~100-fold lower expression of CYP2D6, compared with that in primary hepatocytes. At the same time, DNA methylation level was higher and level of histone H3 trimethylation at lysine 4, an active histone mark, was lower in ESC-hepatocytes. Park et al. also showed that combinatorial inhibition of DNA methyltransferases and histone deacetylases increased transcription of CYP2D6 in ESC-hepatocytes (Park et al. 2015). These results suggest that DNA methylation and histone modification may play a role in CYP2D6 transcriptional

regulation. Regarding miRNA regulation of CYP2D6, miR-101 and miR-128-2 were shown to target and decrease CYP2D6 mRNA levels (Li et al. 2015). Interestingly, miR-101 and miR-128-2 are expressed in the brain and can be upregulated by testosterone in the brain specifically, but not in the liver in mice. The expression level and regulation of miR-101 and miR-128-2 remain unknown in human livers.

As regard to CYP2D6 protein modification, there is only one study available. Phosphorylation of CYP2D6 protein at serine-135 was detected in 2 out of 4 human liver microsomes by using mass spectrometry (Redlich et al. 2008). Ser-135 was also predicted to be phosphorylation site by in silico analysis (i.e. Netphos) (Redlich et al. 2008). This result was consistent with the observation that using peptide containing Ser-135 to quantify CYP2D6 protein always resulted in lower amount when compared with the results using other peptides (Langenfeld et al. 2009).

1.10. Summary and research aims

Results from previous studies suggest that the molecular basis of inter-individual variability in CYP2D6 activity remain to be understood and the extent of variability explained by known factors need to be determined. Good correlation between CYP2D6 expression and activity suggest that differential transcriptional regulation of CYP2D6 may play a role in explaining the inter-individual variability in its activity. Also, studies to determine whether physiological/pathological conditions or environmental factors modulating CYP2D6 transcription factors lead to altered CYP2D6 expression/activity will help identify potential new sources of CYP2D6 inter-individual variability. Therefore, in this thesis research, I aimed to, first, determine the extent of variability in CYP2D6 activity explained by known factors modulating CYP2D6 expression/activity in human liver tissues; and second, to examine the

effect of environmental and physiological factors (i.e. obesity and pregnancy) on the expression of CYP2D6 or mouse *Cyp2d* genes in CYP2D6 humanized mouse.

CHAPTER 2. IDENTIFICATION OF MAJOR CONTRIBUTORS TO INTER-INDIVIDUAL VARIABILITY IN CYTOCHROME 450 2D6-MEDIATED DRUG METABOLISM

2.1. Introduction

Cytochrome P450 (CYP) 2D6 belongs to cytochrome P450 superfamily, an enzyme family capable of catalyzing the oxidative biotransformation of most drugs/xenobiotics and endogenous substances (Zanger and Schwab 2013). As a major phase I drug metabolizing enzyme, CYP2D6 is responsible for the metabolism of over 20% clinically used drugs (He et al. 2015). Substrates of CYP2D6 include tricyclic antidepressants (e.g. desipramine), selective serotonin receptor inhibitors (e.g. paroxetine), β -blockers (e.g. metoprolol), antiarrhythmics (e.g. sparteine), opioid analgesics (e.g. codeine) and anti-cancer agents (e.g. tamoxifen) (He et al. 2015).

CYP2D6-mediated drug metabolism exhibits large inter-individual variability. Urinary metabolic ratio (MR; a ratio of parent drug amount over metabolite amount) estimated after administration of a CYP2D6 substrate (e.g., dextromethorphan) ranges over several orders of magnitude in different individuals (Gaedigk et al. 2008). Large variability in CYP2D6-mediated drug metabolism is also observed in healthy human liver tissues. CYP2D6 activity in 398 and 164 human liver microsomes measured by using dextromethorphan as a probe substrate ranged from no activity to over 1000 pmol/min/mg microsomal protein when (Yang et al. 2010, Temesvari et al. 2012). Based on the results from CYP2D6 phenotyping (i.e., urinary MR), an individual can be classified as ultra-rapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM) and poor metabolizer (PM) (Gaedigk et al. 2008). CYP2D6 phenotype typically shows bimodal or trimodal distribution in European and US population, with PMs accounting for 5-10% of the population (Gaedigk et al. 2008, Bertilsson et al. 2002).

This large variability in CYP2D6-mediated drug metabolism can lead to significant alternations in drug exposure, subsequently therapeutic responses or adverse effects after standard doses of CYP2D6 substrates. For instance, codeine is metabolized by CYP2D6 to pharmacologically active morphine. UM individual can have abnormally high concentration of morphine, subsequently intoxication, after a regular dose of codeine (Gasche et al. 2004). For another instance, nortriptyline is a tricyclic antidepressant with narrow therapeutic index cleared by CYP2D6. Regular doses of nortriptyline (i.e., 75 mg/day) can lead to high plasma drug concentration and cause adverse effect in a PM patient, while 300-500 mg daily dose was needed to achieve therapeutic plasma concentration in a UM patient (Bertilsson et al. 2002). Precision dosing based on CYP2D6 activity level is desirable for CYP2D6 substrates, especially for ones with narrow therapeutic index, to achieve optimum drug responses and to avoid adverse effects.

Genetic polymorphism of CYP2D6 is known as a contributor to inter-individual variability in CYP2D6 activity. Since the discovery that deficiency in debrisoquine 4-hydroxylation (CYP2D6-mediated metabolism) is caused by genetic variation (Mahgoub et al. 1977), CYP2D6 genetic polymorphisms have been intensively studied in the effort to explain inter-individual variability in CYP2D6 activity. Up to date, over 100 CYP2D6 allelic variations have been identified (<http://www.cypalleles.ki.se/cyp2d6.htm>). These CYP2D6 variants can be characterized by the presence of single nucleotide variations, gene hybrids with pseudogene CYP2D7, gene deletion and gene duplications/multiplications (Gaedigk 2013). The outcomes these genetic variations can range from no change (e.g. CYP2D6*2), decreased (e.g. CYP2D6*10), increase (e.g. CYP2D6*1×N) or abrogation (e.g. CYP2D6*4) in CYP2D6 expression/activity. Due to the large number and complexity of CYP2D6 genetic

polymorphisms, a CYP2D6 activity score system was introduced by combining CYP2D6 allelic variants with similar outcomes to simplify CYP2D6 genotype interpretation (Gaedigk et al. 2008). Overall, the mean CYP2D6 activity increases with higher CYP2D6 activity score. Activity score of 0, representing both CYP2D6 alleles non-functional in an individual, can predict poor metabolizer phenotype well. However, substantial inter-individual variability in CYP2D6 activity can be observed in each non-zero AS categories or even in carriers of the same genotype (Gaedigk et al. 2008). This variability among individuals with the same genotype or AS cannot be explained by CYP2D6 genetic polymorphism.

Meanwhile, accumulating evidences have shown that CYP2D6 activity significantly correlates with its expression at protein level (Langenfeld et al. 2009, Lee et al. 2009, Ohtsuki et al. 2012, Zanger et al. 2001) and at mRNA level (Yang et al. 2010, Temesvari et al. 2012, Carcillo et al. 2003, Ohtsuki et al. 2012, Rodriguez-Antona et al. 2001). The correlation between CYP2D6 activity and protein expression in human liver microsomes were consistently high in all the studies (unadjusted linear regression r^2 over 0.7) (Langenfeld et al. 2009, Lee et al. 2009, Ohtsuki et al. 2012). The correlation between CYP2D6 microsomal activity and mRNA expression is also significant, with Pearson or Spearman correlation coefficient of 0.53-0.91 (Yang et al. 2010, Temesvari et al. 2012, Carcillo et al. 2003, Ohtsuki et al. 2012, Rodriguez-Antona et al. 2001). However, there has not been a study that examines CYP2D6 mRNA, protein and activity at the same time in large sample size to determine the extent of variability in CYP2D6 activity explained at each expression level stepwise.

These accumulating evidences suggest important role of regulation of CYP2D6 expression in explaining the inter-individual variability in CYP2D6-mediated drug metabolism, especially that among individuals with the same genotype or AS category. Yet, regulation of

CYP2D6 expression is not well understood. So far, the most well-characterized transcription factor regulating CYP2D6 expression is hepatocyte nuclear factor (HNF) 4 α . HNF4 α as a master transcription factor of hepatic specific transcription (Martinez-Jimenez et al. 2010) binds to a response element (i.e., 253/241) in the CYP2D6 promoter and activate CYP2D6 transcription (Cairns et al. 1996). HNF4 α knockdown in vitro or in vivo (in CYP2D6 humanized mice) led to significant decrease in CYP2D6 expression (Corchero et al. 2001, Jover et al. 2001). Recently, our laboratory identified a new transcriptional regulator of CYP2D6, namely small heterodimer partner (SHP) (Koh, Pan, Shen, et al. 2014, Pan, Lee, and Jeong 2015). SHP is a repressor of CYP2D6 by binding to HNF4 α and inhibiting its activity on CYP2D6 promoter. SHP is a well-known target gene of farnesoid X receptor (FXR) which is activated by bile acids (Lu et al. 2000). Bile acid can induce SHP expression (both at transcription level and protein stability level), which serves important role in negative feedback mechanism of bile acid homeostasis (Miao et al. 2009, Chiang 2009). Indeed, pharmacological (by GW4064) or pathological (i.e. cholestasis) activation of FXR can repress CYP2D6 expression via inducing SHP (Pan and Jeong 2015, Pan, Lee, and Jeong 2015). Together, these suggest potential role of HNF4 α -SHP regulatory pathway in explaining inter-individual variability in CYP2D6 expression.

The goal of this study is to determine the contribution of CYP2D6 genotype/AS, CYP2D6 protein expression, mRNA expression to the inter-individual variability in CYP2D6 activity systematically. This study also sought to determine the extent of variability in CYP2D6 expression that can be explained by known transcriptional regulators of CYP2D6 and their expression/activity modulators.

2.2. Methods

2.2.1. Human liver tissues

Frozen normal human liver tissues were obtained from liver tissue and cell distribution system (LTCDS, funded by NIH Contract # HHSN276201200017C) and Corning Inc. Tissues with poor RNA quality (determined by visual inspection of RNA on agarose gel) were excluded from the study.

2.2.2. CYP2D6 copy number variation (CNV) and rs5758550 genotyping

Genomic DNA was extracted from human liver tissues using Gentra Puregene tissue kit (Qiagen, 158667) following manufacturer instruction. DNA integrity were inspected by gel electrophoresis. DNA concentration was determined using Nanodrop 1000 spectrophotometer (Thermo scientific, Wilmington, DE). Genomic DNA was used in CYP2D6 copy number variation, SNP genotyping and sequencing.

qPCR based Taqman® copy number assays were used to determine CYP2D6 copy number variations. All qPCR assays were performed using StepOnePlus™ Real-Time PCR System. All the samples were tested with two CYP2D6 copy number assays, Hs00010001_cn and Hs04502391_cn (Thermofisher, Foster City, CA), amplifying exon 9 and intron 6 of CYP2D6 gene respectively, and RNase P copy number reference assay (Thermofisher, Foster City, CA) was used for normalization. TaqMan® Universal Master Mix II, no UNG (Thermofisher, Foster City, CA) was used for the reactions. 10 ng of DNA was used per reaction and the assays were repeated in 4 replicates as recommended by the manufacturer. Ct value was set to 0.2 in all assays and the results were analyzed with CopyCaller® 2.0 software to determine copy number for each sample. Samples with copy call confidence below 95% in both assays or copy calls with discrepancy were re-tested with the two assays above and a third one,

Hs04083572_cn, amplifying intron 2 of CYP2D6. The samples with same copy number assignment for intron 2 and intron 6 assay but smaller copy number for exon 9 assay were considered carriers of CYP2D6-CYP2D7 hybrids (e.g. CYP2D6*36 genotype). These additional copies called in intron 6 and 2 assays were assigned as *36 or *36xN in this study.

rs5758550 was examined in human liver tissues using Taqman® SNP genotyping assay, C__29692254_10 (ThermoFisher, Foster City, CA) with TaqMan® Universal Master Mix II, no UNG (ThermoFisher, Foster City, CA), performed using StepOnePlus™ Real-Time PCR System. 10 ng genomic DNA from human liver tissues were used per reaction. Samples without SNP call were re-tested using 20 ng of genomic DNA.

2.2.3. DNA sequencing

SNPs in CYP2D6, SHP (NR0B2) and HNF4α were examined using Ampliseq™ panel for target capture followed by Ion Torrent™ sequencing. The exon regions that cannot be covered by Ampliseq™ panel was sequenced using Sanger sequencing. The coverage was from 4kb upstream regulatory region to 1kb downstream of the CYP2D6 gene (hg 19 chr22:42521501-42530883), 2kb upstream to 1kb downstream of the NR0B2 gene (hg19 chr1:27236975-27242567) and exon and adjunctive intron/regulatory regions of HNF4α gene.

Raw data from Ion Torrent™ sequencing was QCed and aligned to hg19 genome assembly. SNPs was called using PLINK software and all SNPs in all individuals were outputted to NGS_variants_051716.vcf file. All the SNPs were further manually filtered and curated based on sequencing quality and Hardy-Weinberg equilibrium. IGV_2.3.83 software (Broad institute <http://software.broadinstitute.org/software/igv/>) was used for data visualization.

Sanger sequencing results for CYP2D6 and SHP were aligned to hg19 chr22:42525025-42525235 and hg19 chr1:27240340-27240390 regions respectively using novoSNP software

(Weckx et al. 2005). Variations with quality score above 10 were manually inspected to exclude false positive SNP call.

2.2.4. CYP2D6 genotype assignment

CYP2D6 genotype assignment was based on CYP2D6 allele nomenclature (<http://www.cypalleles.ki.se/cyp2d6.htm>) and review article (Gaedigk 2013). Specifically, the detected SNPs or haplotypes for CYP2D6 genotype assignment are listed in Table I.

When CYP2D6 copy number is above 2, individual sequencing results (.BAM files) were examined and heterozygous SNPs were assigned to tandem allele or the other allele based on the read count ratio.

Table I CYP2D6 alleles and corresponding SNPs used in CYP2D6 genotype assignment

CYP2D6 Allele	refSNP ID	Genomic change (hg19 Chr22)	nucleotide change (reference M33388)	protein change (NP_000097.3)
*5		Gene deletion CNV=1 or 0		
*108	rs61736517	42523567T>C	3226A>G	H352R
	rs202102799	42523558T>C	3235A>G	Y355C
*22	rs138100349	42526712G>A	82C>T	R28C
*23	rs267608310	42525838G>A	957C>T	A85V
*12	rs5030862	42526670C>T	124G>A	G42R
	rs16947	42523943A>G	2850C>T	R296C
	rs1135840	42522613G>C	4180G>C	S486T
*6	rs5030655	42525086delA	1707delT	118Frameshift
	rs139779104	42524817C>T	1976G>A	G212E
*17	rs28371706	42525772G>A	1023C>T	T107I
	rs16947	42523943A>G	2850C>T	R296C
	rs1135840	42522613G>C	4180G>C	S486T
*41	rs28371725	42523805C>T	2988G>A	splicing defect
	rs16947	42523943A>G	2850C>T	R296C
	rs1135840	42522613G>C	4180G>C	S486T
*45	rs28371710	42525077C>T	1716G>A	E155K;
	rs28371726	42523539A>G	3254T>C	no change
	rs16947	42523943A>G	2850C>T	R296C
	rs1135840	42522613G>C	4180G>C	S486T
*4	rs3892097	42524947C>T	1846G>A	splicing defect
	rs1065852	42526694G>A	100C>T	P34S
	rs1135840	42522613G>C	4180G>C	S486T
*10	rs1065852	42526694G>A	100C>T	P34S
	rs1135840	42522613G>C	4180G>C	S486T
	Without any SNPs above (i.e. any SNPs in bold from *108 to *4 allele)			
*2	rs16947	42523943A>G	2850C>T	R296C
	rs1135840	42522613G>C	4180G>C	S486T
	Without any SNPs above (i.e. any SNPs in bold from *108 to *10 allele)			
*1	None of the above			

SNPs in bold are allele determining SNP.

2.2.5. CYP2D6 activity score (AS)

CYP2D6 activity score is assigned to each individual sample based on the reported method (Gaedigk et al. 2008). The rationale and assumptions for activity score are as follow: (1) each individual has two alleles of CYP2D6 gene from maternal and paternal heritage respectively; (2) each allele of CYP2D6 gene is assigned with a functional gene dose which reflects the associated activity level of the allele, shown in Table II; (3) activity score (AS) is the sum of the gene dose of both alleles, which assumes equal expression of both alleles.

Table II Gene dose assigned to CYP2D6 alleles used for activity score

Gene dose	Associated activity	Allele variants
0	none	*4, *4xN, *5, *6, *12, *36xN
0.5	decreased	*10, *17, *41, *45, *10xN
1	normal	*1, *2, *22, *23, *108
N	increased	*1xN, *2xN
*22 and *23 are considered as normal function allele based on in vitro activity assay. (Muroi et al. 2014) *108 is considered as normal function by default. No activity information was found for the genotype.		

2.2.6. Preparation of hepatic S9 fraction

Frozen human liver tissue (~150 mg) was used to prepare hepatic S9 fraction. Liver tissues were homogenized in 2 volumes of buffer (50mM Tris-HCl pH 7.0, 150mM KCl, 2mM EDTA and protease inhibitor cocktail) for 30 s in a 5 s on-and-off cycle on ice using PowerGen 125 homogenizer (Fisher Scientific) at medium speed. The homogenate was centrifuged at 9,000

g at 4 °C for 20 minutes. The supernatant was collected, aliquoted and stored at -80 °C until further analysis. Total protein concentration of the S9 fraction were measured with BCA kit (ThermoFisher, Rockford, IL).

2.2.7. Measurement of CYP2D6 activity

CYP2D6 activities in S9 fractions were estimated using the formation rate of dextrophan (from dextromethorphan) as a marker. Incubations were performed in duplicate in a final incubation volume of 100 μ L. A mixture consist of dextromethorphan (5 μ M final concentration), isocitric acid (5 mM final concentration), isocitric dehydrogenase (0.2 Unit/mL final concentration), $MgCl_2$ (5 mM final concentration) and S9 (6 μ L, ~210 μ g protein) in 0.1 M Tris-HCl buffer (pH 7.4), was pre-incubated at 37 °C for 5 minutes. The amount of S9 protein in the reaction mixture was determined in preliminary studies; up to 250 μ g S9 protein, metabolite formation increased linearly. Reactions were initiated by addition of $NADP^+$ (1 mM final concentration) and incubated for 5 minutes at 37 °C with gentle agitation. The reaction condition was optimized and based on literature to keep substrate turnover rate at linear range. After 5 minutes of incubation, the reaction was terminated by adding 100 μ L ice cold acetonitrile with 40 ng/mL dextrophan-D4 (internal standard, IS) and vortexed. The mixture was centrifuged at 16,100 g for 20 minutes at 4 °C and supernatant were collected for LC-MS/MS analysis.

Standard curve samples were prepared using incubation system without $NADP^+$ spiked with different concentration of dextrophan working solution (final concentration range from 0.1 ng/mL to 0.001 ng/mL). 100 μ L ice cold acetonitrile with 40 ng/mL IS was added to the standard samples and processed the same way as incubation samples.

For LC-MS/MS analysis, 3 μ L of sample was injected into Agilent 1200 LC system equipped with Nova-Pak C18 column (3.9 \times 150 mm, 4 μ m, Waters Corp.) for LC separation.

The mobile phase consisted of 30% 5mM ammonium acetate (pH adjusted to 4.7 with acetic acid) and 70% acetonitrile. The flow rate was 0.8 mL/min. The elute was diverted to waste for the first 3 minutes and then to mass spectrometry from 3-9 minutes. The run time was 9 minutes per sample. For MS/MS analysis, AB Sciex 5500 QTRAP with electrospray ion source under positive ion mode was used. Multiple reaction monitoring (MRM) was used to detect dextrophan. CAD of high, ion source (IS) of 5500 V, a source temperature of 700 °C were used. Curtain gas, TurbolonSpray nebulizer (GS1) and heater gas (GS2) flow were set as 10, 55 and 65 psi, respectively. MRM transitions for dextrophan and dextrophan-D4 (IS) were 258.3/157.1 and 261.3/157.0 respectively. Declustering potential (DP)/Collision energy (CE) was 66V/52V and 55V/47V for dextrophan and IS MRM transitions respectively. The dwelling time for each MRM was 100 msec. Data acquisition and quantification was done using Analyst 1.6.2 software.

2.2.8. Western blotting

CYP2D6 relative protein expression was determined by semi-quantitative western blotting. 10 µg of individual hepatic S9 protein or pooled LTCDS S9 protein (for signal normalization) was loaded to 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (4561096 Bio-rad, Hercules, CA). Each sample were duplicated on a separate gel. Different amount of pooled LTCDS S9 protein (0.2-60 µg) as standard was loaded to a third gel on each day. After running, the gels were wet transferred to 0.45 µm nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% skimmed milk in TBST buffer. Mouse monoclonal Gentest™ Anti-Human CYP2D6 Antibody (458246, Corning, Woburn, MA, 1:1000 dilution in 0.5% skimmed milk) was used to detect CYP2D6 protein. Goat anti-mouse IgG-HRP (sc2005, Santa Cruz Biotechnology, CA, 1:5000 dilution in 5% skimmed milk) was used as secondary antibody. The membranes were incubated with SuperSignal™ West Pico Chemiluminescent

Substrate (34080, ThermoFisher, Rockford, IL) and 10 s, 30 s and 1 minute exposures of all three blots at the same time were recorded with FluorChem E system (ProteinSimple, San Jose, CA). The membranes were stripped with Restore™ PLUS Western Blot Stripping Buffer (46430, ThermoFisher, Rockford, IL) following manufacturer instruction. The membranes were re-blotted to detect β -actin as loading control. Mouse monoclonal antibody against β -actin (A1978, Sigma-Aldrich, St Louis, MO, 1:1000 dilution) was used and other conditions were the same as CYP2D6 protein detection. Band signal intensity was quantified using AlphaView software (ProteinSimple, San Jose, CA).

