Transcriptional Regulation of Cytochrome P450 2D6 by Small Heterodimer Partner

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THESIS

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CONTRIBUTION OF AUTHORS

Chapter 1 is a literature review that places my dissertation question in the context of the larger field. My research advisor Dr. Hyunyoung Jeong contributed to the writing of the manuscript. Chapter 2 represents a published manuscript (include complete citation) for which I was the co-first author and major driver of the research. Dr. Kwi Hye Koh performed the experiments shown in Figure 4, Figure 5, and Table IX. Samuel L.M. Arnold at Dr. Nina Isoherranen's lab performed the experiment shown in Figure 15. Dr. Kwi Hye Koh and my research advisor Dr. Hyunyoung Jeong contributed to the writing of the manuscript. Chapter 3 represents a published manuscript (include complete citation) for which I was the first author. My research advisor Dr. Hyunyoung Jeong contributed to the writing of the manuscript. Chapter 4 represents a published manuscript (include complete citation) for which I was the first author. My research advisor Dr. Hyunyoung Jeong contributed to the writing of the manuscript. Chapter 5 represents a series of my own unpublished experiments. I anticipate that this line of research will be continued in the laboratory after I leave and this work will ultimately be published with me as the first-author.

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LIST OF ABBREVIATIONS

3'-UTR	3′-untranslated region
13cRA	13-cis retinoic acid
ALP	alkaline phosphatase
ALT	alanine transaminase
AMPK	AMP-activated protein kinase
ApoC2	apolipoprotein C-II
atRA	all-trans retinoic acid
AUC	area under the curve
CA	cholic acid
CAR	constitutive androstane receptor
C/EBPα	CCAAT/enhancer-binding protein α
ChIP	chromatin immunoprecipitation
CL	clearance
CPR	cytochrome P450 reductase
Cyb5	cytochrome b_5
CYP2D6	cytochrome P450 2D6
DAX-1	dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X chromosome, gene 1
diMet-H3K9	dimethylated lysine 9 of histone 3
EE2	17α-ethinylestradiol
EM	extensive metabolizer
ERα	estrogen receptor α
FGF15/19	fibroblast growth factor 15/19
FXR	farnesoid X receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

LIST OF ABBREVIATIONS

GW4064	3-[2-[2-Chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid
HEK293T	human embryonic kidney 293T
HDAC	histone deacetylase
HNF4α	hepatocyte nuclear factor 4 α
HH	primary human hepatocytes
IM	intermediate metabolizer
LC-MS/MS	liquid chromatography-tandem mass spectrometry
miRNA	microRNA
OSTβ	organic solute transporter β
PKA	protein kinase A
PM	poor metabolizer
Pol II	RNA polymerase II
PXR	pregnane X receptor
qRT-PCR	quantitative real time-PCR
SHP	small heterodimer partner
siRNA	small interfering RNA
SMILE	small heterodimer partner-interacting leucine zipper protein
STAT5a	signal transducer and activator of transcription 5a
UM	ultrarapid metabolizer

SUMMARY

Cytochrome P450 2D6 (CYP2D6) is a major drug-metabolizing enzyme, responsible for eliminating ~20% of marketed drugs (1-3); however, how CYP2D6 expression is regulated remains poorly understood. Of note, CYP2D6-mediated drug metabolism is increased during pregnancy (4-8). Importantly for this project, this clinical finding presents a platform for us to identify and characterize the factors involved in the regulation of CYP2D6 expression.

The mechanisms underlying CYP2D6 induction during pregnancy were completely unknown in part due to a lack of study models that could recapitulate the finding. For the first time, we established an animal model for the study, namely CYP2D6-humanized (Tg-*CYP2D6*) mice, by showing that CYP2D6 expression is induced in the mice at term pregnancy. Tg-*CYP2D6* mice carry human CYP2D6 gene and its 2.5-kb upstream regulatory region in the mouse genome (9). Using Tg-*CYP2D6* mice, we identified a novel transcriptional repressor of CYP2D6 expression–small heterodimer partner (SHP). SHP is known to repress the action of other transcription factors including hepatocyte nuclear factor (HNF) 4α. We showed that SHP expression is downregulated in mouse livers at term pregnancy. Furthermore, we demonstrated in cell culture systems that SHP represses HNF4α transactivation of CYP2D6 promoter. CYP2D6 induction during pregnancy in mice is abrogated in the absence of SHP expression, indicating the essential role of SHP in regulation CYP2D6 expression during pregnancy. Retinoic acids were identified as a potential upstream regulator that is responsible for SHP downregulation during pregnancy: (1) administration of all-trans retinoic acid (atRA; a major bioactive vitamin A metabolite) to Tg-*CYP2D6* mice led to enhanced SHP expression and decreased CYP2D6 expression and (2) hepatic levels of

atRA were decreased at term pregnancy as compared to the pre-pregnancy level in Tg-*CYP2D6* mice. These results are summarized in Chapter 2.