CYP2D6 band intensity of standard samples versus pooled LTCDS S9 protein amount was modeled with Michaelis-Menten equation. CYP2D6 band intensity for individual S9 samples was normalized by that of 10 μ g pooled S9 sample on the same membrane and scaled to standard curve membrane level by multiplying the band signal of 10 μ g pooled S9 on standard curve membrane. Relative CYP2D6 protein level was calculated with the normalized CYP2D6 band intensity. Individual samples with CYP2D6 relative protein level over 20 (when band intensity reaches plateau) were examined again loading 2 μ g S9 protein instead to avoid signal saturation. With dilution assay, CYP2D6 protein level can be determined at a dynamic range from 0.5 to 75 (μ g pooled LTCDS S9 CYP2D6 amount).

2.2.9. RNA isolation and quantitative real time-PCR (qRT-PCR)

Total RNA was extracted using TRIzol® reagent (ThermoFisher, Carlsbad, CA) from human liver tissues. RNA quality was examined by agarose gel electrophoresis. Samples without two sharp bands of rRNA were considered degraded. Livers with degraded RNA indicated poor quality, therefore, were excluded for the study. 2 μ g of RNA were used for cDNA synthesis with High-capacity cDNA reverse transcription kit (ThermoFisher, Carlsbad, CA). Using the cDNA as

template, qRT-PCR was performed using StepOnePlus™ Real-Time PCR System. Primetime® Gene expression assays (Integrated DNA technologies, IDT, Coralville, IA) or Taqman® Gene expression assays (Thermofisher, Foster City, CA) with FastStart Universal Probe Master (Rox) (Roche Diagnostics, Indianapolis, IN) were used for gene expression. The assays are listed in Table III.

The relative mRNA expression level was determined after normalizing the expression levels by those of HPRT1 and relative to one typical subject (ID 1530) using $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008). HPRT1 was selected as endogenous control from several common control genes (GAPDH, B2M, GUSB, TBP, ACTB and HPRT1) and two genes predicted by RefGenes tools in Genevestigator (Hruz et al. 2011) (INO80 and ANAPC7). 6 human liver samples with different levels of GAPDH expression were used to validate the expression of control genes. qRT-PCR results were analyzed with geNorm algorithm in qbase+ software (Biogazelle <https://www.qbaseplus.com/>). HPRT1 was most stably expressed in the samples examined and therefore was chosen as endogenous control.

Table III Probe-based assays used for qRT-PCR in Chapter 2

Gene	Manufacture	Assay ID	Ref Seq ID
CYP2D6	IDT	Hs.PT.56a.45336286.g	NM_000106.5 NM_001025161.2
CYP2D6	ThermoFisher	Hs00164385_m1	NM_000106.5
CYP2D6	ThermoFisher	Hs02576167_m1	NM_001025161.2
HPRT1	ThermoFisher	Hs02800695_m1	NM_000194.2
HNF4A (HNF4 α)	IDT	Hs.PT.58.22303533	NM_178850 (6 more)
NR0B2 (SHP)	IDT	Hs.PT.58.38586840	NM_021969
CYP8B1	IDT	Hs.PT.58.40608207.g	NM_004391
CYP7A1	IDT	Hs.PT.58.21408221	NM_000780
NR5A2 (LRH-1)	IDT	Hs.PT.58.21063641	NM_001276464 (2 more)
GAPDH	IDT	Hs.PT.39a.22214836	NM_002046
TBP	ThermoFisher	Hs00427620_m1	NM_003194.4 (1 more)
B2M	ThermoFisher	Hs00984230_m1	NM_004048.2
GUSB	ThermoFisher	Hs00939627_m1	NM_000181.3 (3 more)
INO80	ThermoFisher	Hs00393757_m1	NM_017553.2
ACTB	ThermoFisher	Hs01060665_g1	NM_001101.3
ANAPC7	ThermoFisher	Hs01067030_m1	NM_001137664.1 (1 more)

2.2.10. Measurement of hepatic bile acid levels

Human liver bile acid levels were measured using published method (Garcia-Canaveras et al. 2012) with minor modification. Briefly, ~100 mg human liver tissue was accurately weighed and homogenized in 0.6 mL cold methanol and 0.2 mL internal standard solution with Precellys soft tissue homogenizing CK14 kit (Bertin Corp., Rockville, MD) and bead beater. The livers were homogenized at 4 °C in maximum speed with 30-second on-and-off cycles twice, or until complete homogenization. The homogenate was centrifuged at 3,000 g for 5 minutes. Supernatant was collected and the pellet were extracted again with 0.4 mL cold methanol with

the same procedure. The supernatant from second extraction were combined with that from the first time. The pooled supernatant was aliquoted into 150 μ L per tube and stored at -80 $^{\circ}$ C until analysis. The aliquots were evaporated to dryness in a Savant Speedvac concentrator (Thermo Scientific) at medium temperature for 1 hour and later reconstituted in 50 μ L of methanol:water (50:50, v/v), vortexed vigorously for 30 s, centrifuged at 16,000 g for 10 min at 4 $^{\circ}$ C, and the supernatant was transferred for LC-MS/MS analysis. Two aliquots were analyzed per liver.

For standard samples, individual bile acid stock solutions (5 or 10 mg/mL) were used to make mixed working solution in methanol:water (50:50, v/v), with final concentration of 100 μ M for each bile acid, except 200 μ M for lithocholic acid. The mixed working solution was serial diluted with methanol:water (50:50, v/v) to concentrations ranging from 5 nM to 50 μ M. Internal standard solution of deoxycholic acid-2,2,4,4-D4 (DCA-D4), glycochenodeoxycholic acid-2,2,4,4-D4 (GCDCA-D4), cholic acid-2,2,4,4-D4 (CA-D4), glycocholic acid-2,2,4,4-D4 (GCA-D4) and lithocholic acid-2,2,4,4-D4 (LCA-D4) were prepared in methanol:water (50:50, v/v) with concentration of 1 μ M for each compound and except 2 μ M for LCA-D4. 25 μ L of standard solution and internal standard solution respectively were added to 100 μ L of cold methanol. The mixture was vacuum dried and reconstituted as described above. Both standard curve samples and human liver samples were prepared in duplicates.

UHPLC separation was performed using 1290 infinity UHPLC system (Agilent) equipped with Acquity UPLC BEH C18 (1.7 μ m, 2.1 \times 100 mm; Waters) column. Column and autosampler temperature was set to 50 and 4 $^{\circ}$ C respectively. 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) were used in gradient elution shown in Table IV with flow rate of 0.5 mL/min. Elute from 5.5 to 21.5 minutes was directed to MS system for analysis, otherwise to waste. 4 μ L was injected for each sample.

Table IV HPLC gradient elution for bile acid profiling

Time (min)	% B
0	5
0.5	5
7	25
16	40
19.5	95
19.9	95
20	5
22	5

QTRAP 6500 MS/MS system (Sciex) with ESI source in negative-ion mode was used for MS analysis. MRM mode was used. CAD of medium, ion source (IS) of -4500 V, a source temperature of 600 °C were used. Curtain gas, TurbolonSpray nebulizer (GS1) and heater gas (GS2) flow were set as 30, 60 and 60 psi, respectively. MRM transitions for individual Bile acids are listed in Table V below. The dwelling time for each MRM was 50 msec. Data acquisition and quantification was done using Analyst 1.6.2 software. Samples with levels of single or multiple bile acid species higher than the upper limit of quantification, were diluted 10 times and analyzed again. Dilution assay results were used only for the bile acid species with levels above quantification limit. The samples were measured over two batches and one additional batch for dilution assays.

Table V MRM condition and internal standard used for bile acid profiling

Analytes	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	CE (V)	Internal standard
GCDCA-D4	452.2	74.2	-100	-52	
GCA-D4	468.4	73.9	-100	-70	
DCA-D4	395.3	395.3	-75	-15	
LCA-D4	379.3	379.3	-118	-15	
CA-D4	411.2	411.2	-100	-25	
CDCA	391.2	391.2	-120	-11	DCA-D4
DCA	391.2	391.2	-120	-11	DCA-D4
UDCA	391.2	391.2	-120	-11	CA-D4
HDCA	391.2	391.2	-120	-11	CA-D4
MuroCA	391.2	391.2	-120	-11	CA-D4
GCDCA	448.2	73.9	-90	-60	GCDCA-D4
GDCA	448.2	73.9	-90	-60	GCDCA-D4
GUDCA	448.2	73.9	-90	-60	GCDCA-D4
GHDCa	448.2	73.9	-90	-60	GCDCA-D4
TCDCA	498.2	80	-50	-70	GCDCA-D4
TDCA	498.2	80	-50	-70	GCDCA-D4
TUDCA	498.2	80	-50	-70	GCDCA-D4
THDCA	498.2	80	-50	-70	GCDCA-D4
CA	407.3	407.3	-130	-15	CA-D4
α MCA	407.3	407.3	-130	-15	CA-D4
β MCA	407.3	407.3	-130	-15	CA-D4
ω MCA	407.3	407.3	-130	-15	CA-D4
HCA	407.3	407.3	-130	-15	CA-D4
TCA	514.4	79.8	-150	-72	GCA-D4
T α MCA	514.4	79.8	-150	-72	GCA-D4
T β MCA	514.4	79.8	-150	-72	GCA-D4
THCA	514.4	79.8	-150	-72	GCA-D4
GCA	464.4	74.1	-60	-50	GCA-D4
GHCA	464.4	74.1	-60	-50	GCA-D4
LCA	375.3	375.3	-40	-10	LCA-D4
GDHCA	458.3	74.1	-100	-66	GCA-D4
GLCA	432.4	73.9	-80	-70	DCA-D4
TDHCA	508.3	80	-120	-120	GCA-D4
TLCA	482.4	79.8	-50	-120	GCA-D4

2.2.11. Measurement of hepatic all-trans retinoic acid (atRA) levels

Hepatic atRA levels were measured by using LC-MS/MS as previously described (Koh, Pan, Shen, et al. 2014, Arnold et al. 2012). Briefly, livers (~100 mg) were homogenized with a 2-mL glass Dounce homogenizer (Kimble Glass) in a 1:1 volume of 0.9% NaCl, and the sample was transferred into a 15-ml glass culture tube. A 2:1 volume of acetonitrile with 0.1% formic acid was added together with 13-cisRA-d5 (internal standard). Retinoic acid was extracted with 10 ml of hexanes, and the organic layer was transferred to a glass tube and dried under nitrogen at 37 °C. The sample was reconstituted in 65 µL of 60:40 acetonitrile/H₂O for LC-MS/MS analysis. Standard curve and quality control samples were generated using UV light-exposed mouse liver homogenate spiked with atRA and 13cRA at concentrations of 0, 2, 5, 10, 15, and 20 nM for the standards and 3, 7.5, and 17.5 nM for the quality control samples. The retinoids were separated using an Agilent 1290 UHPLC (Santa Clara, CA) equipped with a Sigma (St. Louis, MO) Ascentis Express RP Amide column (2.7 µm; 150 mm × 2.1 mm). Gradient elution with a flow rate of 0.5 ml/min using water 0.1% formic acid (A) and acetonitrile/methanol (60/40) (B) was used. The gradient was from initial 60% (A) for 2 min to 45% (A) over 8 min and then to 10% (A) over 7 min. The column was then washed with 95% (B) for 3 min and returned to initial conditions. Samples were kept in the autosampler at 4°C with the light turned off. 10 µL of sample was injected for analysis. Analytes were detected using an AB Sciex 5500 QTRAP Q-LIT mass spectrometer (Foster City, CA) operated in positive ion APCI mode. The compound independent MS parameters were curtain gas: 20; collision gas: low; nebulizer current: 5; temperature: 350°C; ion source gas 1:80. MRM transitions for retinoic acid (all isomers including atRA) and internal standard were 301/205 and 306/116 respectively.

2.2.12. Statistical analysis

To meet the assumptions for parametric analysis, all variables of interest (predictors, outcomes) were assessed for normality of distribution by examining skewness and kurtosis. Non-normal distributions were normalized using log transformations. Unadjusted linear regression analysis was first conducted to determine basic association between different factors. Significant factors were later included in adjusted regression model. In adjusted analysis, non-normal distributions were normalized using natural log transformations and adjusted for age, sex and source of liver. Factors that remained significant after controlling for relevant covariates were included in stepwise multivariate analysis. The cutoff of significance used in multivariate regression model was $p < 0.15$.

2.3. Results

2.3.1. Information of human liver tissues.

A total of 115 snap-frozen adult human liver tissues absent with prominent pathology were obtained from two sources (i.e., LTCDS and Corning Inc.). The demographical information for the human liver tissues is summarized in Table VI. Of note, the age of donors was comparable between the livers from two sources while there were more male than female donors in the Corning group. While age and sex are known not to affect CYP2D6-mediated drug metabolism (Bebia et al. 2004, Yang et al. 2010, Achour, Barber, and Rostami-Hodjegan 2014), these factors (including the source of liver tissues) were included in data analysis as potential covariates.

Table VI Demographic information of human liver tissues used in the study

Source	LTCDS	Corning Inc.	Total
Number of samples	65	50	115
Age (mean \pm SD)	49.4 \pm 16.1	49.4 \pm 14.7	49.4 \pm 15.4
Sex (male/female)	32/33	38/12	70/45
Ethnicity	Not available	41 (Caucasian) 3 (African American) 5 (Asian) 1 (Hispanic)	Not available

2.3.2. Variability in CYP2D6 activity explained by CYP2D6 genotype/activity score.

Genetic polymorphisms of CYP2D6 have been considered to be a major contributor to inter-individual variability in CYP2D6-mediated drug metabolism (Gaedigk et al. 2008). To estimate

the extent of their contribution to CYP2D6 activity, CYP2D6 activity levels in the liver tissues were first estimated by using a CYP2D6 probe drug. To this end, S9 fraction was prepared from individual tissues, and incubated with dextromethorphan, a sensitive CYP2D6 substrate, followed by measurement of dextrorphan (DX, CYP2D6-specific metabolite of dextromethorphan) formation by using LC-MS/MS. CYP2D6 activity in the human liver S9 fractions exhibited large inter-individual variability of over 500 folds (DX formation rate ranging from 0.23 to 74 pmol DX/min/mg S9 protein) (Figure 1), consistent with previous reports (Temesvari et al. 2012, Yang et al. 2010). Notably, the distribution of CYP2D6 activity among individuals followed log-normal distribution.

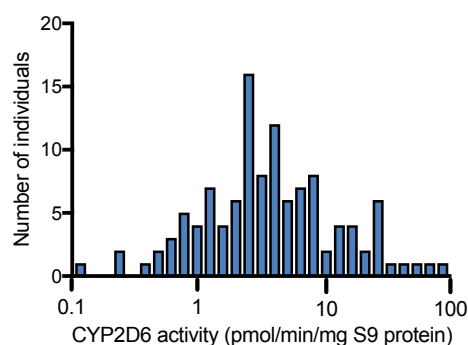


Figure 1. Distribution of CYP2D6 activity levels.

CYP2D6 activity levels in liver S9 fractions were measured by using a CYP2D6 probe drug, dextromethorphan. Data shown are dextrorphan formation rate expressed in pmol/min/mg S9 protein. Note the log-scale in x-axis.

Next, genetic polymorphisms of CYP2D6 gene were identified by (1) the measurement of copy number variation and (2) high-throughput/sanger DNA sequencing. Two Taqman®

assays targeting intron 6 (Hs04502391_cn) or exon 9 (Hs00010001_cn) of CYP2D6 gene were used for copy number determination to ensure accuracy. The majority (82%) of the tissues showed the copy number of 2 (Table VII). The distribution of samples with different copy numbers is consistent with that observed in US population (Beoris et al. 2016). Results from two Taqman® assays were consistent for all except two liver tissues. These two samples exhibited higher copy number when intron 6 assay was used, possibly due to gene conversion (to CYP2D7 hybrid at a locus downstream of intron 6, such as *36 allele, which prohibits the amplification of exon 9 probe).

Table VII Summary of CYP2D6 copy number variations

Copy Number^a	0	1	2	>2
No. of subjects	1	11	94	9 ^b
a. Results of assay targeting intron 6 (Hs04502391_cn).				
b. 2 of the samples were assigned with 2 copies when exon 9 assay (Hs00010001_cn) was used.				

CYP2D6 gene including the regulatory region was sequenced by using high-throughput DNA sequencing complemented with Sanger sequencing. Then, CYP2D6 alleles were assigned based on information on CYP allele nomenclature website (<http://www.cypalleles.ki.se/cyp2d6.htm>). The frequencies of different genotypes (Table VIII) were generally comparable to the reported values in European descendent up to date (Gaedigk et al. 2017).

Table VIII Frequency of CYP2D6 alleles detected in human liver tissues

Genotype	frequency	average frequency in European descendent (Gaedigk et al. 2017)
*1	0.387	0.383
*2	0.178	0.278
*4	0.139	0.182
*5	0.057	0.029
*6	0.03	0.009
*10	0.030	0.024
*12	0.004	0.0001
*17	0.030	0.004
*22	0.004	NA
*23	0.004	NA
*41	0.070	0.074
*45	0.017	NA
*108	0.004	NA
*1xN	0.004	0.008
*2xN	0.017	0.012
*4xN	0.009	0.002
*10xN	0.009	0.0005
*36xN	0.004	NA
NA: not available, not examined by most studies.		

Due to the polymorphic nature of CYP2D6 gene, activity score (AS) was proposed as a simplified genotype scoring system where CYP2D6 activity score is assigned to each CYP2D6 allele so that the sum of CYP2D6 activity scores can be used to predict CYP2D6 phenotype in an individual (Gaedigk et al. 2008). To evaluate the utility of AS in predicting CYP2D6 activity levels in liver tissues, AS was assigned to each liver sample. The distribution of CYP2D6 AS in our liver cohort (Table IX) was comparable to the previous reported values in European descendent (Gaedigk et al. 2017). The median CYP2D6 activity levels in S9 fractions increased

with AS (Table IX and Figure 2), consistent with the previous report (Gaedigk et al. 2008). Of note, large inter-individual variability in CYP2D6 activity levels (ranging ~2 orders of magnitude) was observed within each AS category.

Table IX CYP2D6 activity score, frequency and activity in human liver tissues

AS	Number of samples	Frequency	Reported European population frequency prediction (Gaedigk et al. 2017)	CYP2D6 activity median (range) pmol dextrophan /min/mg S9 protein
0	9	0.078	0.054	1.1 (0.54-2.28)
0.5	8	0.070	0.054	1.8 (0.55-9.2)
1	32	0.278	0.299	2.7 (0.23-14)
1.5	23	0.200	0.148	3.9 (0.14-26)
2	38	0.330	0.446	5.1 (0.7-60)
>2	5	0.043	0.031	17 (2.8-74)

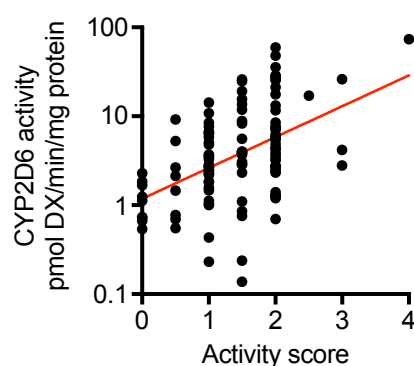


Figure 2. Correlation between CYP2D6 activity and CYP2D6 activity score.

The line was the unadjusted linear regression line of Log CYP2D6 activity versus CYP2D6 activity score.

The extent of variability in CYP2D6 activity that can be explained by CYP2D6 AS was then estimated by using regression analysis with adjustment for covariates (i.e., age, sex and source). , The adjusted r^2 of age, sex and liver source effects on CYP2D6 activity levels were 0.06. Overall, 23% of variability in CYP2D6 activity was explained by AS after adjustment. In the samples exhibiting AS of 1 to 2 (i.e., ~80% of the samples) only, AS accounted for 7.5% of the variability in CYP2D6 activity, indicating that CYP2D6 AS fails to predict CYP2D6 activity levels in the majority of population. Together, these results underscore the need to fully characterize the major sources of CYP2D6 variability especially in AS ≥ 1 groups.

2.3.3. Variability in CYP2D6 activity explained by differential expression of CYP2D6

To identify the sources of CYP2D6 variability, CYP2D6 protein expression in human liver S9 fraction was measured by using semi-quantitative western blots. The distribution CYP2D6 protein expression exhibited bi-modal distribution (Figure 3A). Liver tissues with non-detectable CYP2D6 protein level (n=11) were carrying two non-functional CYP2D6 alleles (specifically *4, *5 and *6), except two samples with one non-functional allele (i.e., *1/*4 and *41/*4).

Overall, CYP2D6 activity was highly correlated with its protein expression (Figure 3B), consistent with previous report (Lee et al. 2009, Ohtsuki et al. 2012, Achour et al. 2014). Results from regression analysis of all liver samples showed that 59% of the variability in CYP2D6 activity can be explained by differential CYP2D6 protein expression when adjusted for sex, age and liver source. The adjusted r^2 of age, sex and liver source effects on CYP2D6 activity levels were 0.07.

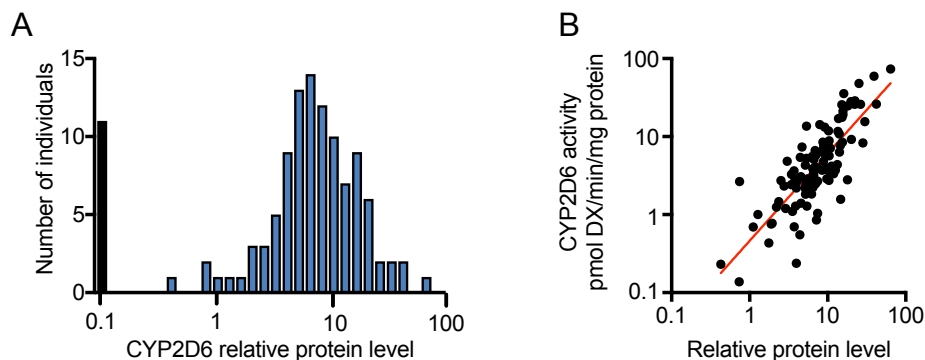


Figure 3. CYP2D6 protein expression in human liver S9 fractions.

A, Distribution of CYP2D6 protein levels. Samples with non-detectable CYP2D6 protein expression is presented as the black bar on the left of the graph. B, overall correlation between CYP2D6 activity and protein expression of liver tissues with CYP2D6 expression. (n=104, unadjusted linear regression of Log CYP2D6 activity vs. Log protein expression, $r^2=0.65$)

Since AS explained only small part of inter-individual variability in CYP2D6 activity in samples with AS 1, 1.5 and 2, we examined CYP2D6 protein-activity correlation separately for each AS group. Interestingly, while CYP2D6 activity and protein levels were well correlated in each AS group, the correlation was poorer in the samples of lower AS (Table X and Figure 4). Within the group of AS 1, the correlation between CYP2D6 activity and protein levels in (the most frequent) *4/wt carriers was better compared to that for the whole AS 1 group (Figure 4C; adjusted $r^2=0.54$ vs. 0.33, for *4/wt and the whole AS=1 group, respectively), suggesting that genetic heterogeneity in AS=1 group led to varying levels of decreased CYP2D6 activity likely diminish the correlation. The positive correlation between CYP2D6 activity and protein levels was lost in AS=0.5 group as expected (Figure 4D), due to small sample size and extreme genetic heterogeneity that each individual have different CYP2D6 alleles. Together, these results indicate that CYP2D6 protein levels are major determinant of CYP2D6 activity levels in most liver tissues.

Table X Adjusted regression analysis of CYP2D6 activity vs. protein expression stratified by CYP2D6 activity score.

AS	1	1.5	2
Number of samples	32	23	38
Overall adjusted r^2	0.4357	0.5138	0.7513
Covariate effect (sex, age, source)	0.1020	0.0393	0.0887
Adjusted r^2	0.3337	0.4745	0.6324

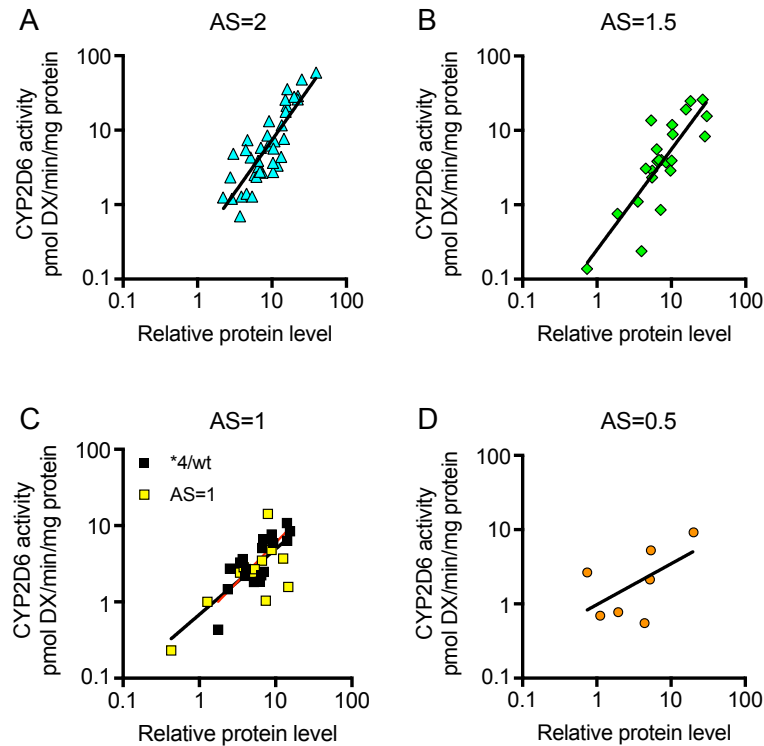


Figure 4. Correlation between CYP2D6 activity and protein expression stratified with AS. (A) AS=2, (B) AS=1.5, (C) AS=1 with *4/wt carriers in black squares, (D) AS=0.5. The black lines are unadjusted linear regression line for Log CYP2D6 activity vs. Log CYP2D6 protein expression for the AS category. The red line is the regression line for *4/wt carriers.

Next, we examined CYP2D6 mRNA expression by qRT-PCR to determine the extent of variability in CYP2D6 protein level that can be explained by differential transcription of the gene. CYP2D6 mRNA levels in liver tissues were measured by two pre-designed probe-based qPCR assays, targeting exon 2-3 region or exon 1 of the gene, respectively. The results from both assays agreed with each other ($r^2=0.96$; data not shown) when the tissues carrying *17 allele (rs28371706; missense mutation on exon 2 that likely interferes with the amplification of exon 2-3) were excluded. CYP2D6 mRNA results obtained from assay targeting exon 1 of CYP2D6 gene were used for later analysis. The distribution of CYP2D6 mRNA levels was not normal, with one tissue exhibiting a very low amplification (i.e., the only *5/*5 carrier in our tissues) (Figure 5A)

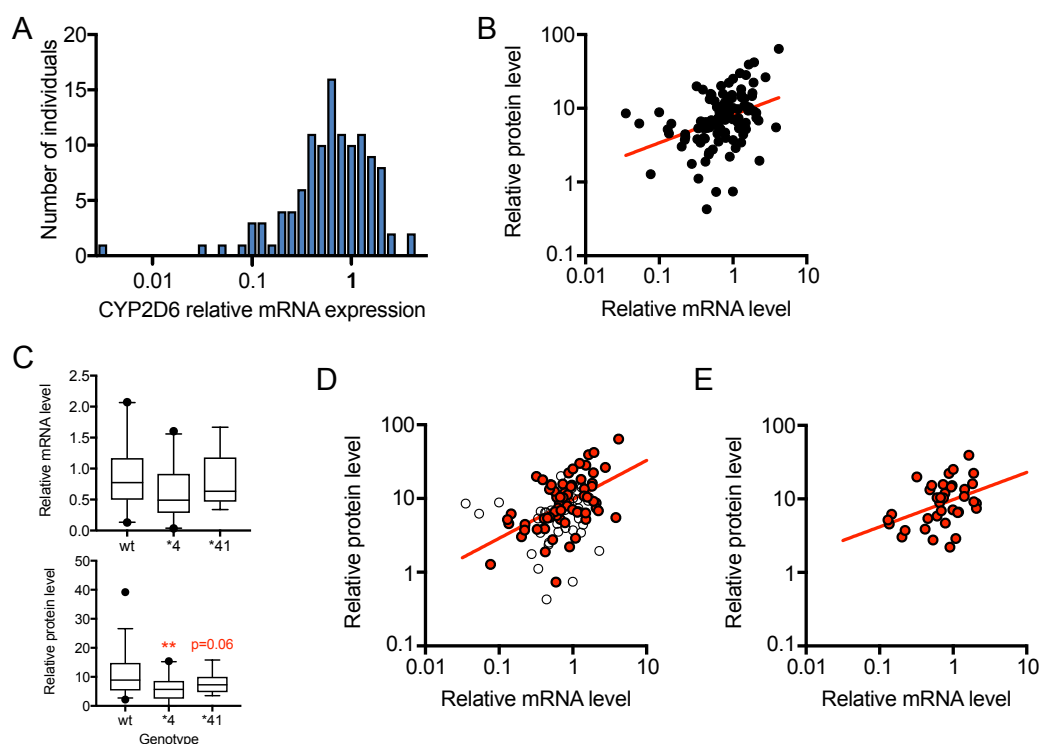


Figure 5. Correlation between CYP2D6 protein expression and mRNA expression.

A, Distribution of CYP2D6 mRNA levels in liver tissues (n=104). B, Overall correlation between CYP2D6 protein and mRNA levels (n=104). C, CYP2D6 mRNA and protein expression in CYP2D6 wt/wt, *4 (i.e., *4/*4, *4/wt) and *41/wt carriers. Welch's t-test were

conducted between wt group and *4 group or wt group and *41 group. D, correlation between CYP2D6 protein and mRNA levels. Open circles were samples (n=41; carriers of *4, *6, *41 *4x2 and *36x2 alleles) excluded from regression analysis. Red dots were the rest of samples (n=63) and included in regression analysis. E. correlation between Log CYP2D6 mRNA and Log protein in *1/*1, *1/*2, and *2/*2 carriers (n=37). Red line was the unadjusted linear regression line between Log CYP2D6 protein level and Log CYP2D6 mRNA level in included samples.

Results from regression analysis of all liver samples showed that CYP2D6 protein level was significantly correlated with its mRNA expression, with adjusted r^2 of 0.11 (Figure 5B). Ohtsuki et al. reported correlation between CYP2D6 microsomal protein level and mRNA expression in 17 human liver tissues with least-squares regression r^2 of 0.52 (Ohtsuki et al. 2012). However, the sample size was small and CYP2D6 genotype was unknown in the study. In a large-scale study (n=398), Spearman correlation between CYP2D6 microsomal activity and CYP2D6 mRNA expression was reported to be 0.53 (Yang et al. 2010), similar to what we observed ($r_s=0.49$, n=115). Of note, the carriers of certain genetic variations of CYP2D6 are expected to exhibit disconnect between mRNA and protein levels inherently. For example, CYP2D6*4 allele (rs3892097 1846G>A) leads to incorrect splicing of mRNA and frameshift in protein translation (Hanioka et al. 1990) and CYP2D6*41 (rs28371725, 2988G>A) leads to increased levels of a nonfunctional mRNA splice variant lacking exon 6, resulting in decreased protein levels without changes in total mRNA levels (Toscano et al. 2006). Indeed, mRNA levels of CYP2D6 in the carriers of only *1 and *2 alleles (i.e., wt carriers) did not differ from those in *4 (i.e., *4/*4 or *4/wt) or *41/wt carriers whereas CYP2D6 protein levels significantly decreased in *4 carriers and showed trend of decrease ($p=0.06$) in *41 carriers when compared with those in wt carriers (Figure 5C). Excluding carriers of alleles that can inherently lead to disconnect between CYP2D6 mRNA and protein levels, we re-examined the correlation between

CYP2D6 protein levels and mRNA levels. In the carriers of *4 (frameshift), *6 (frameshift), *41 (splicing defect) and *36 (CYP2D7 gene hybrid) alleles, CYP2D6 protein and mRNA levels did not correlate ($p = 0.54$). In the remaining samples, the correlation between CYP2D6 mRNA and protein levels improved to the adjusted r^2 of 0.17 (from r^2 of 0.11) (Figure 5D). In only wt allele carriers ($n=37$), CYP2D6 protein expression was still significantly correlated with its mRNA expression with unadjusted r^2 of 0.17 (Figure 5E). Together, these results indicate that a meaningful portion of variability in CYP2D6 protein levels can be explained by differential transcription of CYP2D6 gene, in part consistent with previous report that CYP2D6 activity is significantly correlated with its mRNA levels (Carcillo et al. 2003, Yang et al. 2010, Temesvari et al. 2012).

2.3.4. CYP2D6 mRNA expression explained by its transcriptional regulator expression/activity

Multiple factors were considered as potential contributors to differential CYP2D6 mRNA expression: (1) CYP2D6 copy numbers for transcription, (2) CYP2D6 genetic variations in its regulatory region that alter transcription factor binding or differential expression or activity of its transcriptional regulators, specifically SHP and HNF4 α , (3) expression or activity of CYP2D6 transcriptional regulators that can be altered by genetic variations and endogenous modulators (such as bile acids or retinoids). To explore whether and how much these factors explain the variability in CYP2D6 mRNA expression, contribution of each factor was examined via multivariate regression analysis.

Unadjusted linear regression analysis showed significant positive correlation between CYP2D6 copy number variation and CYP2D6 mRNA levels (Figure 6).

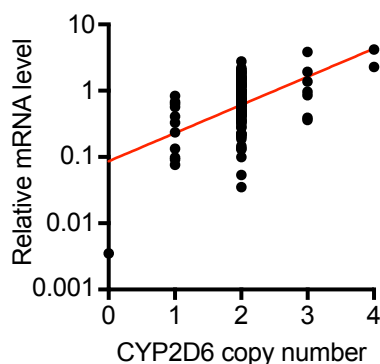


Figure 6. Relationship between CYP2D6 mRNA expression and CYP2D6 copy number variations.

Red line was unadjusted linear regression line for Log CYP2D6 mRNA level vs CYP2D6 copy numbers ($n=115$, $r^2=0.25$, $p<0.0001$).

Table XI Statistics of SNPs detected in CYP2D6, HNF4 α and SHP genes by sequencing

Number detected	CYP2D6	HNF4 α	SHP
All SNPs	86	76	25
Annotated in NCBI SNP database	78	61	19
Not annotated	8	15	6
In exons	19	29	5
In UTR region	1	22	4
Common SNPs^a	26	23	9

A, The percentage of homozygous and heterozygous SNP carriers in the total number of liver tissues is greater than or equal to 15%

To examine the role of SNPs in CYP2D6 regulatory region or in HNF4 α and SHP genes in differential CYP2D6 transcription, SNPs in CYP2D6 (4 kb upstream to 1 kb downstream), SHP (2 kb upstream to 1 kb downstream) and HNF4 α (exons and adjunctive intron/regulatory regions) were detected by high-throughput sequencing. SNPs with perfect linkage disequilibrium ($r^2=1$) were trimmed to reduce analysis redundancy. rs5758550, 115 kb downstream of CYP2D6

was reported to alter CYP2D6 enhancer element activity (Wang, Papp, and Sun 2014), and thus rs5758550 was also detected by Taqman® SNP genotyping assay. The information of SNPs detected in each gene is listed in Table XI.

Lastly, the extent of variability in CYP2D6 mRNA level in the liver tissues explained by differential expression/activity of CYP2D6 transcriptional regulators, namely HNF4 α and SHP, was examined. To this end, HNF4 α and SHP mRNA levels in liver tissues were measured by using qRT-PCR. Due to small sample sizes for the tissues of CNV<2 or >2 (and to exclude potential confounding effects of genetic heterogeneity on CYP2D6 mRNA levels), only the data from CNV=2 samples (n=94) were included in the analysis. In these tissues, CYP2D6 mRNA was positively correlated with HNF4 α expression (Figure 7A, unadjusted linear regression $r^2=0.31$, $p<0.0001$), consistent with previous report that HNF4 α mRNA levels are significantly correlated with CYP2D6 mRNA levels and microsomal activity in human liver tissues (Yang et al. 2010). Interestingly, SHP mRNA expression was also significantly positively correlated with CYP2D6 expression (Figure 7B). Considering that SHP protein expression and activity are functionally modulated by post-translation modification (Miao et al. 2009, Seok et al. 2013, Kim et al. 2016) such that its mRNA levels may not correlate with its activity levels, relationship between mRNA levels of CYP2D6 and CYP8B1 (a target gene of SHP-HNF4 α regulatory pathway (Zhang and Chiang 2001, Inoue et al. 2006)); a marker of overall activity levels of SHP-HNF4 α pathway) was then examined. A prominent positive correlation was observed between CYP2D6 mRNA and CYP8B1 mRNA levels (Figure 7C, unadjusted $r^2=0.50$, $p<0.0001$), suggesting significant roles of SHP-HNF4 α regulatory pathway in explaining variability in CYP2D6 mRNA expression. To further gauge the role of SHP in the transcriptional regulation of CYP2D6, correlation between CYP2D6 and CYP7A1 was also examined. CYP7A1 is another

SHP target gene where SHP represses LRH-1 transactivation of CYP7A1 promoter (Goodwin et al. 2000). Although it was to a smaller degree as compared to the correlation with CYP8B1, a significant positive correlation between CYP2D6 and CYP7A1 expression was observed (Figure 7D, unadjusted $r^2=0.32$, $p<0.0001$), suggesting differential SHP activity may play an important role in determining CYP2D6 expression. To ensure that the observed correlation was not driven by assay quality, (e.g., different RNA/cDNA quality in the samples), correlation between mRNA levels of CYP2D6 and CYP3A4 (a major drug-metabolizing enzyme) was examined. Only marginal association between CYP2D6 and CYP3A4 was observed (Figure 7E, unadjusted $r^2=0.05$, $p=0.02$). Because HNF α is the direct regulator and CYP8B1 shared the same SHP-HNF4 α regulatory pathway, only HNF4 α and CYP8B1 expression were included in the final model of multiple regression modeling.

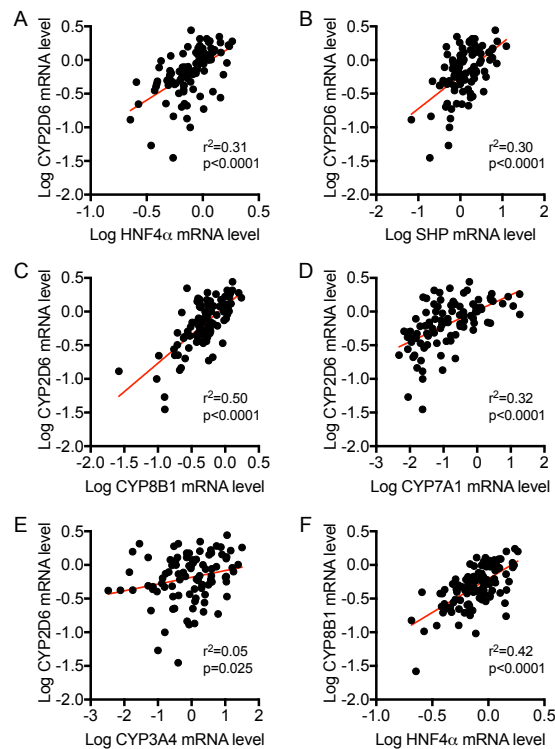
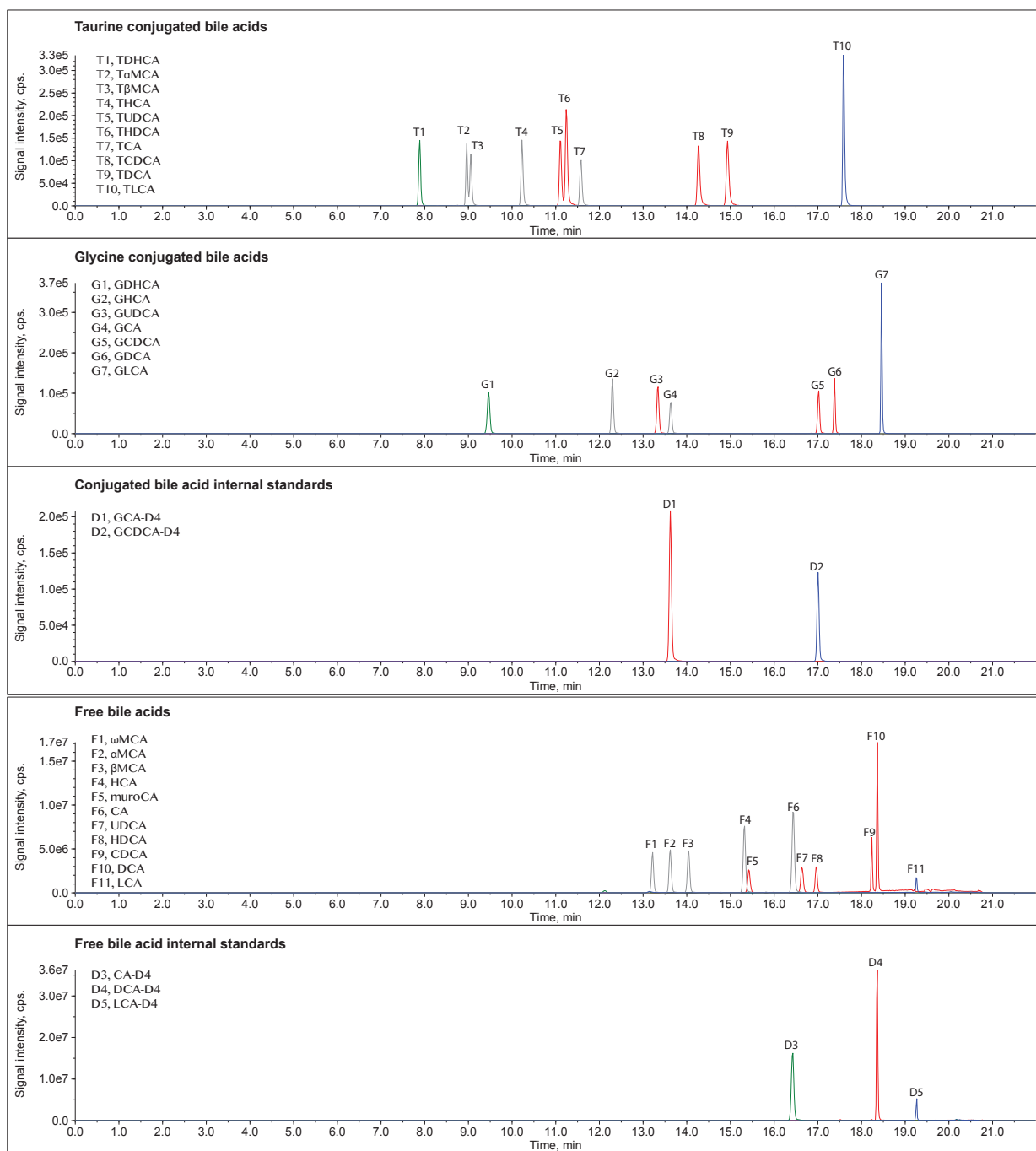


Figure 7. Correlations between mRNA levels in samples with CYP2D6 copy number of 2. Red lines are unadjusted linear regression line between Log CYP2D6 mRNA level and Log mRNA level of the gene of interest. A, CYP2D6 vs. HNF α . B, CYP2D6 vs. SHP. C, CYP2D6 vs. CYP8B1. D, CYP2D6 vs. CYP7A1. E, CYP2D6 vs. CYP3A4. F, CYP8B1 vs. HNF4 α .

SHP expression and/or activity is known to be regulated by bile acids and retinoid acids (Li and Chiang 2015, Cai et al. 2010). To determine whether SHP modulators contribute to inter-individual variability in CYP2D6 expression, hepatic concentration of bile acids (28 primary and secondary bile acids) and all-trans retinoic acid by using LC-MS/MS, and their association with CYP2D6 mRNA expression was examined. Typical chromatography of standard solution (i.e. 250 nM, Figure 8A) and that of individual liver sample (i.e. subject 1534 from LTCDS, Figure 8B) are shown in Figure 8. The accuracy and quantification limits of the bile acid assays are shown in Table XII. Median bile acid pool size was 60 pmol/mg liver (Figure 9A). Over 99% percent of the bile acids in the liver were conjugated with more glycine (G) conjugation over taurine (T) conjugation (Figure 9B). CA, CDCA and DCA and their conjugates were the major bile acid species in human liver, consisting over 95% of the total bile acid pool, consistent with previous reports (Garcia-Canaveras et al. 2012).