The elucidated mechanisms for CYP2D6 induction during pregnancy may provide a basis to better understand CYP2D6-mediated drug metabolism in general populations. Of note, the metabolic activity of CYP2D6 exhibits a significant interindividual variability (up to 1800-fold variation among individuals) (10-13). While genetic polymorphisms of CYP2D6 are known to contribute to the variability in CYP2D6 activity, this is applicable to only ~10% of populations; for the majority of populations, genetic polymorphisms of CYP2D6 alone failed to reliably predict CYP2D6 activity level (3). Previous studies have shown that CYP2D6 mRNA expression is well correlated with CYP2D6 activity (14,15), indicating that differential transcriptional regulation of CYP2D6 may contribute to large interindividual variability in CYP2D6 activity. Based on the results from studying CYP2D6 regulation during pregnancy, we hypothesized that factors modulating SHP expression may alter CYP2D6 expression, and this may in part explain interindividual variability in CYP2D6 activity.

As a proof-of-concept study, we first determined whether a known SHP inducer altered CYP2D6 expression. SHP is a representative target gene of farnesoid X receptor (FXR) (16,17). The effects of a synthetic FXR agonist GW4064 (18) on CYP2D6 expression were examined in Tg-*CYP2D6* mice. Our results showed that GW4064 indeed repressed CYP2D6 expression in Tg-*CYP2D6*, and this repression was abrogated in mice where SHP expression is absent. Similar results (i.e., upregulation of SHP and downregulation of CYP2D6 by GW4064) were obtained in primary human

hepatocytes, indicating that the results from Tg-*CYP2D6* mice are likely translatable to humans. These results are summarized in Chapter 3.

Bile acids are endogenous ligands of FXR (19). To further characterize the effects of FXR activation on CYP2D6 expression, we examined how cholestasis (a condition companied by elevated bile acid level in the liver) altered CYP2D6 expression. Two experimental animal models for cholestasis were used in our study: (1) estrogen-induced cholestasis and (2) cholic acid (CA) feeding. Estrogen-induced cholestasis is a common experimental model to study the pathogenesis of intrahepatic cholestasis (20-23). CA is a major bile acid, the plasma concentration of which increases in human cholestasis (24). CA feeding model is commonly used to mimic the change of bile acid composition under cholestasis (25-28). Estrogen-induced cholestasis was triggered by administration of 17α-ethinylestradiol (EE2) to Tg-*CYP2D6* mice for 5 days. EE2 administration led to increased SHP and decreased CYP2D6 expression in the mice. Results from chromatin immunoprecipitation assays indicate that the upregulation of SHP is in part through transactivation of Shp promoter by estrogen receptor (Chapter 4). On the contrary, CA feeding in Tg-*CYP2D6* mice for 2 weeks led to decreased SHP expression and subsequent increases in CYP2D6 expression. These unexpected results are attributed to post-transcriptional regulation of SHP expression, which has not been reported previously (Chapter 5). Detailed molecular mechanisms underlying SHP regulation in CA-fed mice are currently under investigation.

In conclusion, we revealed SHP as a novel transcriptional regulator of CYP2D6 expression. SHP inducers, such as all-trans retinoic acid, FXR agonist GW4064, and EE2-induced cholestasis, repress CYP2D6 expression and activity. These

results suggest that differential levels of SHP inducers may contribute to interindividual variability in CYP2D6 activity in humans. This study provides a basis to better predict CYP2D6 activity level and thus to enable personalized medicine for CYP2D6 substrates.

1. INTRODUCTION

1.1. Introduction to drug metabolism

1.1.1. Drug metabolism

Drug metabolism is the process of converting drugs to another chemical species that can be readily eliminated into urine or bile. The chemical modification process occurs in two phases (phase I and phase II) and is mediated by multiple drug-metabolizing enzymes (Table I). Phase I reactions involve introduction of a hydrophilic functional group (e.g., hydroxyl group) or cleavage of functional groups through chemical reactions such as dealkylation, hydrolysis, and reduction. Phase II reactions are conjugation of drugs with a bulky functional group such as glucuronic acid, sulfate, acetylate, and glutathione. While these drug-metabolizing enzymes are expressed in many tissues (e.g., liver, kidney, intestine, or brain), liver is the major site of drug metabolism as the largest amounts of the enzymes are expressed.

Hepatic drug metabolism plays a key role in mediating drug elimination, and thus it governs the extent of drug efficacy and/or toxicity. When the pharmacological activity originates mainly from the parent drug (but not from its metabolites), decreased hepatic drug metabolism may lead to accumulation of drug in the body and exaggeration of drug therapeutic effects. This may even lead to undesired drug toxicity as drug exposure increases. On the other hand, if metabolites are more pharmacologically active than the parent drug, opposite clinical outcome may ensue. For example, tamoxifen is an inactive prodrug until it is converted to the active metabolite (e.g., endoxifen). When the activity of drug-metabolizing enzyme responsible for the conversion of tamoxifen to endoxifen is decreased, one may experience decreased drug efficacy.

Table I. Major drug-metabolizing enzymes involved in phase I and phase II reactions.

	Function	Enzymes
Phase I	Hydroxylation	Cytochrome P450
	Dealkylation	Dehydrogenases (alcohol, aldehyde)
	Hydrolysis	Deoxygenase
	Reduction	Epoxide hydrolase
	Epoxidation	Esterase
		Flavin-Containing monooxygenase
		Ketoreductase
		Monoamine oxidase
		Xanthine oxidase
Phase II	Conjugation	Glucuronosyltransferase
		Glutathione S-transferase
		Methyltransferase
		N-acetyltransferase
		Sulfotransferase

Modified from(29).

1.1.2. Cytochrome P450

Cytochrome P450 (CYP) is a class of heme-containing enzymes. In addition to participating in the metabolism of endogenous compounds (e.g., steroid, fatty acid, eicosanoids, and vitamins), CYPs play a key role in detoxification of drugs via oxidative metabolism (29). About 75% of marketed drugs are subject to CYP-mediated metabolism (Fig. 1A). The CYP superfamily is classified into families (numbers) and subfamilies (alphabets) based on the extent of amino acid homology. CYPs that share 40% or more identity at amino acid level fall into the same family, such as CYP1, CYP2, and CYP3. While those sharing more than 55% similarity are defined subfamilies with in the same family. To date, 57 human CYP genes have been identified and divided into 18 families and 43 subfamilies (29-31). Among those, enzymes in CYP1, CYP2, and CYP3 families play predominant roles in drug metabolism. CYP3A4 and CYP3A5 contribute to ~40 % of CYP-mediated drug metabolism, followed by CYP2D6 (20%), CYP2C8/9 (16%) and CYP1A1/2 (10%) (Fig. 1B).