A



B

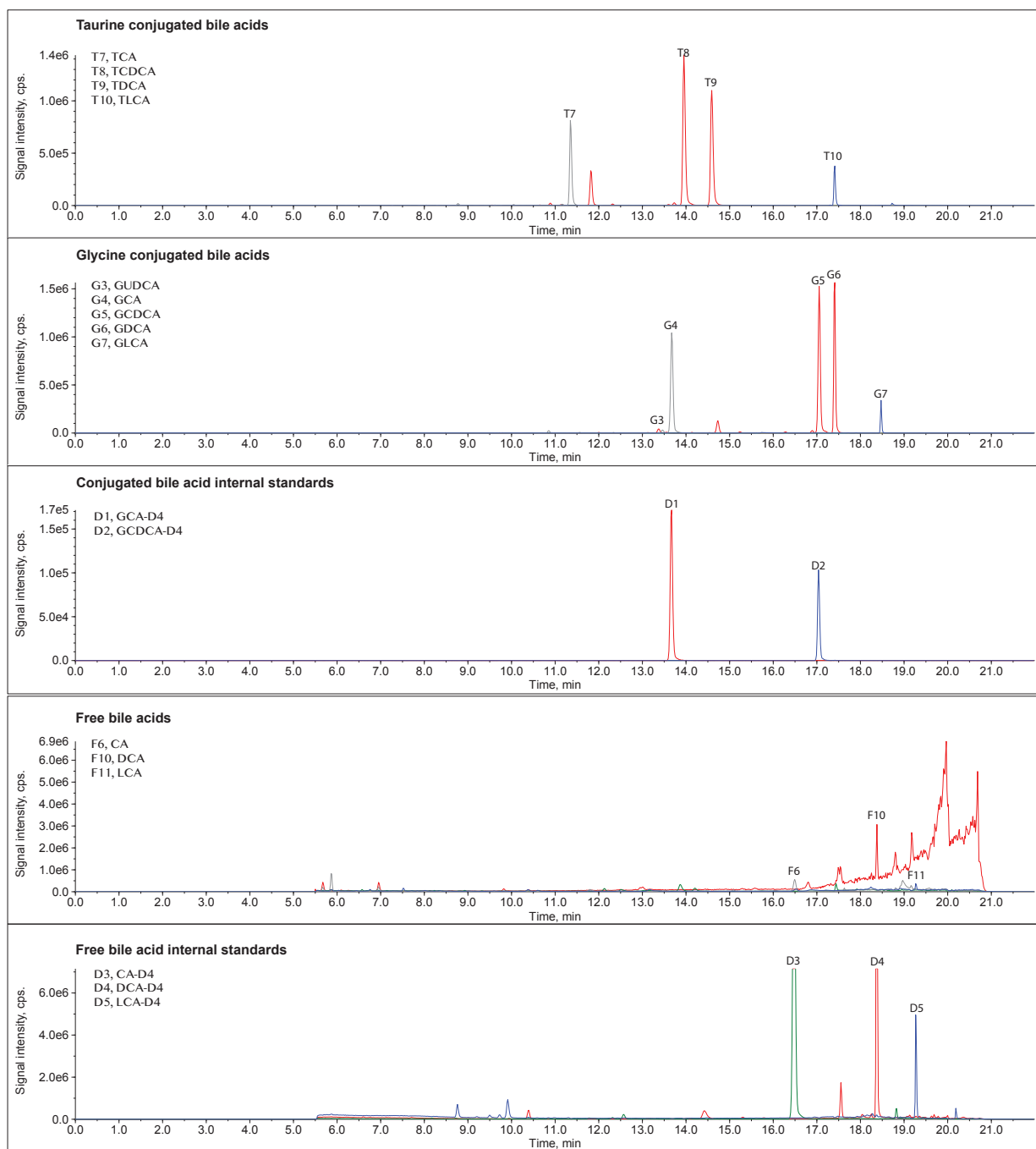


Figure 8. Typical chromatograph for bile acid profiling.

A, chromatographs of mixed standard solution of all the bile acids at 250 nM, except from LCA and LCA-D4 at 500 nM. B, chromatographs of liver extract from subject 1534 from LTCDS with detected bile acids annotated.

Table XII Hepatic bile acid profiling, quantification limits and average accuracy.

Bile acid	Limit of quantification (nM)		Average accuracy for quality control (%)	
	Low	Upper	25 nM (Low)	2500 nM (High)
αMCA	2.5	2500	87	87
βMCA	2.5	2500	106	84
CA	2.5	1000	96	103
GCA	2.5	25000	100	104
GCDCA	5	25000	101	100
GDCA	5	10000	88	101
GDHCA	2.5	25000	108	108
GHCA	5	2500	96	103
GUDCA	5	10000	110	110
HCA	2.5	2500	91	89
HDCA	5	2500	94	84
muroCA	5	2500	96	86
TαMCA	2.5	25000	113	100
TβMCA	2.5	25000	121	100
TCA	2.5	25000	105	104
TCDCa	2.5	25000	100	106
TDCA	2.5	25000	104	107
TDHCA	2.5	25000	93	94
THCA	2.5	25000	106	105
THDCA	2.5	25000	111	100
TLCA	2.5	10000	83	109
TUDCA	2.5	25000	110	103
UDCA	2.5	2500	83	92
ωMCA	10	2500	77	87
CDCA	5	2500	111	111
DCA	5	2500	117	100
GLCA	5	2500	94	96
LCA	50	20000	174	91

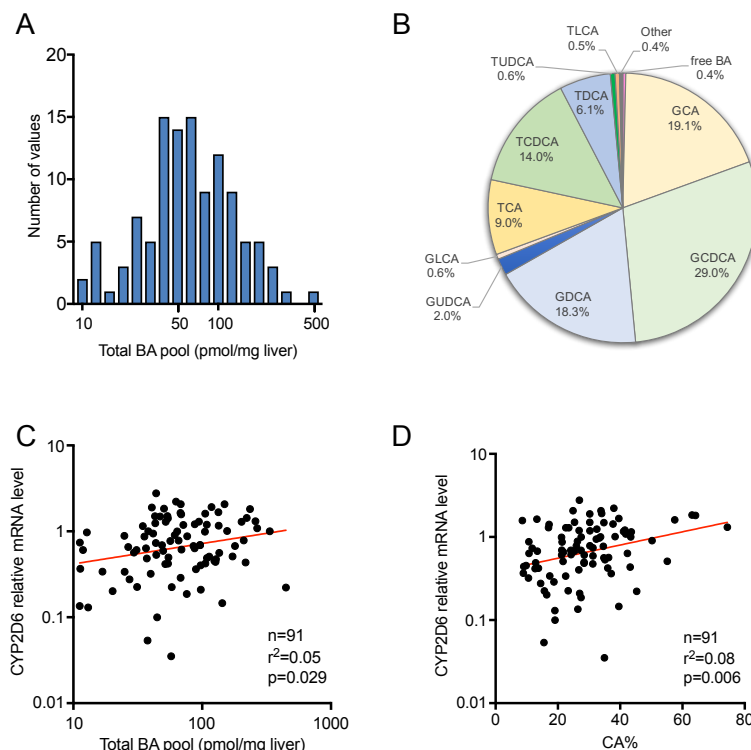


Figure 9. Hepatic bile acids and its correlation with CYP2D6 mRNA levels.

A, Log distribution of total bile acid pool in human liver tissues (n=112). Total hepatic bile acid levels followed log-normal distribution in human liver tissues. B, average composition of bile acid species in human livers. C, correlation between Log CYP2D6 mRNA level and Log total bile acid level in samples with CYP2D6 copy number of 2 (n=91). D, correlation between Log CYP2D6 mRNA level and cholic acid (free and conjugated) percentage (CA%) in samples with CYP2D6 copy number of 2 (n=91).

Considering that (1) CA, CDCA and DCA had similar potency in activating inducing SHP expression/activity (Liu et al. 2014) and (2) conjugated bile acids bind to FXR and stimulate target gene expression (Downes et al. 2003, Ellis et al. 2003), total bile acid levels were used to study the association between bile acid levels and CYP2D6 expression. Interestingly, in samples with CNV of 2, CYP2D6 mRNA expression was positively correlated with total bile acid levels (Figure 9C). Of note, CYP8B1 catalyzes 12-hydroxylation reaction in bile acid synthesis that leads to CA production, and thus the percentage of CA (free CA and its conjugates)

in total bile acids was used as a biomarker of CYP8B1 activity (Li-Hawkins et al. 2002). CA% is significantly correlated with CYP2D6 mRNA expression in samples with CYP2D6 copy number of 2 (Figure 9D).

All-trans retinoic acid (atRA) is the bioactive form of retinoids (Balmer and Blomhoff 2002). The median hepatic atRA level was 41 pmol/g liver and ranged from 8 to 644 pmol/g liver. (Figure 10A). As retinoids are stored in the liver, hepatic atRA level is higher than reported human serum atRA level (i.e. 3 nM) (Arnold et al. 2012). There was a weak negative correlation between hepatic atRA and CYP2D6 mRNA levels (Figure 10B).

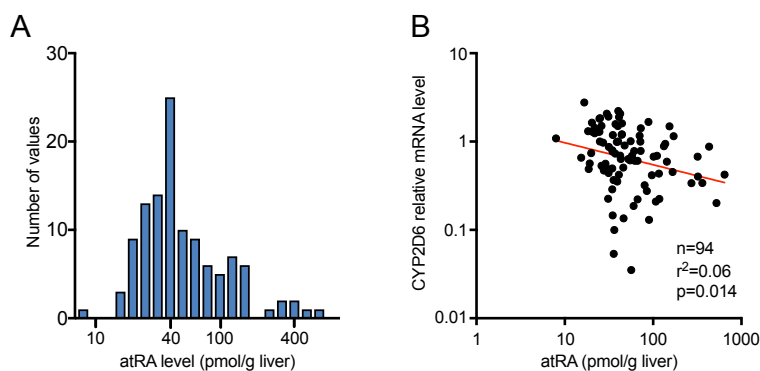


Figure 10. Distribution of hepatic all-trans retinoic acid levels and its correlation with CYP2D6 mRNA expression.

A, distribution of Log atRA levels in human liver tissues (n=115). B, correlation between Log CYP2D6 mRNA expression and Log atRA level in livers with CYP2D6 copy number of 2 (n=94).

At the end, the following parameters (i.e., all common SNPs and factors significantly correlated with CYP2D6 mRNA expression in unadjusted analysis) were included in the

multivariate regression analysis: CYP2D6 CNV, 58 common SNPs, CYP8B1 mRNA expression, HNF4 α mRNA expression, hepatic total bile acid level, CA% and hepatic atRA level. Results of correlation analysis after adjusting covariate effects (using *p*-value cutoff of 0.05) revealed that the following variables remain significantly associated with differential mRNA expression: CYP8B1, HNF4 α , total bile acid, CA%, rs16947A, rs3892097T, and rs28371703T (Table XIII).

Notably, of 58 common SNPs included in this analysis, only 4 SNPs (in part due to small sample sizes for minor alleles) were significantly associated with differential CYP2D6 mRNA levels in the adjusted regression analysis: rs16947 (2850C>T in CYP2D6*2-like alleles (Gaedigk 2013)), rs3892097 (1846G>A in CYP2D6*4 allele), rs28371703 (974C>A in CYP2D6*4 allele) and chr22:42529256_42529257insT (2.3 kb upstream of CYP2D6). The three annotated SNPs are either nonsynonymous SNP or intronic SNP with known function located in CYP2D6 coding region while chr22:42529256_42529257insT is novo SNP. However, this SNP was not validated by Sanger sequencing and therefore was not included in stepwise regression model.

Table XIII Significant predictors of CYP2D6 mRNA expression

Variables	β	95% CI	Adjusted p-value	Adjusted r^2
Log CYP8B1 mRNA	0.48	0.31 to 0.64	<0.0001	0.19
Log HNF4 α mRNA	0.38	0.21 to 0.55	<0.0001	0.13
Log total bile acid	0.23	0.06 to 0.41	0.008	0.05
CA%	0.23	0.06 to 0.41	0.009	0.05
rs16947A	0.19	0.01 to 0.35	0.037	0.01
rs3892097T	-0.27	-0.43 to -0.10	0.002	0.05
rs28371703T	-0.27	-0.43 to -0.10	0.002	0.05
chr22:42529256_42529257insT	0.25	0.08 to 0.41	0.004	0.04

Stepwise regression with p value cutoff of 0.15 was conducted with variables listed in Table XIII. The final regression model showed Log CYP8B1 mRNA level, Log HNF4 α mRNA level, Log total bile acid level, and rs3892097 were significant predictors of CYP2D6 mRNA expression, with 32% of the variability explained. The model results were shown in Table XIV.

Table XIV Final regression model of CYP2D6 mRNA expression (p<0.15)

Variable	Parameter estimate	SE	p value	Stepwise adjusted r ²
Intercept	0.0183	0.0690	0.79	
Male	-0.0730	0.0726	0.32	0.122
Age	0.0702	0.0702	0.30	
Corning	-0.1321	0.0784	0.095	
Log CYP8B1 mRNA expression	0.3744	0.0984	0.0002	0.372
rs3892097T	-0.2453	0.0712	0.0008	0.426
Log HNF4 α mRNA expression	0.1707	0.0966	0.080	0.435
Log total BA	0.1197	0.0732	0.105	0.443

2.4. Discussion

In this study, we measured CYP2D6 genotype, mRNA expression, protein expression and activity collectively in over 100 snap-frozen healthy human liver tissues to identify the major sources of inter-individual variability in CYP2D6 activity levels in a systematical manner. To our knowledge, this is the first study where CYP2D6 is fully characterized at mRNA and protein expression as well as enzyme activity levels in a large number of healthy human liver tissues, along with comprehensive CYP2D6 genotype information.

Since CYP2D6 genetic polymorphisms are known to contribute to inter-individual variability in CYP2D6-mediated drug metabolism, we first determined the extent of variability explained by this known contributing factor. We sequenced the whole CYP2D6 gene and complemented it with qPCR based copy number variation assay to genotype CYP2D6. The sequencing read length is ~300 bp for the high-throughput sequencing method (ion-torrent). Due to the intrinsic limitation of the sequencing method, long range haplotypes for each allele was assembled based on reported haplotypes on CYP allele website. For two liver tissues, CNV assay targeting exon 9 called 2 copies of CYP2D6 gene while CNV assays targeting intron 2 and 6 called more than 2 copies, suggesting CYP2D6-CYP2D7 hybrid after intron 6 assay region. Although the exact hybrid alleles cannot be determined in these two samples, hybrid alleles will not produce functional CYP2D6 protein and are assigned as 0 for functional gene dose with confidence. Of note, samples with uncertain genotype assignments were rare in this study. Therefore, uncertainty in CYP2D6 genotyping was not likely a confounding factor in the subsequent analysis.

We detected 18 CYP2D6 allelic variants and subsequently 37 CYP2D6 diplotypes in our cohort of human liver tissues. Due to the complexity of CYP2D6 genotypes and low frequencies

of some alleles, AS was used as a semi-quantitative system to group CYP2D6 alleles with similar functions into the same categories. We observed significant positive correlation between AS and CYP2D6 activity in this study, consistent with in vivo observations using dextromethorphan urinary metabolic ratio or metoprolol AUC as markers of CYP2D6 activity levels in vivo (Matthaei et al. 2015, Gaedigk et al. 2008). Despite the significant correlation, AS only explained 23% of the inter-individual variability in CYP2D6 activity in all human liver tissues. This is in part consistent with results from a recent twin study. A clinical PK study of metoprolol in identical and fraternal twins showed that 91% of the variability in metoprolol AUC (obtained after 5 mg i.v. single dose) is attributed to inheritable factors, but CYP2D6 genotype only explained 39% of the variability in metoprolol AUC (Matthaei et al. 2015). The finding that CYP2D6 genetic variations only explained a minor portion of the variability in metoprolol AUC supports our observation in human liver tissues. Of note, in individuals with 1 to 2 functional CYP2D6 alleles (i.e., AS 1, 1.5, or 2; >80% of samples), AS only played a minor role in determining CYP2D6 activity (7.5%). This is not surprising that median CYP2D6 activity doubled when AS doubled from 1 to 2 but CYP2D6 activity within each AS category varied at the level of 2 magnitudes. Similar results were also observed in previous in vivo studies where CYP2D6 activities in AS 1, 1.5 and 2 categories were largely overlapped (Matthaei et al. 2015, Gaedigk et al. 2008). This suggests that CYP2D6 genotypes play a limited role in explaining inter-individual variability in CYP2D6 activity in EM population, and that the overall CYP2D6 genotype-phenotype association is mainly driven by non-function CYP2D6 allele carriers (i.e., PMs), at least in European descendent population. Indeed, non-function alleles of CYP2D6 (i.e. CYP2D6*4, *5, *6 and *12) alone accounted for 14.4% of the variability in CYP2D6 activity, comparing with 23% using AS system with all CYP2D6 genotypes included. Nevertheless, AS

remains a significant contributor to CYP2D6 inter-individual variability and genotyping for non-function CYP2D6 alleles is informative to identify CYP2D6 poor metabolizers.

The assumptions made to combine different CYP2D6 diplotypes into AS categories may create some bias in the analysis. First, each CYP2D6 allele is assigned with a functional gene dose. CYP2D6*45 allele with three amino acid substitutions (i.e. E155K, R296C and S486T) is associated with normal or decreased CYP2D6 function (<http://www.cypalleles.ki.se/cyp2d6.htm>). *45 allele was originally classified as decreased-function allele with score of 0.5 in the activity score system (Gaedigk et al. 2008) which was used in this study. In a more recent report, *45 was classified as normal-function allele with score of 1 (Gaedigk et al. 2017). In this cohort of human liver tissues, we only observed 4 heterozygous CYP2D6*45 carriers. Assigning score of 0.5 or 1 for *45 allele did not affect the correlation between AS and CYP2D6 activity significantly (data not shown). We also observed one carrier of CYP2D6*108 allele, characterized with H352R and Y355C amino acid substitution. Since no functional analysis of *108 allele is available, *108 allele was assumed to be normal-function with a score of 1. Due to the rareness of this allele, AS assignment for *108 carrier is not likely to affect the overall association between AS and CYP2D6 activity in this study. However, for cohorts with more carriers of this allele, it might be useful to conduct functional analysis for the allele before assigning AS to it. Another rationale for AS system is that AS for CYP2D6 diplotype is the sum of the scores for paternal and maternal alleles. This leads to the same AS assignment of 1 for carriers with two decreased-function alleles ($0.5+0.5$ for AS) and carriers with one non-function and one normal-function alleles ($0+1$ for AS). Whether these two types of carrier have similar CYP2D6 activity level is yet to be studied in a larger sample size. Together, using AS system to represent CYP2D6 genotype information may bring some bias in the analysis, however, the

impact may be small. Large portion of variability in CYP2D6 activity remains to be explained by factors other than CYP2D6 genotypes.

In liver tissues with AS equal or over 1, CYP2D6 activity is highly correlated with its protein expression, indicating that CYP2D6 protein levels govern its activity levels in these tissues. Over 60% of the variability in CYP2D6 activity can be explained by differential CYP2D6 protein expression in different individuals. The remaining variability can be explained by multiple factors. First, the inherent limitation of dextromethorphan as a CYP2D6 probe drug can be one factor. Although dextromethorphan O-demethylation to form dextrorphan is primarily mediated by CYP2D6, other CYPs can also mediate this biotransformation at a slower rate (Takashima et al. 2005, Yu and Haining 2001). This is consistent with our observation that a small amount of dextrorphan was still formed in liver tissues with 2 non-function CYP2D6 alleles. Second, differential expression and/or activity of CYP partners may in part explain the remaining inter-individual variability in CYP2D6 activity. Cytochrome P450 oxidoreductase (POR) is required for electron transfer in CYP-mediated reactions (Riddick et al., 2013). POR activity was reported to be associated with CYP2D6 activity in human liver microsomes, although the degree of association was weak when compared with those with some other CYPs examined in the study (Hart et al. 2008). Cytochrome b5, also a well-known partner of CYPs, has conflicting effect on CYP2D6 activity. In recombinant system in vitro, the presence or absence of cytochrome b5 did not affect CYP2D6 activity (Bart and Scott 2017, Yamazaki et al. 1997). Yet, liver microsomes from CYP2D6 humanized mice with mouse cytochrome b5 knockout showed 40-50% of decrease in the metabolism of debrisoquine and bufuralol (both CYP2D6 substrates) (Henderson et al. 2015). Third, CYP2D6 antibody used in this study to measure CYP2D6 protein amount may not detect certain CYP2D6 variants. We used a

commercial monoclonal antibody against wild-type CYP2D6 protein to determine CYP2D6 protein levels in human liver S9 fractions. Since the epitope of the antibody is not mapped, it is not known whether the antibody has similar binding affinity to different CYP2D6 alleles (e.g. CYP2D6*10, *12 and *17 with amino acid substitutions). We did observe CYP2D6 protein band detected by the antibody in CYP2D6*17/*17 carriers, but antibody binding affinity was not known. Although it's less likely, we cannot completely rule out the possibility that antibody affinity to different CYP2D6 alleles may affect the association between CYP2D6 activity and protein levels.

To trace the source of variability in CYP2D6 protein levels, we examined the correlation between CYP2D6 protein and mRNA expression. The CYP2D6 protein expression was significantly correlated with its mRNA level with adjusted r^2 of 0.11. This result is in part consistent with the previous findings. The only study of CYP2D6 mRNA and protein correlation reported r^2 of 0.521 after least-square regression in 17 human liver tissues (Ohtsuki et al. 2012). The correlation between CYP2D6 activity and mRNA levels were reported more often. The sample size and the degree of correlation varied largely, with Spearman coefficient ranging from 0.53 in 398 livers with all CYP2D6 genotypes (Yang et al. 2010) to 0.91 in ~100 liver tissues without CYP2D6*3, *4 and *6 carriers (Temesvari et al. 2012). Interestingly, the study observing strong correlation between CYP2D6 activity and mRNA expression also observed strong correlation between activity and mRNA levels in other CYPs examined (Temesvari et al. 2012). Studies observing weaker CYP2D6 activity-mRNA correlation also observed weaker activity-mRNA correlation for other CYPs (Yang et al. 2010, Ohtsuki et al. 2012). This suggests that poor tissue quality may confound the results such that CYP2D6 mRNA-protein correlation is driven by differential levels of mRNA/protein integrity. In this study, we checked RNA

integrity in all the tissue samples and only liver tissues with good RNA quality (examined by using gel electrophoresis) were used for further analysis.

The qRT-PCR assay used in this study was amplifying exon 1 region of CYP2D6 mRNA. This assay will pick up all splicing variants of CYP2D6, which better reflects total transcription level of CYP2D6 gene. High correlation ($r^2=0.96$) between results from this assay and another assay targeting wild-type CYP2D6 mRNA (NM_000106) exon 2-3 junction indicates that CYP2D6 mRNA level measurement was reliable, without genomic DNA contamination. On the other hand, this assay will also detect CYP2D6 non-functional splicing variants, naturally occurring ones as well as ones caused by CYP2D6 polymorphism. As a result, some CYP2D6 genetic variations that lead to non-functional mRNA variant production (i.e. *4, *4×N, *6, *41 and *36×N genotypes) can interfere with CYP2D6 mRNA-protein correlation. Indeed, in carriers of these CYP2D6 alleles, the correlation between CYP2D6 mRNA and protein levels was diminished. A stronger correlation was observed in non-carriers when comparing with that in overall samples.

When considering the dynamic biological process, protein-mRNA correlation is not as simple as protein-activity correlation where CYP2D6 enzymatic activity is directly proportional to functional protein level. CYP2D6 mRNA level is controlled by its synthesis rate and degradation rate. Similarly, CYP2D6 protein level is also controlled by protein synthesis rate and degradation rate. CYP2D6 mRNA level affect CYP2D6 protein level by regulating its synthesis. CYP2D6 mRNA half-life is 4.8 hours in HepG2 cells (Wang et al. 2014) while CYP2D6 protein half-life is estimated to be at 51-70 hours (Yang et al. 2008, Venkatakrishnan and Obach 2005). Assuming mRNA and protein half-lives are accurate and CYP2D6 protein synthesis rate is proportional to its mRNA level, any change in mRNA synthesis will lead to a new steady-state

for mRNA expression in ~15 hours but it would take 150-210 hours for protein. It is likely that CYP2D6 mRNA and protein does not correlate well only because the liver tissues were collected at different stage of mRNA-protein change. For example, a mechanistic PK/PD model of CYP3A induction by dexamethasone in rats showed that CYP3A1 mRNA half-life was estimated to be 3 hours and mRNA induction peaked at ~25 hours after inducer dosing, while CYP3A1 protein half-life was estimated to be 26 hours and protein expression peaked at ~40 hours (Li et al. 2012). Due to the sequence of regulation and longer time to reach steady-state for protein regulation, the correlation between CYP protein and mRNA levels would not be perfect even when mRNA level was the only factor modulating protein expression, if not at steady state for both mRNA and protein. This delayed response will be more prominent with CYP2D6 protein because of its longer half-life. Since human liver tissues only provide a snapshot view of this molecular dynamics, one cannot rule out the importance of differential mRNA expression in explaining inter-individual variability in CYP2D6 protein levels.

Whether and how miRNA or protein phosphorylation plays a role in regulating CYP2D6 mRNA and protein levels remains unknown. miR-101 and miR-128-2 have been shown to regulate CYP2D6 expression in cell culture (Li et al. 2015). Based on miRmine miRNA expression profile database (Panwar, Omenn, and Guan 2017), miR-101 is expressed in the liver, while miR-128-2 is not detected. The physiological significance of miRNA in regulating CYP2D6 expression and its role in explaining inter-individual variability in CYP2D6 expression is yet to be studied. For CYP2D6 protein modification, only one previous study observed CYP2D6 protein phosphorylation at Ser135 in 2 out of 4 human liver tissues examined (Redlich et al. 2008). It is unknown whether this Ser135 phosphorylation has any physiological functions, such as modulating protein localization, stability, or enzymatic activity.

Results from our laboratory and others using CYP2D6-humanized mice (Corchero et al. 2001, Koh, Pan, Shen, et al. 2014, Pan and Jeong 2015, Pan, Lee, and Jeong 2015) indicated that expression/activity change in CYP2D6 transcriptional regulators can lead to serial changes in CYP2D6 mRNA, protein and eventually activity levels. Thus, we tried to gauge the role of transcriptional regulation in explaining inter-individual variability in CYP2D6 mRNA levels. Differential CYP2D6 transcriptional activity can be results of both cis- (i.e. CYP2D6 copy number variations and SNPs affecting transcription factor binding) and trans- (i.e. differential expression/activity of transcription factors) regulations. We first examined the effect of CYP2D6 copy number variation in CYP2D6 mRNA expression. CYP2D6 mRNA expression is known to significantly associate with CNV (Wang et al. 2011). We also observed positive correlation between CYP2D6 mRNA level and CNV in un-adjusted analysis, however, the correlation was not significant after covariant (age, sex, liver source) adjustment. This is likely due to relatively low frequency of gene deletion and multiplication in this study (in European descendent population), compared with that of Chinese population in the previous study. We next examined if SNPs in CYP2D6 its regulators (i.e. HNF4 α and SHP) are associated with CYP2D6 mRNA expression. Of all the SNPs detected, only 58 SNPs with frequency over 0.15 were examined. Only 3 SNPs were significantly associated with CYP2D6 mRNA expression. Interestingly, all 3 SNPs were ones in coding region of CYP2D6 gene. Two of the SNPs were in CYP2D6*4 allele. *4 allele associates with lower CYP2D6 mRNA expression maybe because rs3892097 (1846G>A) causes incorrect splicing of mRNA and frameshift in protein translation, subsequently triggering nonsense-mediated mRNA decay (Popp and Maquat 2016). The last SNP, rs16947 (2850C>T), is the SNP that defines CYP2D6*2-like alleles (Gaedigk 2013), including CYP2D6*2, *12, *17, *41 and *45 detected in this study. rs16947 has been associated

with higher CYP2D6 expression but not by direct effect. It was suggested that rs5758550, in high linkage disequilibrium with rs16947, was the functional SNP that impacts CYP2D6 transcriptional activity at downstream enhancer region (Wang, Papp, and Sun 2014, Wang et al. 2014). We also detected rs5758550 by using Taqman® SNP assay. Interestingly, this SNP did not show significant association with CYP2D6 mRNA expression in our study. rs1080985 (-1584C>G), also commonly found in CYP2D6*2 alleles, was associated with increased metabolism of CYP2D6 substrate in vivo (Dorado et al. 2009, Llerena et al. 2013). We did not observe significant association between rs1080985 and CYP2D6 mRNA levels either. Only rs3892097 was the significant genetic variation included in the final stepwise regression model accounting for 5% of the variability in CYP2D6 mRNA expression in this study.

Next, we examined whether expression or activity of CYP2D6 regulators can predict CYP2D6 mRNA expression. As a key transcription factor in the liver, HNF4 α expression is tightly controlled in the liver. We only observed a 9-fold range of expression for HNF α expression. Despite small inter-individual variability in HNF4 α expression, expression of CYP2D6 and CYP8B1, both target genes of HNF4 α , were positively correlated with that of HNF4 α with unadjusted r^2 of 0.31 and 0.42 respectively, suggesting important role of HNF4 α expression in regulating basal expression of both genes. SHP is a recently identified repressor of CYP2D6 gene (Koh, Pan, Shen, et al. 2014). We examined the association between CYP2D6 mRNA levels and SHP mRNA levels. Interesting, we observed a positive correlation between the expression of two genes. Considering the fast turnover rates for both SHP mRNA and protein (Langhi et al. 2008, Miao et al. 2009) and that SHP post-translational modifications modulate SHP protein stability and activities (Seok et al. 2013, Miao et al. 2009), SHP mRNA expression at a snapshot may not reflect SHP activity in repressing target gene expression at the time. Since

SHP also represses its own expression (Lu et al. 2000), the positive correlation we observed between CYP2D6 mRNA and SHP mRNA levels might be the results of co-regulation the two genes by SHP. To better probe SHP activity level in the livers, we used CYP8B1 expression as a surrogate of SHP activity of repressing HNF4 α transactivation of target genes. Intriguingly, we observed the highest positive correlation ($r^2=0.5$) between CYP2D6 mRNA and CYP8B1 mRNA levels. This is consistent with the observation in a previous study by Yang et al that CYP2D6 mRNA expression is best correlated with that of CYP8B1 among all CYPs (Yang et al. 2010). This suggests that the shared regulatory pathway between CYP2D6 and CYP8B1, most likely HNF4 α -SHP pathway, plays a key role in controlling the expression of CYP2D6. The association between CYP2D6 mRNA and HNF4 α or CYP8B1 remained strong (p value below 0.0001) after adjustment for covariates. In stepwise regression analysis, CYP8B1 mRNA level was the most significant predictor of CYP2D6 expression, explaining 25% of the overall variability in CYP2D6 mRNA expression. Although CYP2D6 CNV was not included in stepwise analysis, conducting the stepwise analysis in CNV=2 samples revealed higher portion (i.e. 36%) of CYP2D6 mRNA variability explained of CYP8B1 expression. Adding HNF4 α expression into the analysis after CYP8B1 mRNA only gained 1% additional variability explained, suggesting that the portion of CYP2D6 mRNA variability explained by differential HNF4 α is likely included in the portion explained by CYP8B1 expression.

Intrigued by this finding, we measured hepatic levels of known SHP modulators, i.e. bile acids and all-trans retinoic acid to explore their roles in explain inter-individual variability in CYP2D6 mRNA expression. The physiological role and regulation of SHP have been intensively studied to understand bile acid homeostasis. Briefly, hepatic bile acids can bind to FXR receptor and induce SHP expression directly (Goodwin et al. 2000); FXR activation also induce FGF19

(Song et al. 2009) and FGF19 signaling can cause SHP protein modification and subsequently changes in protein level and activity (Miao et al. 2009, Seok et al. 2013). Increase expression/activity of SHP will lead to more repression on expression of bile acid synthesis genes. A weak positive association was observed between hepatic total bile acid levels and CYP2D6 mRNA expression. The direction of association was opposite to what we hypothesized. Nevertheless, we included it for stepwise regression analysis. Total bile acid levels only predicted about 1% of the variability in CYP2D6 mRNA expression in the final model and it dropped out as non-significant when only CNV=2 samples were considered. all-trans retinoic acid also has been shown to induce SHP expression (Cai et al. 2010). Hepatic atRA level was only weakly correlated with CYP2D6 expression in unadjusted analysis and no longer significant after covariate adjustment. Thus, it was not carried further for stepwise regression analysis.

Since sterol 12 α -hydroxylation by CYP8B1 controls cholic acid composition in the bile acid pool, we used CA% in hepatic bile acid pool as biomarker of CYP8B1 activity. The association between CA% and CYP8B1 mRNA expression was weak, likely due to the same reason that leads to relatively weak CYP2D6 mRNA-activity association. Still, we observed significant positive correlation between CYP2D6 mRNA expression and CA% with or without covariate adjustment. CA% could not be added to stepwise regression model after CYP8B1 expression was added, suggesting that predictive effect of CA% on variability in CYP2D6 mRNA expression is mediated through CYP8B1 expression.

Sex, age and source of liver tissues were included as covariates in adjusted analysis. Male, age and Corning Inc. as tissue source explained 6%, 7% and 12% of the variability observed in CYP2D6 activity, protein and mRNA levels. Sex and age are known to have no effect on CYP2D6 activity (Bebia et al. 2004, Yang et al. 2010, Achour, Barber, and Rostami-Hodjegan

2014). Indeed, sex and age were not associated with CYP2D6 mRNA, protein and activity levels in our study. Interestingly, livers obtained from two different sources had significantly different expression and activity levels for CYP2D6. Specifically, livers obtained from Corning Inc. had consistently lower mRNA, protein and activity levels for CYP2D6 comparing with those in livers from LTCDS. Tissue quality was checked for all the liver samples, so this is less likely due to different tissue qualities. The cause of the difference is unknown.

In conclusion, we studied the source of inter-individual variability in CYP2D6 activity and expression systematically in human liver tissues. Our results showed that (1) CYP2D6 activity score, representing its genotype information, only explains 23% percent of the variability in CYP2D6 activity; (2) the major driving force of variability in CYP2D6 activity is differential CYP2D6 functional protein expression, explaining over 60% of the variability; (3) transcriptional regulation of CYP2D6 may partially explain the variability in CYP2D6 protein expression, however, mathematical modeling with time information may be needed to calculate the exact contribution of CYP2D6 mRNA expression to inter-individual variability in CYP2D6 protein expression.

CHAPTER 3. OBESITY EFFECT ON CYTOCHROME P450 2D6 EXPRESSION

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3.1. Introduction

Obesity is a prevalent medical condition that 35% of adults and 17% of children in the United States are overweight (Ogden et al. 2014). Obesity is associated with increased incidences of chronic comorbidities, such as diabetes, cardiovascular diseases, and non-alcoholic fatty liver diseases (Fujioka 2015), which often require drug treatment. Yet, our understanding on whether and how obesity alters the rate and extent of drug disposition, especially hepatic drug metabolism, remains incomplete. Clinical reports suggest that the effect of obesity on hepatic drug metabolism is metabolic pathway-specific; however, the directional changes for cytochrome P450 (CYP)-mediated metabolism are largely inconclusive (Brill et al. 2012).

Molecular changes accompanying obesity include altered expression and/or activity of different transcriptional regulators of drug-metabolizing enzymes (DMEs). Specifically, high fat diet (HFD) feeding in mice has been shown to alter expression levels of nuclear receptors that are involved in the regulation of major DME expression, including HNF4 α , SHP, pregnane X receptor (PXR), and constitutive androstane receptor (CAR). HNF4 α is the master transcription factor for hepatic gene expression, including CYPs (Kamiyama et al. 2007, Gonzalez 2008, Jover, Moya, and Gomez-Lechon 2009, Jover et al. 2001). In mice, HFD induces hepatic expression of microRNA (miR)-34a that targets HNF4 α and thus decreases HNF4 α expression (Xu et al. 2015). On the other hand, SHP represses gene expression by interfering the actions of other transcription factors such as HNF4 α (Koh, Pan, Shen, et al. 2014, Pan, Lee, and Jeong

2015). SHP protein levels were shown to be higher in the obese mice of genetic-based or diet-induced models, likely due to decreased proteasomal degradation of SHP (Miao et al. 2009). PXR and CAR are transcriptional activators of DMEs, and HFD was shown to decrease expression of PXR (and its target gene *Cyp3a11*) in mice (Ghose et al. 2011). The effects of obesity on CAR expression appear inconsistent (Yoshinari, Takagi, Sugatani, et al. 2006, Ghose et al. 2011, Xu et al. 2012, Chartoumpekis et al. 2013).

Previous studies have shown important roles of HNF4 α and SHP in transcriptional regulation of CYP2D6 (Cairns et al. 1996, Koh, Pan, Shen, et al. 2014, Pan, Lee, and Jeong 2015). HNF4 α transactivates CYP2D6 promoter by binding to a proximal promoter region (Cairns et al. 1996) while SHP represses CYP2D6 expression by inhibiting HNF4 α transactivation of CYP2D6 promoter (Koh, Pan, Shen, et al. 2014, Pan, Lee, and Jeong 2015). Despite the reports of altered expression of HNF4 α and SHP accompanying in obesity, whether the changes in the major regulators of CYP2D6 expression affect CYP2D6 expression remains unclear. In this study, we examined whether HFD feeding modulates CYP2D6-mediated drug metabolism by using Tg-CYP2D6 mice. Furthermore, we explored how endogenous mouse CYP gene expression is altered in HFD-fed mice to provide insights into the effects of obesity on hepatic drug metabolism in general.

3.2. Materials and Methods

3.2.1. Animals.

Tg-CYP2D6 transgenic mice were previously described (Corchero et al. 2001, Koh, Pan, Shen, et al. 2014). The mice are in the background of C57BL/6 that are known to be susceptible to obesity upon HFD feeding (Kanuri and Bergheim 2013, Podrini et al. 2013). Male Tg-CYP2D6 mice (6-8 week-old; n = 7-9 per group) were fed with either Adjusted Calories Diet (HFD, TD.88137, 42% kJ from fat, Teklad), or a matching low fat control diet (TD.08485, 13% kJ from fat, Teklad; reduced contents of fat, sugar, cholesterol, and calorie as compared to HFD) for 18 weeks in specific pathogen-free environment. The mice had access to the diet and water ad libitum. The mice were housed 2-5 per cage in an environment with 12-h light and dark cycles. Mouse body weight was measured every two weeks. After 18 weeks of feeding, livers from the mice were collected, weighed, snap-frozen and stored at -80 °C until further analysis. All procedures were approved by the Institutional Animal Care and Use Committee in the University of Illinois at Chicago.

3.2.2. RNA isolation and quantitative real time-PCR (qRT-PCR).

Total RNAs were isolated from mouse liver tissues using Trizol (Life Technologies, Carlsbad, CA) and used as template for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA). Using the cDNA as template, qRT-PCR was performed using StepOnePlus™ Real-Time PCR System. Primetime® Gene expression assays (Integrated DNA technologies, Coralville, IA) for CYP2D6 and mouse Gapdh, or Taqman® Gene expression assays (Life technologies, Foster City, CA) for mouse endogenous CYP genes and Pck1 were used with FastStart Universal Probe Master (Rox) (Roche Diagnostics, Indianapolis, IN). Car, Gapdh, Hes6, Hnf4 α , Il1b, Il6, Oatp1a, Pxr, Shp, and Tnf α

mRNA levels were determined using Power SYBR® Green Real Time PCR Master Mix (Life technologies, Foster City, CA) and primer sets listed in Table XV. The relative mRNA expression level was determined after normalizing the expression levels by those of mouse Gapdh ($2^{-\Delta\Delta C_t}$ method) (Schmittgen and Livak 2008).

Table XV Primers used in qRT-PCR assays in Chapter 3

Car	Forward	5'- CCCTGACAGACCCGGAGTTA -3'
	Reverse	5'- GCCGAGACTGTTGTTCCATAAT -3'
Gapdh	Forward	5'- AGGTCGGTGTGAACGGATTTG -3'
	Reverse	5'- TGTAGACCATGTAGTTGAGGTCA -3'
Hnf4 α	Forward	5'- CACGCGGAGGTCAAGCTAC -3'
	Reverse	5'- CCCAGAGATGGGAGAGGTGAT -3'
Hes6	Forward	5'- TTTGGGCATTCTGAGGATCTA -3'
	Reverse	5'- CGCAACTGTGTTACAAACGA -3'
Il1b	Forward	5'- AACCTGCTGGTGTGTGACGTTT -3'
	Reverse	5'- CAGCACGAGGCTTTTTTGTGT -3'
Il6	Forward	5'- AGAAGGAGTGGCTAAGGACCAA -3'
	Reverse	5'- AACGCACTAGGTTTGCCGAGTA -3'
Oatp1a	Forward	5'- TAATCGGGCCAACAATCTTC -3'
	Reverse	5'- ACTCCCATAATGCCCTTGG -3'
Pxr	Forward	5'- GATGGAGGTCTTCAAATCTGCC -3'
	Reverse	5'- GGCCCTTCTGAAAAACCCCT -3'
Shp	Forward	5'- GGCCTCTACCCTCAAGAACAT -3'
	Reverse	5'- TGTCAACGTCTCCCATGATAGG -3'
Tnfa	Forward	5'- AGCCCCCAGTCTGTATCCTT -3'
	Reverse	5'- GGTCACGTGTCAGCATCTT -3'

3.2.3. Western blot.

Mouse liver lysates were prepared using RIPA buffer, and were resolved using 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hercules, CA). CYP2D6, HNF4 α ,

and SHP protein expression levels relative to that of β -actin were determined using the following antibodies: CYP2D6, BD GentestTM catalog #458246; HNF4 α , R&D system K9218; SHP, Santa Cruz sc-30169; and β -actin, Sigma A1978. The CYP2D6 antibody was shown not to cross-react with mouse endogenous CYP2Ds (Miksys et al. 2005).

3.2.4. Hepatic S9 fraction.

Liver tissues (~200 mg) were accurately weighed for S9 preparation. The samples were homogenized on ice using tissue homogenizer (PowerGen 125, Fisher Scientific) in 0.05 M Tris-HCl (pH 7.0) buffer containing 150 mM KCl, 2 mM EDTA and protease inhibitor cocktail (Sigma, P8340) (0.2 mL buffer per 100 mg liver). The homogenate was centrifuged at $9,000 \times g$ for 20 min at 4 °C. The supernatant was collected and volume measured. Protein content in S9 fraction was measured by using BCA protein assay (Pierce Biotechnology, Rockford, IL). Total S9 protein amount per liver = (S9 protein concentration \times S9 volume / liver weight used for S9 preparation) \times total liver weight

3.2.5. CYP2D6 and CYP3A activity.

CYP2D6 activity was measured as previously described (Koh, Pan, Shen, et al. 2014). Briefly, for the measurement of CYP2D6 activity, S9 fractions (~3 mg/mL) were incubated with debrisoquine (200 μ M) for 15 min (based on preliminary results that show a linear increase in metabolite production), and the reaction was stopped by adding one volume of ice-cold acetonitrile with internal standard (paraxanthine, 800 ng/mL). The concentration of 4-hydroxydebrisoquine was measured by LC-MS/MS (Agilent 6410 Triple Quadrupole LC/MS) using electrospray ion source in positive mode. Multiple reaction monitoring data acquisition was employed: m/z 192.3/132.2 for 4-hydroxydebrisoquine and 181.1/124.1 for the internal standard.

For CYP3A activity, triazolam was used based on its specificity for mouse CYP3As (Perloff et al. 2000). Mouse liver S9 (~0.6 mg/mL) was incubated with triazolam (23 μ M) for 5 minutes and the reaction was stopped by adding one volume of ice-cold acetonitrile containing internal standard (α -hydroxytriazolam-D4, 40 ng/mL). The concentrations of α - and 4-hydroxytriazolam were simultaneously measured by LC-MS/MS (Agilent 1200 HPLC with AB Sciex QTRAP® 5500 System) using electrospray ion source in positive mode. Multiple reaction monitoring transitions for α -, 4-hydroxytriazolam and α -hydroxytriazolam-D4 were 359.4/176.3, 359.4/273.1 and 363.2/335.0, respectively.

3.2.6. Cytochrome P450 reductase activity.

The enzyme activity in hepatic S9 fractions was measured by using Cytochrome c Reductase (NADPH) Assay Kit following manufacture's protocol (Catalog #CY0100, Sigma).