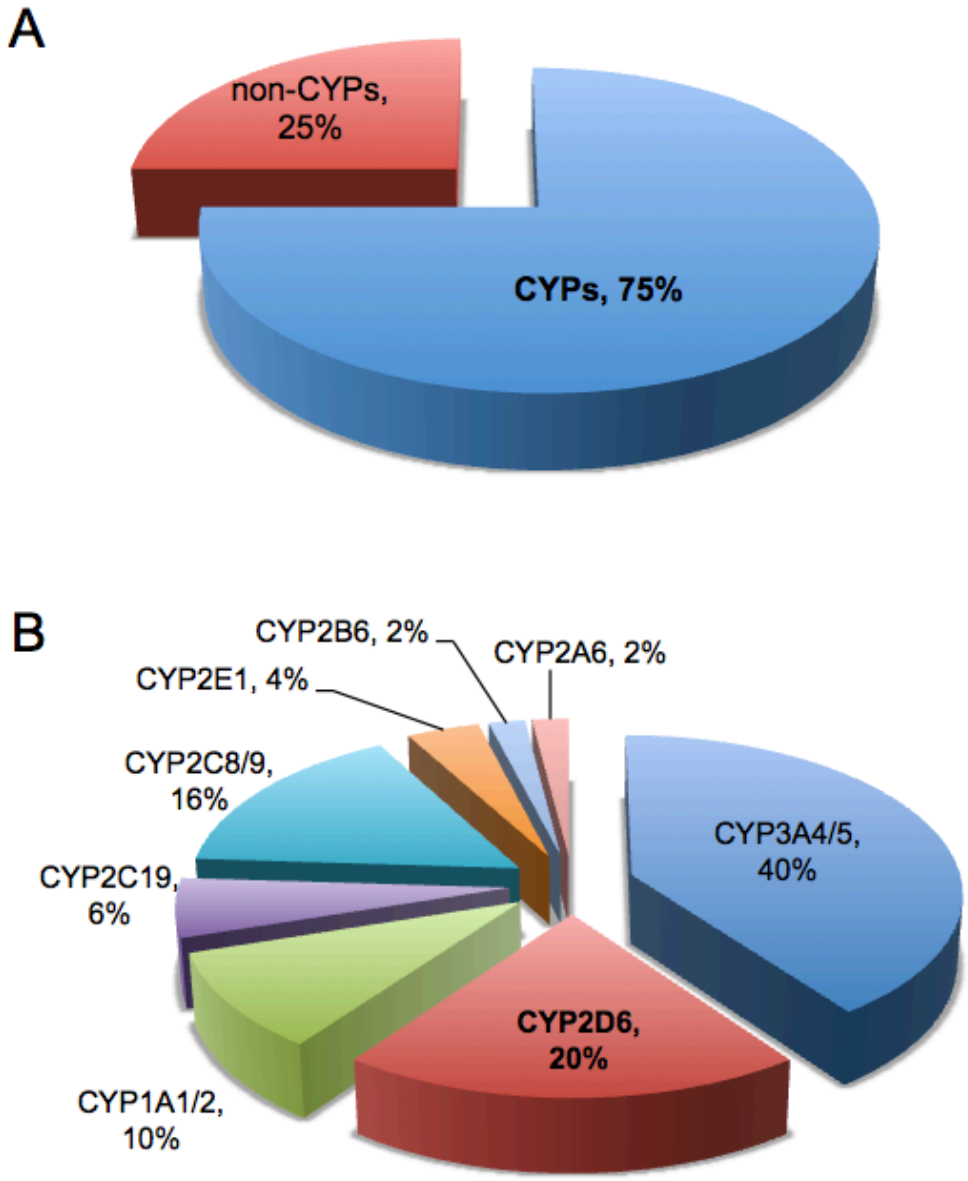

Figure 1. Relative contribution of (A) CYP vs. non-CYP enzymes and (B) different CYP enzymes to overall drug metabolism.
Modified from (3,29,32,33).

All CYPs undergo the same oxidative reaction cycle that requires several key components: heme (in CYP), electron donors (NAPDH and/or NADH), electron transfer proteins, and molecule oxygen (29). Stoichiometric conversion of oxygen to a substrate drug is accompanied by electrons transfer. Electrons from NADPH are transferred to CYP via cytochrome P450 reductase. Besides, cytochrome b_5 accepts electron from NADH through cytochrome b_5 reductase and further transfers it to CYP (Fig. 2).

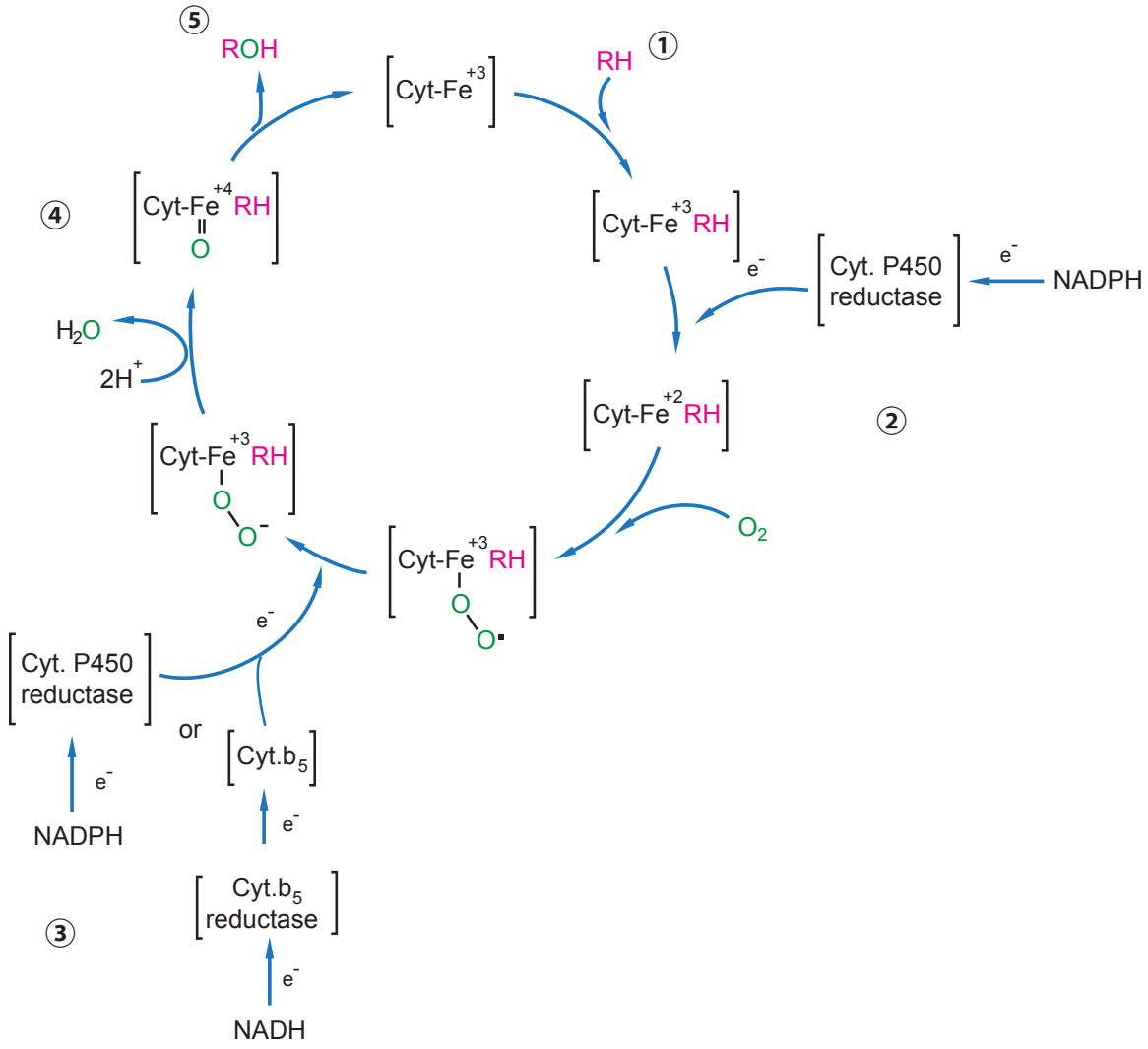

Figure 2. CYP reaction cycle.
Step 1, ferric cytochrome P450 binds to substrate, RH. Step 2, CYP-substrate complex is reduced by cytochrome (Cyt.) P450 reductase to oxyferous P450, followed by binding of oxygen. Step 3, the “second electron” is then transferred to the oxyferrous P450 by either cytochrome P450 reductase or Cyt.b_5, followed by the cleavage of the oxygen bond. This results in the formation of the active oxidizing species (Step 4). Step 5, the more hydrophilic product (ROH) is next dissociated from the active site of CYP enzymes.

Modified from (29).

1.2. CYP2D6-mediated drug metabolism

1.2.1. Introduction

CYP2D6 is a major drug-metabolizing enzyme and responsible for metabolizing approximately 20% of marketed drugs (Table II). Typical CYP2D6 substrates contain a nitrogen atom and a flat hydrophobic region of negative electrostatic potential; CYP2D6-mediated oxidative reaction occurs at 5-10 Å from the nitrogen atom (34-36). CYP2D6 is mainly expressed in the liver, but it is also expressed in extra-hepatic organs including brain, kidney, and intestine (37). While CYP2D6 in liver plays a key role in drug elimination, the roles of CYP2D6 in other tissues remain unclear. Of interest, previous studies have shown that CYP2D6 mediates the conversion of 5-methoxytryptamin to serotonin (38,39). Serotonin is a neurotransmitter that is involved in multiple biological and behavioral functions in central nervous systems, including hormonal secretion, sleep-wake cycle, impulsive aggression, depression, suicidal behavior, and substrate abuse (40,41). CYP2D6 is also responsible for the formation of neurotransmitter dopamine (42) and detoxification of neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT) (43). Of note, the latter is implicated in the development of Parkinson's disease (44,45). Indeed, studies have shown that decreased CYP2D6 activity level is associated with a significantly increased susceptibility to Parkinson's disease in Caucasians, especially in British Caucasian subjects (45,46). Taken together, these suggest the potential physiologic and pathophysiologic roles of CYP2D6 in the brain.

Table II. Examples of CYP2D6 substrate drugs.

Therapeutic class	Drug		
Analgesics	Codeine	Ethylmorphine	Oxycodone
	Dextromethorphan	Hydrocodone	
	Dihydrocodeine	Norcodeine	
Anti-ADHD	Atomoxetine		
Anti-arrhythmics	Aprindine	Mexiletine	Propafenone
	Encainide	N-propylamine	Sparteine
	Flecainide	Procainamide	
Anti-cancer	Tamoxifen		
Anti-dementia drugs	Galanthamine	Nicergoline	
Anti-depressants	Amitriptyline	Fluvoxamine	Mirtazapine
	Clomipramine	Imipramine	Nortriptyline
	Citalopram	Maprotiline	Paroxetine
	Desipramine	Mianserin	Venlafaxine
	Fluoxetine	Minaprine	
Anti-diabetic	Phenformine		
Anti-emetics	Dolasetron	Ondansetron	Tropisetron
Anti-histamines	Mequitazine	Promethazine	
Anti-hypertensives	Debrisoquine	Guanoxan	Indoramin
	Haloperidol	Risperidone	
Anti-psychotics	Thioridazine	Perphenazine	
β-adrenergic	Alprenolol	Bupranolol	Timolol
blocking agents	Bufuralol	Metoprolol	
	Bunitrolol	Propranolol	
Calcium antagonist	Perhexiline		
MAO-inhibitors	Amiflamine	Brofaromine	
Vasodilators	Cinnarizine	Flunarizine	

Modified from (3,33).