3.2.7. Statistical analysis.

All data were presented as mean \pm S.D. Statistical analyses for qRT-PCR and western blot results were performed using student t-test between control and HFD group. A p -value < 0.05 was considered statistically significant. For correlation study, Pearson's correlation test and linear regression was used as appropriate.

3.3. Results

3.3.1. Diet-induced obesity model in Tg-CYP2D6 mice.

Tg-CYP2D6 mice, harboring human CYP2D6 gene along with its regulatory region (Corchero et al. 2001), have been successfully used as an *in vivo* model for studying transcriptional regulation of CYP2D6 expression (Koh, Pan, Shen, et al. 2014, Koh, Pan, Zhang, et al. 2014, Pan and Jeong 2015, Pan, Lee, and Jeong 2015). To evaluate the effect of obesity on CYP2D6 expression *in vivo*, we first established a diet-induced obesity model in Tg-CYP2D6 mice by chronic HFD feeding. Male Tg-CYP2D6 mice were fed with either HFD or a matching control diet for 18 weeks *ad libitum*. Throughout the course of feeding, mice in HFD group gained more weight than those from control diet group since week 8 (Figure 11A). After 18 weeks of feeding, the livers from HFD-fed mice were pale and enlarged, suggesting hepatic steatosis (Figure 11B). The mouse liver weight was 1.68 ± 0.38 and 3.62 ± 1.02 g in control and HFD groups, respectively. Liver-to-body weight ratio was also increased significantly in HFD mice (Figure 11C). The total protein yield (in S9 fractions) per liver was ~60% higher in HFD-fed mice (Figure 11D), indicating increased numbers and/or sizes of parenchymal cells upon HFD.

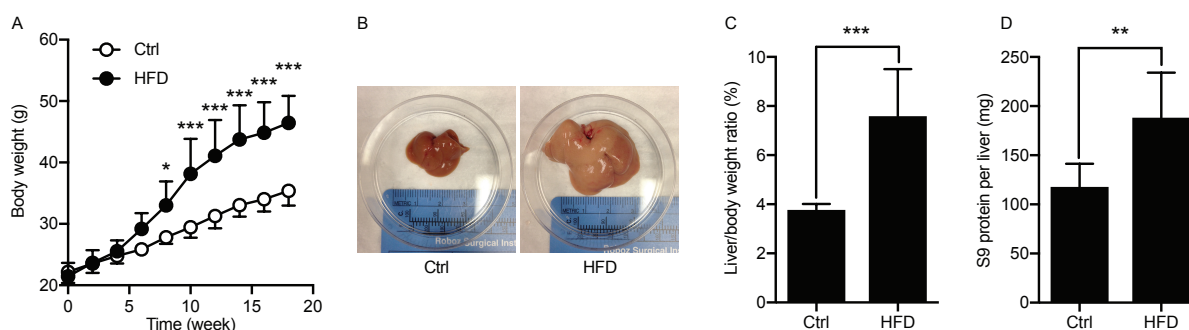


Figure 11. Diet induced obesity model in Tg-CYP2D6 mice.

(A) Body weight over the course of HFD feeding in Tg-CYP2D6 mice. (B) Representative image of the mouse livers from control (Ctrl) and high-fat diet (HFD) group after 18 weeks of feeding. (C) Liver to body weight ratio in control group vs. HFD group. (D) Total S9 protein amount per liver in control group vs. HFD group. (n=7-8 per group). Mean \pm S.D.; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.3.2. CYP2D6 expression did not differ between HFD and control mice.

Previous studies have shown that HFD feeding decreases HNF4 α and increases SHP expression in mouse liver (Xu et al. 2015, Miao et al. 2009). To validate if expression of these transcription regulators is altered in obese Tg-CYP2D6 mice, hepatic mRNA and protein levels of HNF4 α and SHP were examined in HFD-fed Tg-CYP2D6 mice. HNF4 α mRNA and protein levels decreased significantly (by 1.5-fold) in HFD group in Tg-CYP2D6 mice (Figure 12A). This was accompanied by decreased expression of known HNF4 α target genes in the liver [i.e., organic anion–transporting polypeptides 1a (Oatp1a) and phosphoenolpyruvate carboxykinase 1 (Pck1)] (Hayhurst et al. 2001, Martinez-Jimenez et al. 2010, Fang et al. 2012, Ning et al. 2015) (Figure 12B). The changes in mRNA expression of hes family bHLH transcription factor 6 (Hes6) did not reach statistical significance. Contrary to the previous report, SHP mRNA and protein levels did not differ between HFD and control groups (Figure 12C).

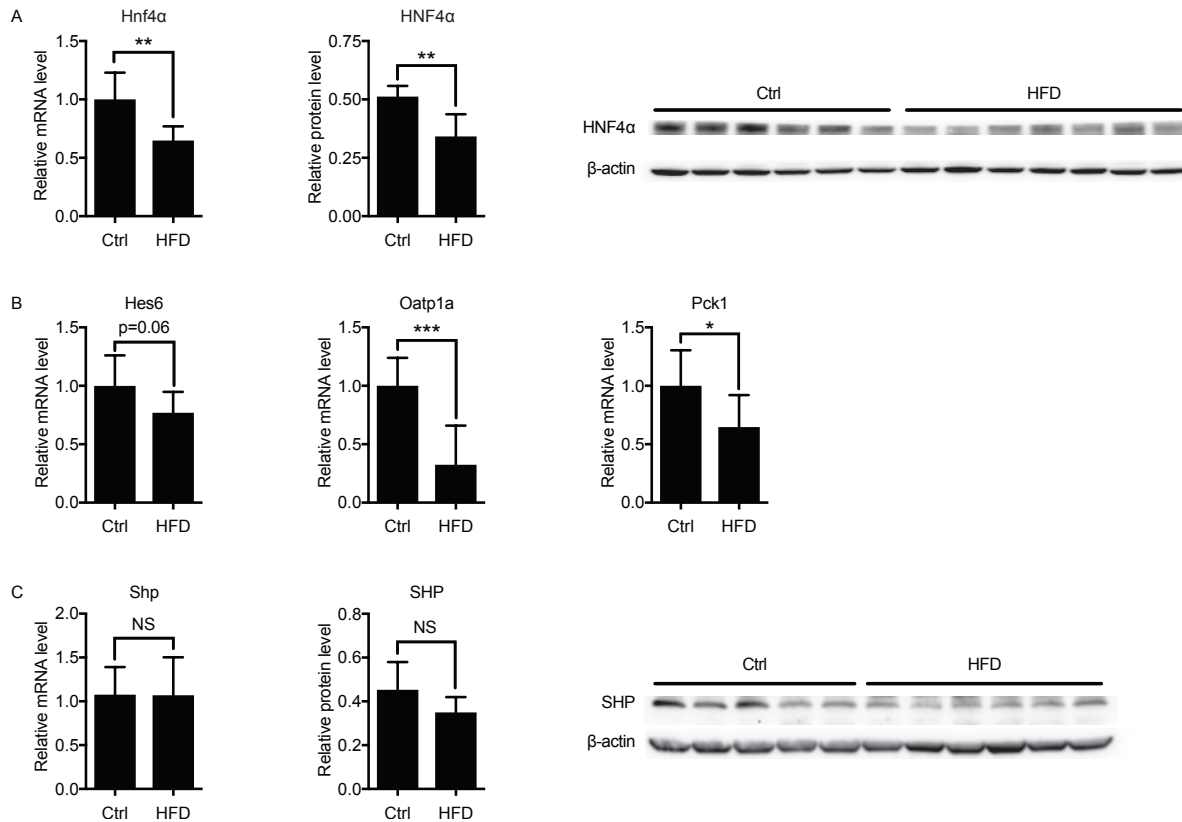


Figure 12. Decreased HNF4 α expression and activity in HFD-fed Tg-CYP2D6 mice.

Liver tissues were collected from Tg-CYP2D6 mice after 18 weeks of HFD or matching control (Ctrl) diet feeding. (A) Hepatic mRNA and protein levels of HNF4 α were determined by qRT-PCR and western blot, respectively. Relative Hnf4 α mRNA levels (left) were normalized by that of control group. Relative HNF4 α protein levels (right) were normalized against β -actin band intensities. (B) Hepatic mRNA levels of representative HNF4 α target genes, Pck1, Hes6 and Oatp1a, were determined by qRT-PCR and normalized by that of control group. (C) Hepatic mRNA and protein levels of SHP were determined by qRT-PCR and western blot respectively. Relative SHP mRNA levels (left) were normalized by that of control group. Relative SHP protein levels (right) were normalized against β -actin band intensities. n=7-8 for qRT-PCR. Mean \pm S.D.; NS, not significant, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Importantly, hepatic mRNA levels of CYP2D6 decreased to a small extent in HFD group (Figure 13A). Hepatic CYP2D6 protein and activity levels did not differ between HFD and control groups (Figure 13B and 13C) although the total CYP2D6 activity per liver was significantly higher in HFD-fed mice due to the larger liver size (Figure 13D).

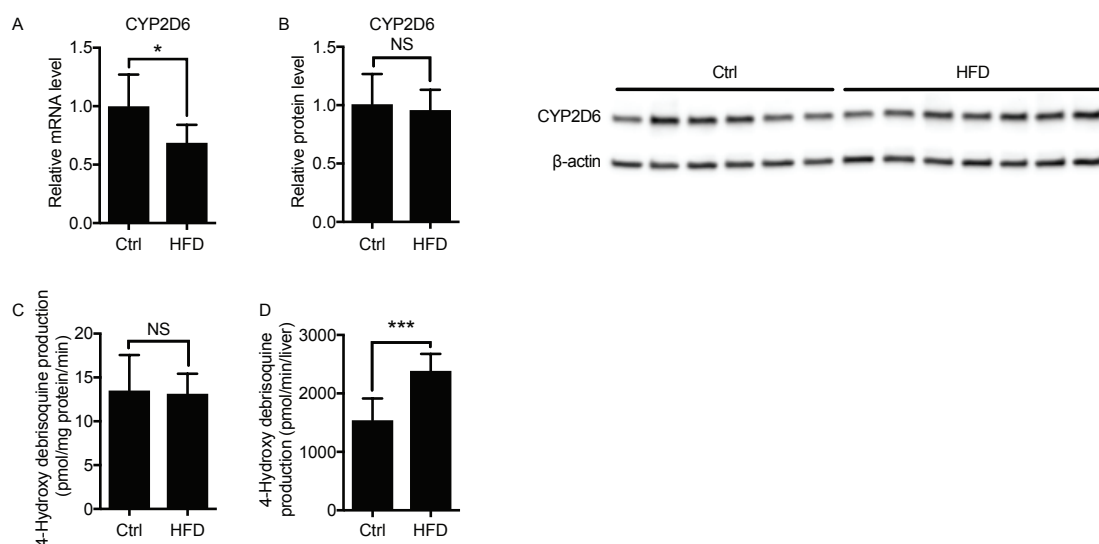


Figure 13. CYP2D6 expression and activity in Tg-CYP2D6 mice after HFD feeding.

Liver tissues were collected from mice after 18 weeks of HFD or matching control (Ctrl) diet feeding. (A) Hepatic mRNA level of CYP2D6 was determined by qRT-PCR and normalized by that of control group. (B) Liver CYP2D6 protein level in S9 fraction was determined using western blot normalized against β -actin band intensities. (C) CYP2D6 activity in mouse liver S9 fraction was measured using debrisoquine as a substrate. CYP2D6 activity is presented as 4-hydroxydebrisoquine formation rate per mg S9 protein. (D) Total CYP2D6 activity per liver was the product of CYP2D6 activity per mg S9 protein and total S9 protein level per liver. $n=6-8$ per group for qRT-PCR and activity assay. Mean \pm S.D.; NS, not significant, *, $p<0.05$, ***, $p<0.001$.

3.3.3. Obesity had differential effects on mouse endogenous CYPs.

Considering that HNF4 α is a global regulator of hepatic genes including those encoding CYPs (Jover, Moya, and Gomez-Lechon 2009), we examined the mRNA expression of representative mouse endogenous CYPs, i.e., Cyp1a2, Cyp2b10, Cyp2c37, Cyp2e1, and Cyp3a11. qRT-PCR results showed pathway-dependent changes in their expression in HFD-fed mice (Figure 14A). Cyp1a2 and Cyp2c37 expression decreased significantly in HFD-fed mice while Cyp2e1

expression did not differ between the groups. Of note, Cyp3a11 expression increased significantly in HFD-fed mice.

Considering that Cyp3a11 is a mouse homolog of CYP3A4, the most important DME in humans, the changes in Cyp3a11 expression were further verified at the enzyme activity level using triazolam as a probe substrate. The results showed that hepatic CYP3A activity in mouse S9 fraction was significantly higher in mice fed with HFD (Fig. 14B). CYP3A activity correlated well with Cyp3a11 mRNA levels ($r = 0.81$) (Figure 14C), suggesting that increased CYP3A enzyme activity in HFD mice is in part attributable to enhanced Cyp3a11 expression. Considering that PXR and CAR are major transcriptional activators of Cyp3a11 expression in mice (Li et al. 2016), hepatic expression of Pxr and Car was examined. qRT-PCR results showed that mRNA expression of neither Pxr nor Car increased in HFD-fed mice (Figure 14D), suggesting that altered expression of these transcription factors is not responsible for Cyp3a11 induction in HFD-fed mice. Cytochrome P450 reductase is required for electron transfer in CYP-mediated reactions (Riddick et al. 2013). To determine whether HFD alters cytochrome P450 reductase activity and thus CYP-mediated drug metabolism, cytochrome P450 reductase activity was measured in hepatic S9 fractions of HFD and control groups. The enzyme activity did not differ between the groups (data not shown).

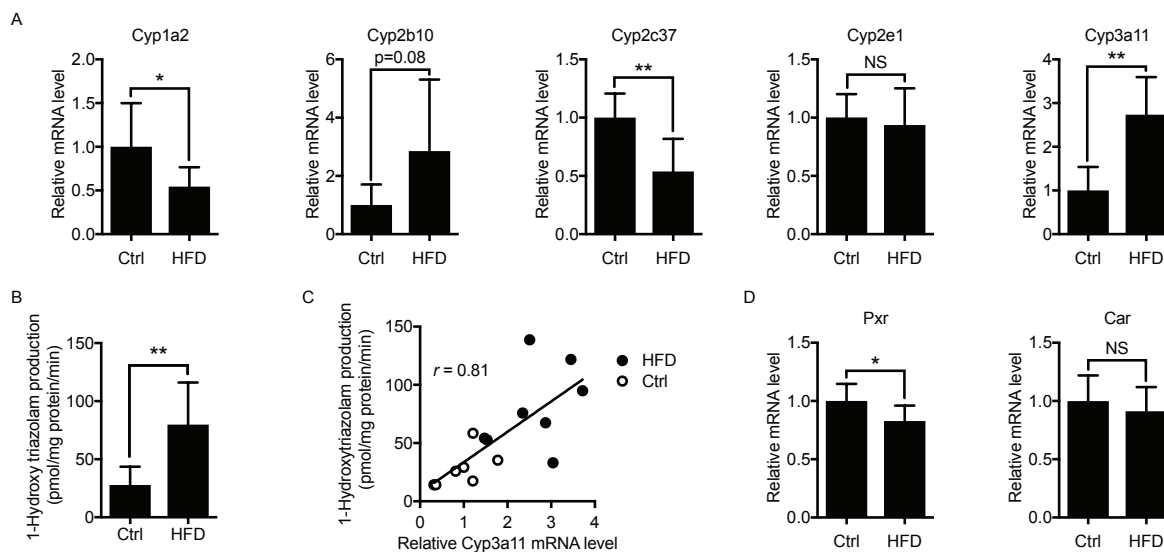


Figure 14. Mouse endogenous CYP expression upon HFD feeding in Tg-CYP2D6 mice.

Liver tissues were collected from mice after 18 weeks of HFD or matching control (Ctrl) diet feeding. Hepatic mRNA levels of drug-metabolizing CYPs were measured using qRT-PCR and normalized to those of the control group. (B) Mouse CYP3A activity in liver S9 fraction was measured using triazolam as a substrate. CYP3A activity is presented as 1-hydroxytriazolam formation rate. (C) Mouse CYP3A activity correlated with Cyp3a11 relative mRNA level (Pearson correlation efficiency $r = 0.81$, $p < 0.001$). (D) Hepatic mRNA levels of Pxr and Car were determined using qRT-PCR normalized by those of the control group. $n = 7-8$ per group; Mean \pm S.D.; NS, not significant, *, $p < 0.05$, **, $p < 0.01$.

3.4. Discussion

Obesity is prevalent and associated with increased incidences of chronic comorbidities, which often require drug treatment. Yet, our understanding on whether and how obesity alters hepatic drug metabolism remains incomplete. Based on previous results indicating altered expression of HNF4 α and SHP in the mouse models of obesity, we examined whether HFD feeding affects CYP2D6-mediated drug metabolism using Tg-CYP2D6 mice. Our results showed that HFD feeding decreased CYP2D6 mRNA levels. This is in line with the previous report showing that HFD induces hepatic expression of miR-34a that targets HNF4 α and thus decreases HNF4 α expression (Xu et al. 2015). Of note, the decrease in CYP2D6 transcript levels in HFD-fed mice was to a small extent (i.e., 1.5-fold) that it did not lead to decreased protein or enzyme activity levels of CYP2D6 in the liver tissues. Importantly, HFD feeding triggered significant increases (~60%) in liver mass, resulting in greater total protein amounts or activities of CYP2D6 per liver in HFD-fed mice. These results suggest that CYP2D6-mediated drug metabolism will likely be greater in obese mice, mainly due to enlarged liver size.

Results from currently available clinical data suggest insignificant changes in the metabolic rates of CYP2D6 substrate drugs in the obese as compared to non-obese subjects. For example, Cheymol et al. reported no differences in the clearance of dexfenfluramine (a CYP2D6 substrate of hepatic extraction ratio ~0.3) between obese vs. non-obese subjects after intravenous infusion (Cheymol et al. 1995). On the other hand, plasma clearance of nebivolol after intravenous bolus administration was reported to be significantly higher in obese subjects (Cheymol et al. 1997). Notably, nebivolol is a high hepatic extraction ratio drug (Hilas and Ezzo 2009), indicating that the changes in nebivolol clearance reflect altered hepatic blood flow (rather than altered intrinsic metabolic rate) in obese subjects (Knibbe et al. 2015). These results

suggest that obesity has minimal effects, if any, on hepatic CYP2D6-mediated drug metabolism in humans, i.e., a result contradicting to our results in HFD-fed Tg-CYP2D6 mice. Interestingly, obesity is known to cause hepatomegaly in humans to a less extent as compared to mice (Lewis et al. 2006, Bian et al. 2014); the liver volume (determined by using magnetic resonance imaging) in obese to morbidly obese subjects is 1.25- to 1.5-fold larger (Lewis et al. 2006, Bian et al. 2014) than that in subjects with normal body weight (Johnson et al. 2005). In Tg-CYP2D6 mice, HFD caused 2.2-fold increases in liver size, accompanied by 1.6-fold increases in protein amounts (in S9 fractions/liver). Considering that the effects of obesity on CYP2D6-mediated hepatic drug metabolism is minimal in humans, it appears plausible that increased liver size in humans is due to accumulating fats in the liver rather than the expansion of parenchymal cells.

Contrary to a previous report that HFD feeding increases SHP protein levels by enhancing SHP protein stability (Miao et al. 2009), protein level of SHP did not increase in HFD-fed mice in this study. Differences in mouse study design may explain the apparent discrepancy. Different mouse strains (i.e., CV57 and C57BL/6 mice in the previous and current study, respectively) were used. Also, while a matching low fat control diet (TD.08485, 13% kJ from fat, Teklad) was used in this study, the diet used for the control group was not specified in the previous study and thus unknown. Different diet and strain have shown to cause differential composition of gut microbiota in mice (Magnusson et al. 2015, Perry et al. 2016, Ussar et al. 2015). Accumulating evidence indicates that gut microbiota modulates multiple aspects of physiology, including the composition of bile acids and subsequent intestinal farnesoid X receptor (FXR) signaling (Wahlstrom et al. 2016). The products of intestinal FXR signaling such as fibroblast growth factor (FGF) 15 can alter SHP protein stability in the liver (Miao et al. 2009).

HFD feeding led to gene-specific changes in mouse endogenous CYP genes. Notably, the expression and activity of Cyp3a11 (the most abundant Cyp3a isoform in mice (Cui, Renaud, and Klaassen 2012)) was enhanced by 3-folds in the liver from HFD-fed mice, consistent with the previously reported increases in Cyp3a11 expression in mouse obesity model (Kudo et al. 2009, Kim et al. 2004). On the other hand, others have reported that HFD feeding decreased hepatic Cyp3a11 expression or activity in mice (Yoshinari, Takagi, Yoshimasa, et al. 2006, Ghose et al. 2011, Wahlang et al. 2014). Differences in diet composition and housing condition may have contributed to the discrepancy. For instance, feeding of mice with HFD containing saturated fat (as in our HFD) induced Cyp3a11 expression while HFD containing unsaturated fat decreased it (Geng et al. 2015). Also, mice housed in conventional condition are reported to exhibit low-grade inflammation in the liver upon HFD feeding although mice in specific pathogen-free environment do not (Muller et al. 2016). Hepatic inflammation is known to cause CYP3A down-regulation in humans and mice (Morgan et al. 2008). Indeed, increased hepatic expression of inflammation markers (e.g., IL1 β , IL6 and TNF α) in HFD-fed mice was accompanied by decreased Cyp3a11 expression (Yoshinari, Takagi, Yoshimasa, et al. 2006, Ghose et al. 2011, Wahlang et al. 2014). Of note, hepatic expression of the inflammation marker genes did not differ between HFD and control groups in our study (data not shown) where mice were housed in specific pathogen-free environment.

The underlying mechanisms for increased Cyp3a11 expression in HFD-fed mice shown in our study and others are unclear. PXR and CAR are ligand-activated nuclear receptors and key transcriptional regulators of Cyp3a11 expression (Li et al. 2016). mRNA levels of Pxr and Car were not higher in HFD-fed mice, suggesting that altered expression of these nuclear receptors is not responsible for Cyp3a11 induction in the mice. The possibility remains that HFD feeding

modulates (e.g., increases) the hepatic levels of their endogenous ligands (e.g., bilirubin, bile acids, and steroid hormones) (Bjorkholm et al. 2009). Indeed, HFD feeding was shown to cause adrenal cortical hyperplasia in mice, leading to higher plasma levels of corticosteroids (i.e., PXR activators) (Swierczynska et al. 2015). How obesity alters the hepatic concentrations of different bile acids or bilirubin remains unknown.