1.2.3. Interindividual variability in CYP2D6 activity

CYP2D6-mediated drug metabolism is known to exhibit large interindividual variability (10,11). CYP2D6 activity levels can be determined clinically by measuring urinary metabolic ratio of parent drug and metabolite concentration after a single administration of a known CYP2D6 substrate (e.g., sparteine). Up to 1800-fold difference in sparteine oxidation mediated by CYP2D6 was observed in humans (10). Based on the urinary metabolic ratio ([parent drug]/[metabolite]), patients are classified into four distinct phenotypes—ultrarapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM), and poor metabolizer (PM) (Table III). The EM phenotype is expressed in the majority of the population (75-80% in Caucasians). The IM (1-5% in Caucasians) phenotype has impaired but detectable residual CYP2D6 activity. The PM (5-10% in Caucasians) phenotype is with complete absent CYP2D6 activity, leading to an increase of undesired side effects, and lower efficacy for drugs requiring CYP2D6 activation. In UM (6-10% in Caucasians) phenotype, CYP2D6-mediated drug metabolism is extremely fast, leading to a loss of therapeutic efficacy at standard doses.

Table III. Interethnic differences in CYP2D6 phenotype.

Phenotype	CYP2D6 activity	Ethnic Differences			
		Caucasians	Asians	African-Americans	Ethiopians and Saudi Arabians
Poor Metabolizer	None	6-10%	1-5%	2-8%	1-5%
Intermediate Metabolizer	Low	1-5%	51%	—	3-9%
Extensive Metabolizer	Normal	75-80%	40-50%	80-90%	60-80%
Ultrarapid Metabolizer	High	5-10%	1%	5%	21%

Modified from [47].

1.3. Factors governing CYP2D6 activity

1.3.1. Genetic polymorphisms of CYP2D6

The large inter-individual variability in CYP2D6-mediated drug metabolism is in part explained by genetic polymorphisms of CYP2D6. CYP2D6 is located on chromosome 22q13. CYP2D6 genetic polymorphisms are associated with altered CYP2D6 activities in individuals (Table IV). Since the initial discovery of CYP2D6 genetic polymorphisms responsible for the PM phenotype of CYP2D6 was made in 1990 (48), over 100 different genetic polymorphisms of CYP2D6 have been identified (http://www.cypalleles.ki.se). Some of these polymorphisms are associated with low or minimal expression of CYP2D6 protein (e.g., due to frame-shift and premature stop codon in the open-reading frame) or expression of nonfunctional CYP2D6 proteins, leading to absent CYP2D6 activity (PM phenotype) (3). On the other hand, individuals with multiple copies of functional CYP2D6 alleles (e.g., CYP2D6 *2) were found to be associated with UM phenotype (10-13,49).

Table IV. Major variant alleles of CYP2D6 associated with altered CYP2D6 activity.

Major variant alleles	Mutation	Consequence	Allele frequencies (%)			
			Caucasians	Asians	African Americans	Ethiopians and Saudi Arabians
CYP2D6 * 2xn	Gene duplication /multiduplication	Increased enzyme activity	1−5	0−2	2	10−16
CYP2D6 * 4	Defective splicing	Inactive enzyme	12−23	1	2	1−4
CYP2D6 * 5	Gene deletion	No enzyme	2−7	6	4	1−3
CYP2D6 * 10	P34S, S486T	Unstable enzyme	1−2	38-70	6	3−9
CYP2D6 * 17	T107I, R296C, S486T	Altered affinity for substrates	0	0	20−35	3−9

Modified from [50].

Despite the increasing amount of knowledge on the genetic polymorphisms of CYP2D6, the information of CYP2D6 genotypes alone fails to reliably predict CYP2D6 phenotype in the majority of populations (3). PM phenotypes may be predicted based on the genotype information of CYP2D6, but these phenotypes comprise only a small portion (~10%) of populations (10-13,49,51,52). In the rest of populations, the relationship between CYP2D6 activity levels and CYP2D6 genotypes is ambiguous. For example, individuals carrying two functional CYP2D6 alleles display more than 30-fold variability in CYP2D6 activity (10), some exhibiting the UM phenotype. And individuals carrying only one functional allele of CYP2D6 (instead of two) may present EM phenotype (3,10,53). Taken together, genetic polymorphisms of CYP2D6 cannot fully convey the large interindividual variability in CYP2D6 activity; other sources of CYP2D6 variability remain elusive (Fig. 3).

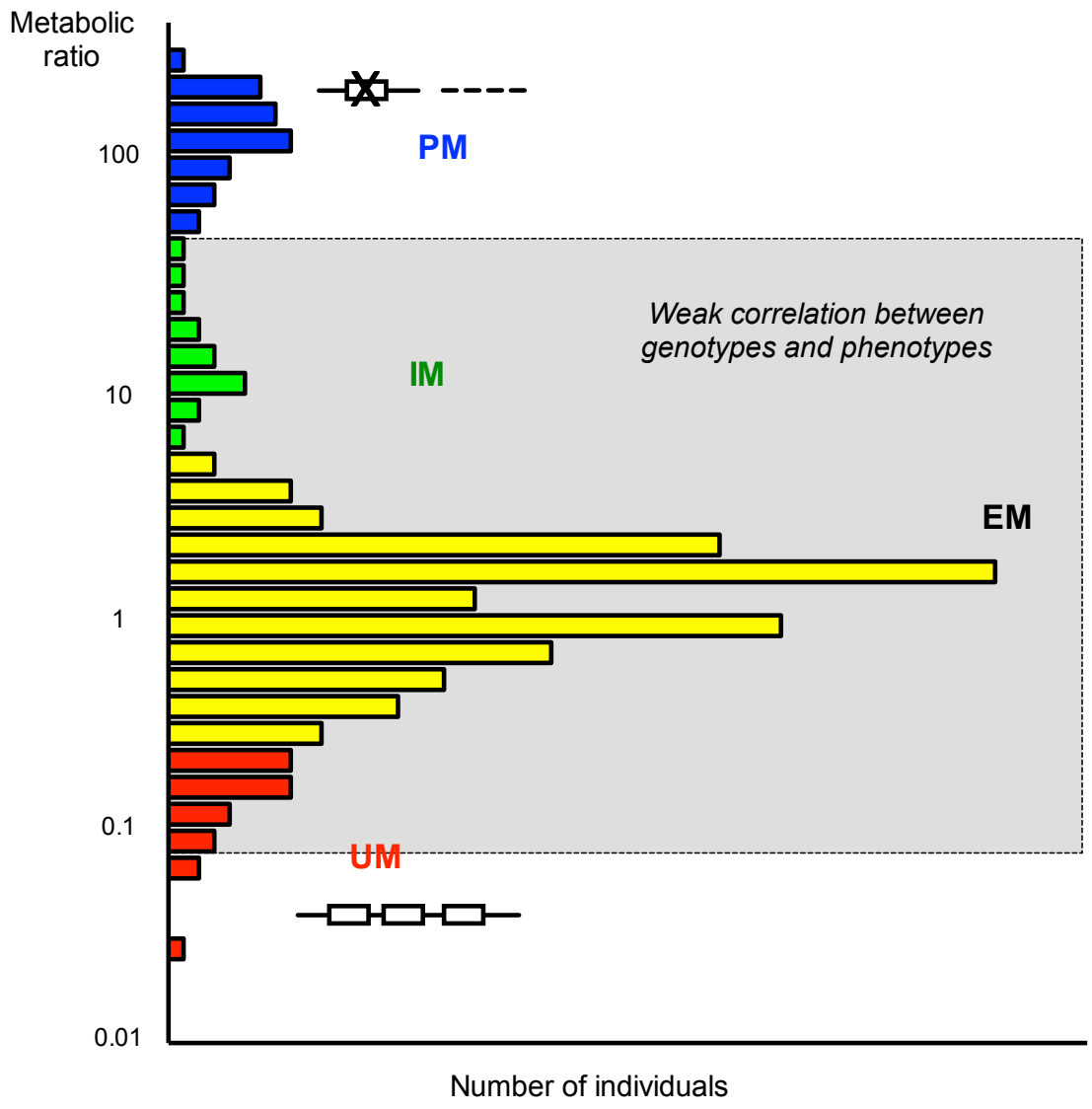

Figure 3. CYP2D6 genotype and phenotype relationship.
This figure illustrates the prevalence of four distinct CYP2D6 phenotypes that identified based on CYP2D6 activity levels—ultrarapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM), and poor metabolizer (PM). All PM phenotype and ~10% of UM phenotype could be certainly predicted by testing the genotype of CYP2D6. The correlation between genotypes and phenotypes is week in the UM, EM, and IM populations.