Clinical evidence is conflicting on whether and how obesity affects CYP3A4-mediated drug metabolism in humans. A population PK analysis of midazolam [a CYP3A4 substrate with hepatic extraction ratio 0.3-0.5 (Trouvin et al. 1988)] in morbidly obese patients showed no change in systematic clearance but decreased oral clearance of midazolam as compared with those in healthy volunteers (Brill et al. 2014). On the other hand, the systemic clearance of triazolam (a CYP3A4 substrate of intermediate hepatic extraction ratio) after intravenous dose did not differ between obese subjects and those with normal weight (Derry et al. 1995). In human liver tissues, CYP3A4 protein levels in liver biopsies collected from obese patients (during laparoscopic gastric bypass or biliopancreatic diversion surgery) were negatively correlated with patients BMI (Ulvestad et al. 2013), indicating repressive effects of obesity on CYP3A4 expression. Considering that morbid obesity is often accompanied by co-morbidity (e.g., diabetes) that incurs chronic hepatic inflammation, decreased CYP3A4 expression in the liver tissues may be in part due to underlying inflammation in obese subjects. Definitive evidence on how increased body weight impacts hepatic CYP3A4 expression in the absence of inflammation in humans is currently lacking.

To summarize, results from our diet-induced obesity model in Tg-CYP2D6 mice suggest that obesity increases overall CYP2D6-mediated drug metabolism largely via causing hepatomegaly. HFD feeding in the mice also led to pathway-specific changes in hepatic drug

metabolism. Together, results from this study provide insight into how obesity may impact hepatic drug disposition.

CHAPTER 4. CYTOCHROME P450 2D6 INDUCTION AND UNDERLYING MECHANISM DURING MOUSE PREGNANCY

This chapter was previously published as Ning M, Koh KH, Pan X, Jeong H. (2015) Hepatocyte nuclear factor (HNF) 4 α transactivation of cytochrome P450 (Cyp) 2d6 promoter is enhanced during pregnancy in mice. *Biochem Pharmacol.* 94(1), 46-52.

4.1. Introduction

Cytochrome P450 2D6 (CYP2D6) is a major drug-metabolizing enzyme and expressed in the liver, intestine, kidney, and brain (Miksys et al. 2005, Siegle et al. 2001). CYP2D6 mediates hepatic metabolism of approximately 25% of marketed drugs including antidepressants and antipsychotics (Zhou 2009). The roles of CYP2D6 in altered biological functions in extrahepatic tissues (such as brain) remains unclear, in part due to a lack of animal models to study CYP2D6 function *in vivo*. Tg-CYP2D6 mice that carry human CYP2D6 gene in the mouse genome exhibit robust CYP2D6 expression in liver and intestine. However, CYP2D6 expression is absent in the brain of the transgenic mice (Miksys et al. 2005). Identification and characterization of mouse Cyp2d homologs whose expression or activities are co-regulated as CYP2D6, if any, may potentially enable establishing study models to explore the biological functions of CYP2D6 in *in vivo* systems.

CYP2D6-mediated drug metabolism is increased during human pregnancy (Hogstedt et al. 1985, Wadelius et al. 1997, Tracy et al. 2005). We have recently shown that CYP2D6 expression is enhanced by 4-fold at term pregnancy (as compared to the pre-pregnancy or postpartum period) in Tg-CYP2D6 mice, and this was accompanied by increased transactivation of CYP2D6 promoter by HNF4 α (Koh, Pan, Shen, et al. 2014). Our studies also demonstrated that the increased HNF4 α transactivation of CYP2D6 promoter is in part attributed to two transcription

factors, namely SHP (NR0B2) and Krüppel-like Factor 9 (KLF9), whose hepatic expression is differentially regulated during pregnancy (Koh, Pan, Shen, et al. 2014, Koh, Pan, Zhang, et al. 2014). Specifically, hepatic SHP expression decreases, and this leads to de-repression of CYP2D6 promoter during pregnancy. On the other hand, KLF9 transactivates CYP2D6 promoter in synergy with HNF4 α . During pregnancy, hepatic KLF9 expression increases, further potentiating HNF4 α transactivation of CYP2D6 promoter (Koh, Pan, Zhang, et al. 2014). Importantly, these results provide a potential platform to identify and characterize mouse Cyp2d homologs that are regulated similarly as CYP2D6.

Humans express only one functional CYP2D (i.e., CYP2D6), but mouse genome harbors the following nine Cyp2d homologs: *Cyp2d9*, *2d10*, *2d11*, *2d12*, *2d13*, *2d22*, *2d26*, *2d34*, and *2d40*. How the expression of mouse Cyp2d genes is regulated remains largely unknown. Also unknown is whether the regulatory mechanisms leading to CYP2D6 induction during pregnancy is conserved for endogenous mouse Cyp2d genes. In this study, we identified Cyp2d40 as an endogenous mouse Cyp2d gene whose expression is induced during pregnancy. We further evaluated the role of HNF4 α and its modulators (i.e., SHP and KLF9) in the regulation of Cyp2d40 expression during pregnancy, to explore conserved physiological/functional significance of CYP2D induction during pregnancy.

4.2. Materials and Methods

4.2.1. Animals.

Tg-CYP2D6 and Hnf4 α /AlbCre transgenic mice were previously described (Koh, Pan, Shen, et al. 2014). Adult female (8 weeks old) mice were mated with male mice of similar age. Mating between adult mice was confirmed by the presence of vaginal plugs (day 0). Male mice were separated from female mice immediately after a vaginal plug was found. Virgin mice were group-housed so that their estrous cycles were suppressed. Male C57BL/6J mice were injected with GW4064 (Sigma-Aldrich, St. Louis, MO) at 15 mg/kg/day or vehicle (olive oil) for 5 days. Livers were collected 6 hours after the last injection for examination of gene expression. All procedures were approved by the Institutional Animal Care and Use Committee in the University of Illinois at Chicago.

4.2.2. Plasmids.

The upstream region of Cyp2d40 (GenBank accession no. NC_000081.6) was PCR-amplified using genomic DNA of C57BL/6J mouse and the primer set listed in Table XVI. The PCR product was digested by *Kpn*I and *Sac*I restriction enzymes (New England Biolabs, Ipswich, MA) and cloned into promoterless pGL3-basic vector (Promega, Madison, WI) digested by the same enzymes, yielding pGL3-Cyp2d40 (-2000/+6). For cloning of 5'-deletion constructs, different regions of Cyp2d40 promoter were PCR-amplified using pGL3-Cyp2d40 (-2000/+6) as template and respective forward primers listed in Table XVI. The PCR product was digested by *Kpn*I and *Sac*I restriction enzymes and cloned into pGL3-basic vector digested by the same enzymes. To construct mutant vectors of Cyp2d40 promoter at HNF4 α binding site, pGL3-CYP2d40 (-171/+6) was PCR-amplified by using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) and 5'-phosphorylated primers harboring mutation or deletion of

the HNF4 α binding site listed in Table XVI. The PCR products were then ligated by using T4 ligase (New England Biolabs, Ipswich, MA).

Table XVI Primers used in the Chapter 4

Cloning primers		
pGL3-Cyp2d40 (-2000/+6)	Forward	5' – ATCGGGTACCCACAGGACTGCAAAGCACTC –3'
	Reverse	5' – ATCGGAGCTCCCACTGCTTCCCAGGCTTCA –3'
pGL3-Cyp2d40 (-1451/+6)	Forward	5' – ATCGGGTACCAGCATCCTGAGACTGTCA –3'
pGL3-Cyp2d40 (-981/+6)	Forward	5' – ATCGGGTACCGGATCCAGCAAGGACATG –3'
pGL3-Cyp2d40 (-471/+6)	Forward	5' – ATCGGGTACCCTCTTCCAATGCCAAGAG –3'
pGL3-Cyp2d40 (-171/+6)	Forward	5' – ATCGGGTACCCTGGCCTGTCTCTACACT –3'
pGL3-Cyp2d40 (-86/+6)	Forward	5' – ATCGGGTACCAAGGTGGTAGGATCCAAG –3'
pGL3-Cyp2d40 (-171/+6) HNF4 α site mutation	Forward	[Phos] 5' – AATTCACACCTGGACACTCCTTTATAAGG –3'
	Reverse	[Phos] 5' – GTCAATCTGCCCCCAACCCCAATCCT –3'
pGL3-Cyp2d40 (-171/+6) HNF4 α site deletion	Forward	[Phos] 5' – ACCTGGACACTCCTTTATAAGG –3'
	Reverse	[Phos] 5' – CTGCCCCCAACCCCAATCCT –3'
qRT-PCR primers		
Shp	Forward	5' – GGCCTCTACCCTCAAGAACAT –3'
	Reverse	5' – TGTCAACGTCTCCCATGATAGG –3'
Gapdh	Forward	5' – AGGTCGGTGTGAACGGATTTG –3'
	Reverse	5' – TGTAGACCATGTAGTTGAGGTCA –3'
ChIP PCR primers		
Cyp2d40	Forward	5' – TCTCTGGCCTGTCTCTACAC –3'
	Reverse	5' – GCTTGGATCCTACCACCTTA –3'

4.2.3. Cell culture and luciferase reporter assay.

HEK293T cells were cultured in RPMI-1640 media supplied with 10 mM Hepes buffer, 100 μ M non-essential amino acids, 5,000 U penicillin/streptomycin (Life Technologies, Carlsbad, CA) and 10% fetal bovine serum (Gemini Bio-products, West Sacramento, CA). For assays, HEK293T cells were seeded in 24-well plates at a density of 1×10^5 cells per well. On the next

day, the cells were transfected with 0.3 µg of luciferase construct, 0.1 µg of expression plasmid (or empty vector as a control), and 0.002 µg of *Renilla* expression vector (Promega, Madison, WI) per well, using Fugene HD transfection reagent (Promega, Madison, WI) according to the manufacturer's protocol. After 48 hours, the transfected cells were harvested for determination of luciferase activities using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI).

4.2.4. RNA isolation and quantitative real time-PCR (qRT-PCR).

Total RNAs were isolated from mouse liver tissues using Trizol (Life Technologies, Carlsbad, CA) and used as template for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA). Using the cDNA as template, qRT-PCR was performed using StepOnePlus™ Real-Time PCR System and the following TaqMan® Gene expression assays (Integrated DNA technologies, Coralville, IA) for Cyp2ds and mouse Actb. Shp and Gapdh mRNA levels were determined using SYBR® Green Real Time PCR Master Mix (Life technologies, Foster City, CA) and primer sets listed in Table XVI. The relative expression in mRNA levels was determined after normalizing the gene expression levels by those of mouse Gapdh or Actb ($2^{-\Delta\Delta C_t}$ method).

4.2.5. Chromatin Immunoprecipitation (ChIP) assay.

ChIP assays with mouse liver were performed as previously described (Koh, Pan, Shen, et al. 2014). Briefly, livers were finely minced and incubated in PBS containing 1% formaldehyde at room temperature for 15 min, and glycine was added to stop the crosslinking reaction. Cell pellets were resuspended in hypotonic buffer (15 mM HEPES, 60 mM KCl, 2 mM EDTA, 0.5% BSA, 0.15 mM spermine, 0.5 mM spermidine, 0.32 M sucrose, pH 7.9) and lysed by homogenization. Nuclei were pelleted and resuspended in nuclei lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 1% SDS, pH 8.0). The samples were sonicated to shear DNA at the length

from 100 to 500 bp. After centrifuge, the chromatin samples were immunoprecipitated using magnetic beads coated with 2 µg antibody (HNF4α, sc-6556x, Santa Cruz Biotechnology, Dallas, TX) or immunoglobulin G (IgG, sc-2028, Santa Cruz, Dallas, TX) at 4°C overnight. The immune complex on the magnetic beads was collected and extensively washed, and the bound chromatin was eluted. Genomic DNA was purified by phenol chloroform extraction followed by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). qRT-PCR was performed using the genomic DNA and the primer set listed in Table XVI.

4.2.6. Electrophoretic mobility shift assay (EMSA).

EMSA was performed as previously described (Koh et al. 2012). Briefly, recombinant HNF4α protein (with C-terminal MYC/DDK tag; 0.2 µg; TP317863, OriGene Technologies. Rockville, MD) or nuclear protein extracts (5 µg) from HEK293T cells transfected with HNF4α expression vector (or empty vector) were pre-incubated with binding buffer (4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5) at room temperature in the presence or absence of unlabeled DNA probes or HNF4α antibody (2 µg, sc-6556x, Santa Cruz). After 10 min, the binding reaction was initiated by adding 0.035 pmol of 5'-end ³²P-labeled probes harboring Cyp2d40 putative HNF4α-binding sequence or CYP2D6 HNF4α-binding sequence. The reaction mixture was incubated at room temperature for 20 min. Protein-bound probes were separated from free probes on 4% (w/v) non-denaturing polyacrylamide gel. The gel was dried, and radioactivity visualized using PhosphorImager (GE Healthcare Bio-Sciences, Pittsburgh, PA).

4.2.7. Statistical analysis.

All data were presented as means \pm SD. Statistical analyses were performed using one-way ANOVA followed by Dunnett's post-hoc test. A value of $p < 0.05$ was considered statistically significant.

4.3. Results

4.3.1. Cyp2d40 expression is enhanced during pregnancy.

To identify the mouse Cyp2d genes that exhibit similar upregulation as CYP2D6 does during pregnancy, we examined the mRNA expression levels of the following Cyp2d homologs in livers of Tg-CYP2D6 or wild type mice at different gestational time points: *Cyp2d9*, *2d10*, *2d12*, *2d13*, *2d22*, *2d26*, *2d34*, and *2d40*. Cyp2d11 was not examined in this study because its expression is male-specific (Wong et al. 1989). Cyp2d12 and Cyp2d34 expression was not detectable in the mouse liver tissues (data not shown). In the livers of non-pregnant mice, Cyp2d26 expression was most abundant, followed by Cyp2d10, Cyp2d22, Cyp2d13, Cyp2d9 and Cyp2d40 (Figure 15A). Among the six Cyp2d isoforms, Cyp2d40 expression increased significantly at term as compared to pre-pregnancy level in both Tg-CYP2D6 and wild-type mice (by 6- and 4-fold, respectively) (Figure 15B and 15C), suggesting that the presence of transgene (i.e., CYP2D6) does not impact Cyp2d40 regulation during pregnancy. On the other hand, mRNA expression of the remaining five Cyp2d isoforms slightly decreased during pregnancy (data not shown).

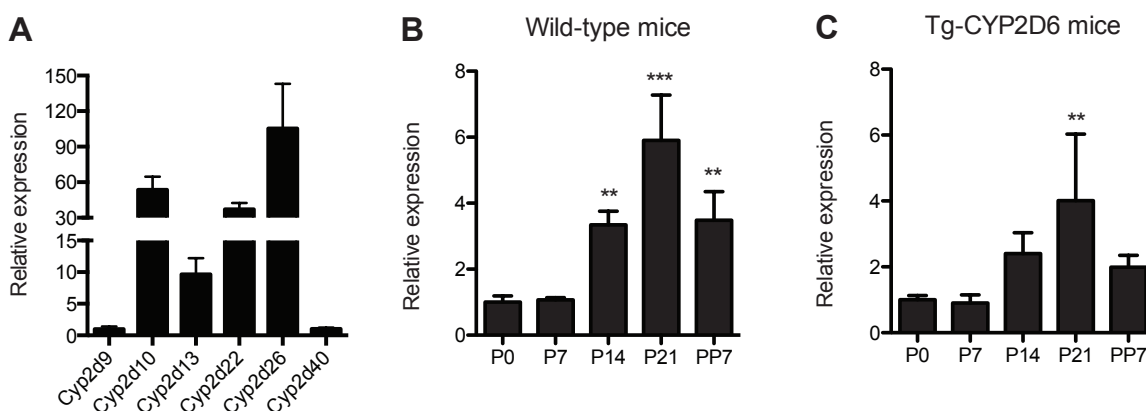


Figure 15. Hepatic Cyp2d40 is induced in both wild-type and Tg-CYP2D6 mice during pregnancy.

A, Liver tissues of wild-type nonpregnant female mice were collected (n=4). mRNA expression levels of Cyp2ds were determined by qRT-PCR and normalized by Cyp2d40 expression. B and C, Liver tissues of wild-type (B) and Tg-CYP2D6 (C) mice were collected at different gestational time points: pre-pregnancy (P0), 7, 14, or 21 days of pregnancy (P7, P14, P21, respectively), 7 days postpartum (PP7). mRNA levels of Cyp2d40 were determined by qRT-PCR and normalized by that in the pre-pregnancy group (n=4, mean \pm S.D.; **, $p < 0.01$, ***, $p < 0.001$ versus P0 group).

4.3.2. HNF4 α is critical for Cyp2d40 induction during pregnancy.

We have previously shown that CYP2D6 induction during pregnancy is attributable to enhanced transactivation of CYP2D6 promoter by HNF4 α (Koh, Pan, Shen, et al. 2014). To define the role of HNF4 α in Cyp2d40 induction during pregnancy, we examined whether reduced hepatic HNF4 α expression alters the extent of Cyp2d40 up-regulation seen at term pregnancy. To this end, the pregnancy-mediated changes in Cyp2d40 expression were compared between mice carrying two vs. one copy of *Hnf4 α* allele in the liver (noted as *Hnf4 α (wt/wt)* and *Hnf4 α (-/wt)* mice, respectively). Hepatic HNF4 α protein expression level in *Hnf4 α (-/wt)* mice was previously shown to be about half of that in *Hnf4 α (wt/wt)* mice (Koh, Pan, Shen, et al. 2014). In *Hnf4 α (-/wt)* mice, the basal mRNA expression of Cyp2d40 decreased dramatically as compared to that in *Hnf4 α (wt/wt)* mice (Figure 16), indicating that HNF4 α is a major regulator of basal Cyp2d40 expression. More importantly, Cyp2d40 induction during pregnancy was abrogated in *Hnf4 α (-/wt)* mice, suggesting a key role of HNF4 α in Cyp2d40 induction during pregnancy.

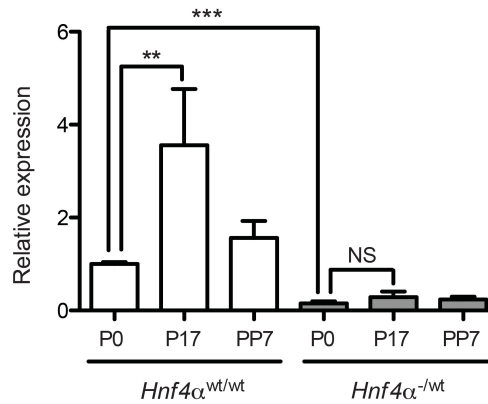


Figure 16. HNF4 α is critical for Cyp2d40 basal expression and induction during pregnancy. Liver tissues were collected from *Hnf4α*(wt/wt) and *Hnf4α*(-/-) mice at pre-pregnancy (P0), 17 days of pregnancy (P17), and 7 days post-partum (PP7). mRNA levels of Cyp2d40 were determined by qRT-PCR and normalized by that of *Hnf4α*(wt/wt) mice at P0 (n=4, mean \pm S.D.; **, p<0.01; ***, p<0.001; NS, not significant).

4.3.3. HNF4 α transactivates Cyp2d40 promoter.

To further characterize the role of HNF4 α in the regulation of Cyp2d40 expression, transient transfection and luciferase reporter assays were conducted. Transfection of HNF4 α in HEK293T cells led to a dramatic increase in *Cyp2d40* promoter activity (Figure 17A), indicating that HNF4 α transactivates *Cyp2d40* promoter. To map the *cis*-element(s) responsible for HNF4 α transactivation, a series of luciferase vectors harboring 5'-truncated upstream regulatory region of *Cyp2d40* were constructed, and luciferase assays were performed. Ectopic HNF4 α expression led to transactivation of *Cyp2d40* promoter as short as -171 to +6 (Figure 3B). However, the transactivation by HNF4 α was completely abrogated when the region between -171 and -86 was deleted (Figure 17B), indicating the presence of key *cis*-element(s) for HNF4 α action within the region. *In silico* promoter analysis revealed a putative HNF4 α binding site in this region, which is conserved between CYP2D6 and Cyp2d40 (Figure 17C). To further validate the putative HNF4 α binding site in *Cyp2d40*, mutation and deletion assays were performed. HNF4 α

transactivation of Cyp2d40 promoter was completely abolished when the putative binding site of HNF4 α was deleted or mutated (Figure 17D).

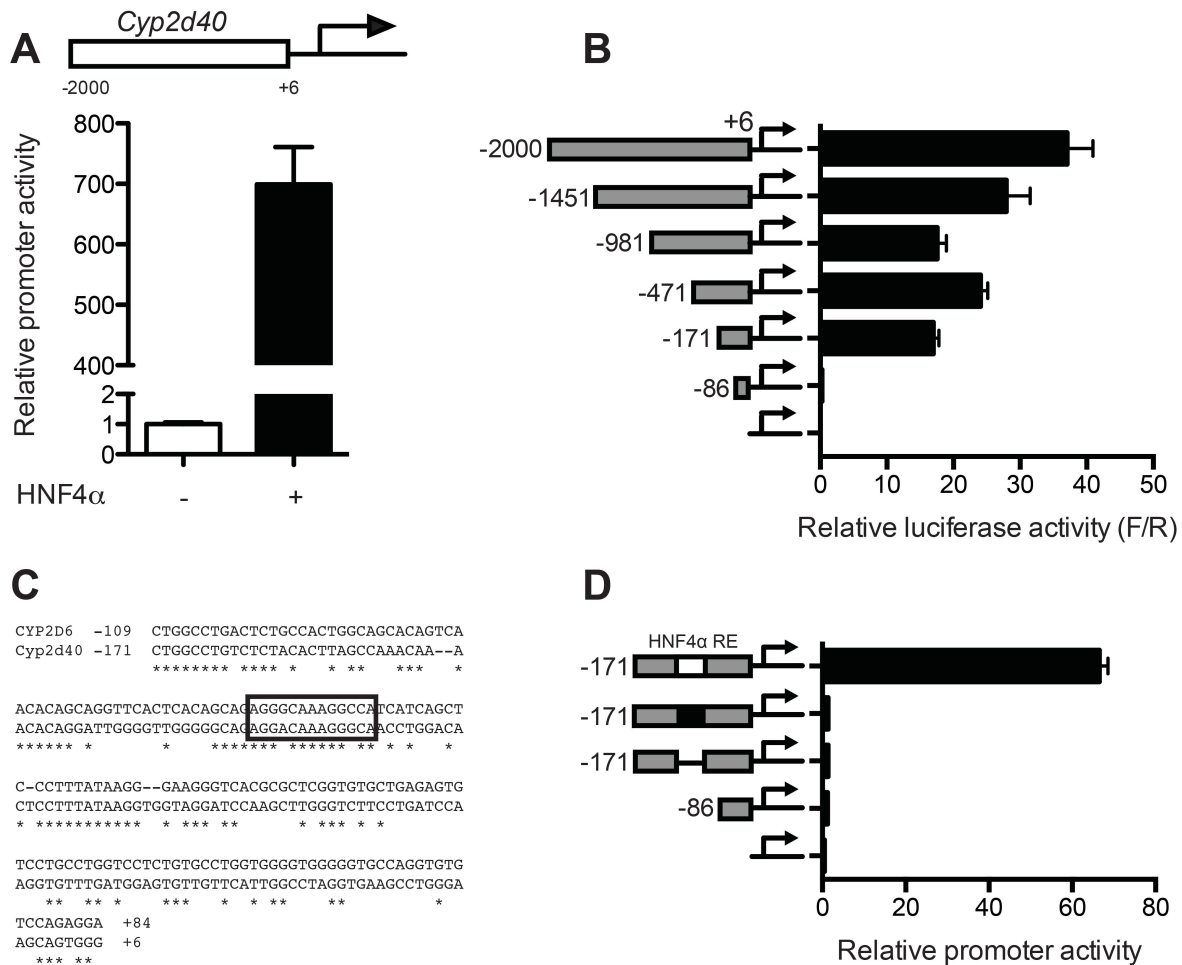


Figure 17. HNF4 α transactivates Cyp2d40 promoter where a putative HNF4 binding site is critical for the activation.

(A) HEK293T cells were co-transfected with pGL3-Cyp2d40 with HNF4 α expression plasmid (or empty vector as a control), and dual luciferase assays were performed (n=3, mean \pm S.D.). (B) HEK293T cells were co-transfected with HNF4 α expression plasmid and one of 5'-truncated Cyp2d40 promoter luciferase vectors, and dual luciferase assays were performed (n=3, mean \pm S.D.). (C) A putative HNF4 binding site in Cyp2d40 upstream regulatory region (marked using a box) is aligned with the known HNF4 α binding site in CYP2D6. (D) HEK293T cells were co-transfected with HNF4 α expression plasmid, along with a luciferase construct where HNF4 α response element (RE) of Cyp2d40 (marked using a white box) was mutated (marked using a black box) or deleted, and dual luciferase assays were performed (n=3, mean \pm S.D.).

To verify direct interaction between HNF4 α and the putative response element identified above, EMSA was performed. Recombinant HNF4 α protein or nuclear extracts from HEK293T cells transfected with HNF4 α expression vector (or empty vector as a control) were incubated with radiolabeled DNA probes harboring HNF4 α response element of CYP2D6 (as positive control) or that of Cyp2d40, and the reaction mixtures were resolved on non-denaturing gels. A shifted band was observed when recombinant protein was incubated with either probe (Figure 18, lane 2 and 10 for CYP2D6 and Cyp2d40 probe, respectively). Of note, a weaker signal was observed for Cyp2d40 than CYP2D6 (Figure 18, lane 2 vs. 10), suggesting differences in binding affinity of HNF4 α to the DNAs. Shifted bands at the similar location were obtained when nuclear extracts from HEK293T cells were used (Figure 18, lane 7 and 15 for CYP2D6 and Cyp2d40 probe, respectively). The signal intensity of shifted bands for CYP2D6 and Cyp2d40 probes significantly decreased when unlabeled CYP2D6 or Cyp2d40 probe was added to the reaction mixture as a competitor (Figure 18, lane 3, 4, 11, and 12). On the other hand, the probe harboring mutated sequences of HNF4 α binding site failed to compete for the binding (Figure 18, lane 5 and 13 for CYP2D6 and Cyp2d40, respectively). Addition of HNF4 α antibody to the reaction mixture led to a super-shift of the band (Figure 18, lane 6, 14 and 16). These EMSA results indicate that HNF4 α directly binds to the putative binding site of Cyp2d40.

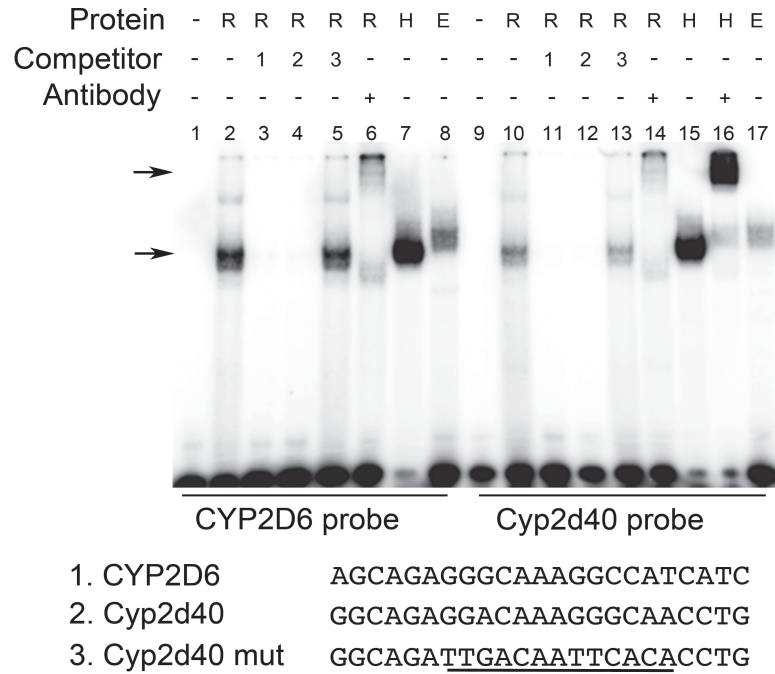


Figure 18. HNF4 α physically interacts with the putative HNF4 binding site in Cyp2d40 promoter.

Recombinant HNF4 α protein (R, 0.2 μ g) or nuclear proteins (5 μ g) prepared from HEK293T cells transfected with HNF4 α expression vector (H) or empty control vector (E) were incubated with 32P-labeled DNA probes harboring putative HNF4 α cis-element of Cyp2d40 or the known HNF4 α binding sequence of CYP2D6, in the presence or absence of HNF4 α antibody or indicated unlabeled DNA probes as competitors (in 50-fold excess). The mixture was resolved on non-denaturing gel. The lower arrow indicates the location of shifted bands by apparent HNF4 α binding to DNA. The upper arrow indicates the super-shift complex. The probe sequences are shown below the gel image, with the mutated sequence underlined.

4.3.4. HNF4 α recruitment to Cyp2d40 promoter increases at term pregnancy.

To examine the pregnancy-mediated changes in HNF4 α transactivation of Cyp2d40 promoter *in vivo*, ChIP assays were performed using mouse liver tissues collected at different gestational time points. The amount of HNF4 α -bound DNA was measured by qRT-PCR using a primer set that can detect the HNF4 α response element of Cyp2d40. The results showed that HNF4 α recruitment to Cyp2d40 was increased by 2.3-fold at term pregnancy as compared to pre-

pregnancy (Figure 19), suggesting that enhanced transactivation of Cyp2d40 promoter is in part responsible for Cyp2d40 induction during pregnancy.

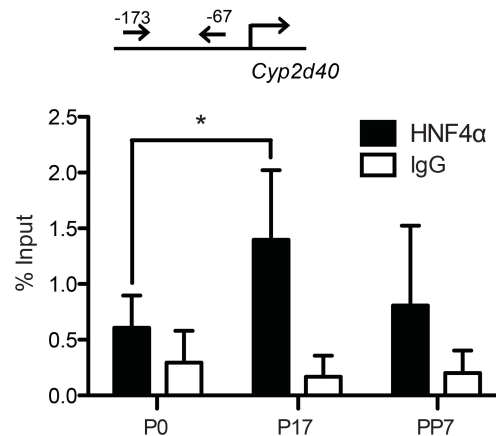


Figure 19. HNF4 α recruitment to Cyp2d40 promoter increases at term pregnancy.

Liver tissues were collected from Tg-CYP2D6 mice at pre-pregnancy (P0), 17 days of pregnancy (P17), and 7 days post-partum (PP7). ChIP assays were performed using HNF4 α antibody (or IgG as a control), and the pulled-down DNA was quantified by qRT-PCR using a set of primers that bind -171/-67 of Cyp2d40 (n=7, mean \pm S.D.; *, p<0.05).

4.3.5. HNF4 α activity on CYP2D6 and Cyp2d40 promoter is differentially regulated during pregnancy.

Previously, we have shown that SHP represses and KLF9 potentiates HNF4 α transactivation of CYP2D6 promoter (Koh, Pan, Shen, et al. 2014, Koh, Pan, Zhang, et al. 2014). To examine whether SHP and KLF9 modulate HNF4 α transactivation of Cyp2d40 promoter (similarly to CYP2D6 promoter), we performed promoter reporter assays in HEK293T cells. HEK293T cells were co-transfected with pGL3-Cyp2d40 along with HNF4 α and/or SHP expression vectors, and luciferase activity was measured. The results showed that SHP

overexpression led to repressed HNF4 α transactivation of Cyp2d40 promoter in HEK293T cells (Figure 20A, lane 3 vs. 4). Considering that findings in HEK293T cells could result from super-physiological levels of SHP upon transient transfection, the role of SHP in Cyp2d40 regulation was further studied *in vivo*. To this end, mice were treated with GW4064, an inducer of SHP expression (Goodwin et al. 2000), and mRNA expression levels of Cyp2d40 were measured. Cyp8b1 expression is known to be repressed when SHP is upregulated (Zhang and Chiang 2001), and thus Cyp8b1 was included as a positive control. Unexpectedly, Cyp2d40 mRNA was not affected when SHP expression was induced in mice, whereas the expression of Cyp8b1 was significantly repressed in mice (Figure 20B), suggesting that SHP does not modulate Cyp2d40 expression in *in vivo* systems. Results from promoter reporter assays for KLF9 showed that KLF9 did not affect HNF4 α transactivation of Cyp2d40 promoter (Figure 20C, lane 3 vs. 4), unlike the previously-shown potentiation of HNF4 α transactivation of CYP2D6 promoter by KLF9 (Koh, Pan, Zhang, et al. 2014). Together, these indicated that neither SHP nor KLF9 modulates Cyp2d40 expression, and the mechanism(s) underlying increased HNF4 α transactivation of Cyp2d40 promoter likely differ from that of CYP2D6.

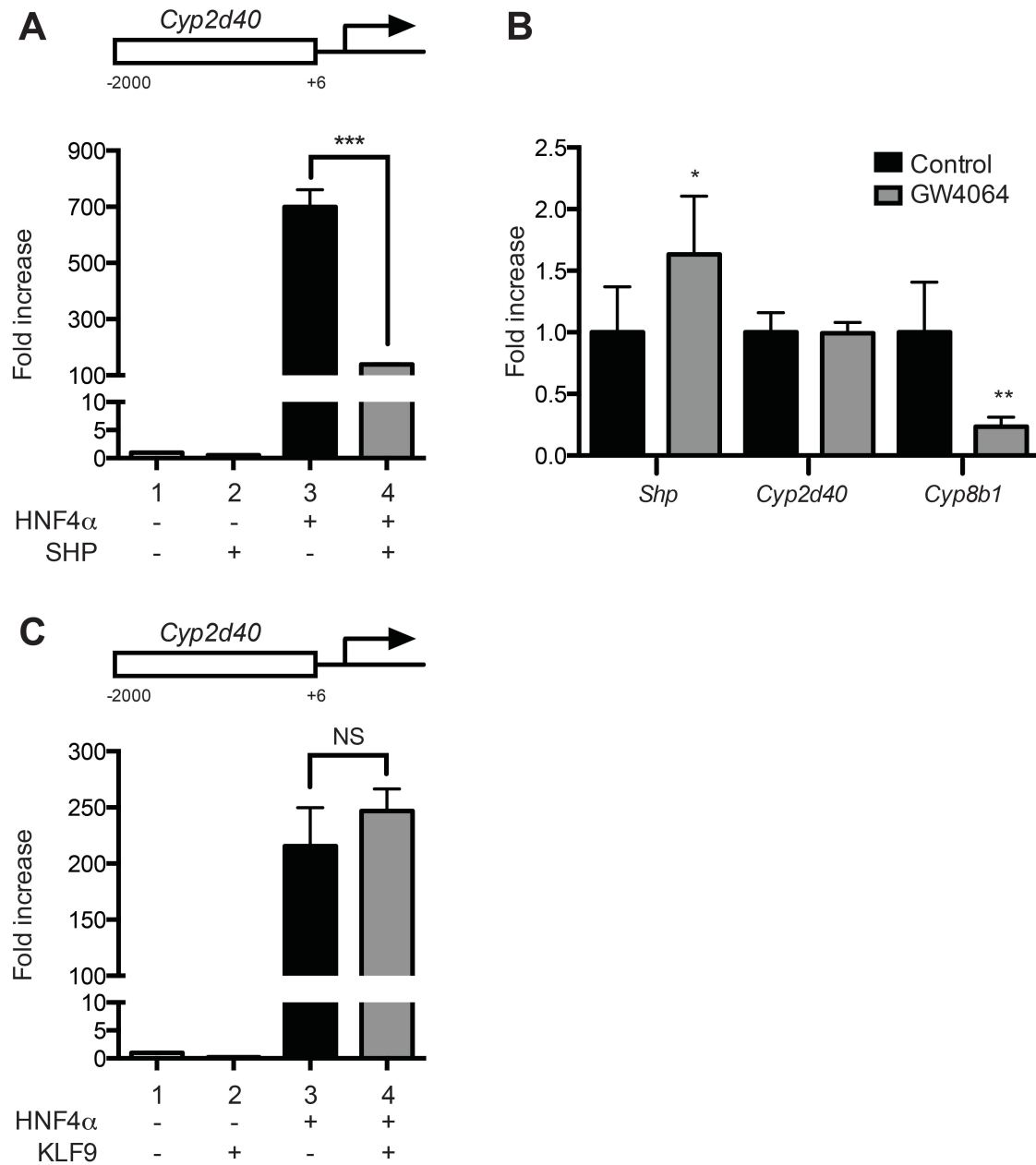


Figure 20. SHP and KLF9 do not modulate HNF4α transactivation of Cyp2d40.

A and C, HEK293T cells were transfected with pGL3-Cyp2d40 with HNF4α and/or SHP expression vectors (A) or KLF9 expression plasmid (C), and dual luciferase assays were performed (n=3, mean ± S.D.). ***, p<0.001; NS, not significant. B, C57BL/6J mice were injected with 15mg/kg GW4064 or olive oil as control for 5 days, and liver mRNA expression levels 6 hours after last injection were determined by qRT-PCR (n=3, mean ± S.D.; *, p<0.05, **, p<0.01 versus siRNA-Control).

4.4. Discussion

Our previous study in Tg-CYP2D6 mice showed that CYP2D6 induction during pregnancy is potentially triggered by enhanced HNF4 α transactivation of CYP2D6 promoter, attributable to decreased expression of transcriptional repressor (SHP) and increased expression of transcriptional activator (KLF9) during pregnancy (Koh, Pan, Shen, et al. 2014, Koh, Pan, Zhang, et al. 2014). We hereby examined whether this regulatory mechanism is conserved for mouse endogenous Cyp2d genes.

Among the Cyp2d homologs, we found that Cyp2d40 expression is significantly up-regulated during pregnancy. This result is in part consistent with the previous report where expression of most Cyp2d genes examined in the study (i.e., Cyp2d11, 2d22, 2d26, and 2d40) was shown increased during pregnancy (Topletz et al. 2013). Interestingly, our results showed that expression of most Cyp2ds (except Cyp2d40) decreased during pregnancy. The discrepancy is potentially due to differences in mouse strains used in the studies (FVB vs. C57BL/6). While the directional changes in the expression of other mouse Cyp2ds remain to be verified, both our and the previous studies clearly demonstrated that mouse Cyp2d40 expression is increased during pregnancy similarly to human CYP2D6.

The finding of enhanced CYP2D6 and Cyp2d40 expression during pregnancy suggests that the physiological roles of these two enzymes may be conserved in mice and humans. CYP2D6 in the liver metabolizes and eliminates a variety of xenobiotics, and its enhanced activity during pregnancy may be an evolutionary consequence of providing protection against potentially harmful xenobiotics in developing fetus. On the other hand, CYP2D6 expressed in the brain has been implicated in the synthesis of neurotransmitters (Yu et al. 2003, Hiroi, Imaoka, and Funae 1998) although the roles of CYP2D6 in brain physiology remains to be verified.

Whether pregnancy alters CYP2D6 expression in brain is currently unknown. CYP2D40 is highly homologous (i.e., 81%) to CYP2D6, and this suggests that CYP2D40 may play similar roles as CYP2D6 by participating in hepatic drug detoxification and modulating brain functions. Indeed, CYP2D proteins have been detected in mouse brain (Miksys et al. 2005), and the contribution of different mouse CYP2D isoforms (especially CYP2D40) to the CYP2D proteins expressed in the brain is yet to be determined.

Our previous study has shown that CYP2D6 induction during pregnancy is due to enhanced transactivation of CYP2D6 promoter by HNF4 α (Koh, Pan, Shen, et al. 2014). Results from this study showed that HNF4 α also plays a key role in enhanced expression of *Cyp2d40* during pregnancy; *Cyp2d40* induction during pregnancy was abrogated in mice with decreased hepatic HNF4 α expression, and HNF4 α recruitment to *Cyp2d40* promoter increased at term pregnancy. We further showed that *Cyp2d40* is a direct target gene of HNF4 α , and mapped a HNF4 α response element to -117/-105 of *Cyp2d40*. Interestingly, the HNF4 α binding site was found conserved in the promoters of the other mouse *Cyp2d* genes (except *Cyp2d22*) (data not shown). This suggests that the presence of HNF4 α binding sequence in *Cyp2d40* promoter alone does not explain the unique feature of *Cyp2d40* regulation during pregnancy. HNF4 α transactivation of its target gene promoters is likely modulated in a *Cyp2d* gene-specific manner during pregnancy, similarly to other HNF4 α target genes whose expression is altered during pregnancy in a pattern different from that for CYP2D6.

Enhanced HNF4 α transactivation of CYP2D6 promoter is triggered in part by decreased SHP and increased KLF9 expression during pregnancy. However, results from this study suggest that neither SHP nor KLF9 plays a key role in modulating HNF4 α transactivation of *Cyp2d40* promoter. This is potentially due to differences between CYP2D6 and *Cyp2d40* in the promoter

sequences, resulting in altered interplay among SHP, KLF9, and HNF4 α . Firstly, our EMSA results indicate that HNF4 α binding to Cyp2d40 promoter may be of lower affinity than that to CYP2D6 promoter. In an *in vivo* system, this may lead to dampened SHP action on HNF4 α transactivation of Cyp2d40 promoter. In accordance with this notion, although SHP significantly repressed Cyp2d40 promoter activity in HNF4 α -overexpressing HEK293T cells, such finding was not recapitulated in mice. Secondly, whereas *CYP2D6* harbors multiple binding sites for KLF9 that enhances HNF4 α transactivation of the promoter (Koh, Pan, Zhang, et al. 2014), *Cyp2d40* lacks such KLF9 binding sites in its promoter (based on *in silico* promoter analysis; data not shown). Our results from promoter reporter assays also correspond to the lack of KLF9 binding to *Cyp2d40*. The detailed molecular mechanisms underlying enhanced HNF4 α transactivation of Cyp2d40 promoter during pregnancy remain unclear. The roles of other known regulators of HNF4 α activity (Rha et al. 2009), such as PGC1 α , in the regulation of HNF4 α activity on Cyp2d40 promoter warrant further investigation.

Taken together, we have shown that mouse Cyp2d40 expression is enhanced in the liver during pregnancy, similarly to CYP2D6. The enhanced expression of CYP2D6 and Cyp2d40 during pregnancy is attributed to an increase in HNF4 α transactivation of both promoters. These results suggest as-yet-unknown, evolutionarily conserved physiological functions of human CYP2D6 and mouse CYP2D40 during pregnancy.

CONCLUSION AND FUTURE DIRECTIONS

In this thesis research, I determined the major sources of inter-individual variability in CYP2D6 activity in 115 healthy human liver tissues. The results indicate that CYP2D6 genotypes only partially explain the variability in CYP2D6 activity and its role in non-PM population was limited. CYP2D6 activity level in the liver is predominantly governed by its protein amount. Also, CYP2D6 mRNA expression at least partially explains the variability in CYP2D6 protein levels suggesting that a meaningful portion of variability in CYP2D6-mediated drug metabolism may be explained by differential regulation of CYP2D6 transcription.

I also studied CYP2D6 or mouse Cyp2d40 regulation under different physiological states (i.e. obesity and pregnancy). High-fat diet feeding, accompanying obesity, has negligible effect on CYP2D6 expression and activity. During pregnancy, Cyp2d40 expression is enhanced in mice through increased HNF4 α activity, similarly to that of CYP2D6.

For future directions, mechanistic studies of CYP2D6 regulation will provide more information regarding differential basal expression of CYP2D6 in different individuals. Also, modeling the dynamics between CYP2D6 mRNA and protein levels may help determine the exact contribution of CYP2D6 transcription to variability in CYP2D6 protein levels.

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APPENDICES

Appendix A Permission letter for manuscript used in Chapter 1



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April 12, 2017

Miaoran Ning

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Dear Miaoran Ning:

This is to grant you permission to include the following article in your dissertation entitled "Molecular basis of inter-individual variability in Cytochrome P450 2D6-mediated drug metabolism" for the University of Illinois at Chicago:

X Pan, M Ning, and H Jeong (2017) Transcriptional Regulation of CYP2D6 Expression, *Drug Metab Dispos*, 45(1):42-48; DOI: <https://doi.org/10.1124/dmd.116.072249>

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