1.3.2. Transcriptional regulation of CYP2D6 expression

Previous studies have shown that the mRNA expression and enzyme activity levels of CYP2D6 are well correlated (14,15). For example, CYP2D6 activity in 134 human liver tissues correlates well with CYP2D6 mRNA levels (correlation coefficient = 0.91) (14). This level of correlation was similar to that shown for CYP3A4 (coefficient 0.95) (54) whose activity is known to be governed at the transcriptional level (55-59). These results indicate that differential transcriptional regulation of CYP2D6 may contribute to the large individual variability in CYP2D6 activity. However, transcriptional regulation of CYP2D6 remains poorly understood as compared to other drug-metabolizing enzymes including CYP3A4. To date, hepatocyte nuclear factor (HNF) 4α and CCAAT/enhancer-binding protein (C/EBP) α are the only two transcription factors known to be involved in constitutive expression of CYP2D6.

HNF4α is a master regulator of hepatic genes involved in the regulation of hepatocyte differentiation, lipid homeostasis, glucose metabolism, insulin secretion, and blood coagulation (60-62). Previous studies have shown that HNF4a plays a key role in regulating basal expression of CYP2D6 (63,64); HNF4α binds to a response element at -53/-41bp of *CYP2D6* and transactivates the promoter (63). Knock-down of HNF4α in human hepatocytes and HepG2 cells leads to decreased CYP2D6 expression (63,65,66). Also, in CYP2D6-humanized mice where wild-type CYP2D6 gene and its 2.5-kb upstream regulatory region were inserted into the mouse genome, conditional knockout of HNF4α led to a >50% decrease in CYP2D6 activity (9). These studies indicate critical roles of HNF4α in maintaining basal CYP2D6 expression.

C/EBPα is a transcription factor involved in the regulation of cell growth, cell differentiation, and energy homeostasis (67). Stable transfection of C/EBPα increased

the mRNA expression of CYP2D6 in HepG2 cells (68). Also, a putative binding site for C/EBPα has been identified at -1231 to -1220 bp region of CYP2D6 promoter (69), and knock-down of C/EBPα expression led to decreased CYP2D6 expression in HepG2 cells (69). These results suggest that C/EBPα is a potential activator of CYP2D6 expression. Whether C/EBPα indeed regulates CYP2D6 expression in vivo remains unknown.

1.3.3. Drug-drug interactions (CYP2D6 phenocopying)

Co-administered drugs may decrease CYP2D6 activity, sometimes resulting in changes in CYP2D6 phenotype. Many drugs are known to inhibit catalytic activity of CYP2D6 (Table V). The inhibition may occur through reversible inhibition (e.g., reversibly binding to catalytic sites) and/or mechanism-based inhibition (e.g., forming a metabolite intermediate complex) of CYP2D6 metabolic activity (70,71). The latter typically leads to persistent inactivation of CYP2D6 enzyme through irreversible binding between metabolite intermediate complex and the enzyme. Administration of strong inhibitors of CYP2D6 may result in a change of apparent CYP2D6 phenotype, also known as phenocopying. For example, paroxetine (a strong CYP2D6 inhibitor (72)) can convert individuals with UM phenotype to EM phenotype and decrease elimination of CYP2D6 substrate drugs such as nortriptyline (73). Furthermore, co-administration of paroxetine and tamoxifen leads to substantial decreases in the metabolism of tamoxifen (74-76), attenuated clinical efficacy (77), and increased mortality in breast cancer patients (78). The effects of CYP2D6 inhibitors on CYP2D6 phenotypes can be dose-dependent. Clinical studies in five UM subjects showed that giving paroxetine (20 mg daily) for 2 weeks resulted in a change in all UM individuals to the EM phenotype. While, doubling the paroxetine dose (to 40mg daily) in four UM subjects for 2 weeks converted them to PM phenotype (73).

Table V. Examples of CYP2D6 inhibitor drugs.

Strong Inhibitors ≥ 5-fold increase in AUC or > 80% decrease in CL	Bupropion Fluoxetine	Paroxetine Quinidine
Moderate inhibitors ≥ 2 but < 5-fold increase in AUC or 50-80% decrease in CL	Cinacalcet Duloxetine	Terbinafine
Weak inhibitors ≥ 1.25 but < 2-fold increase in AUC or 20-50% decrease in CL	Amiodarone Celecoxib Cimetidine Desvenlafaxine Diltiazem Diphenhydramine Echinacea Escitalopram Febuxostat Gefitinib Hydralazine	Hydroxychloroquine Imatinib Methadone Oral contraceptives Propafenone Ranitidine Ritonavir Sertraline Telithromycin Verapamil

Modified from (79). AUC, area under the curve; CL, clearance.

1.3.4. CYP2D6 induction (pregnancy)

Transcriptional regulation of CYP2D6 remains poorly understood, in part because CYP2D6 has been considered as a “non-inducible” gene. Prototypical inducers of CYP expression such as phenobarbital, rifampin, or dexamethasone enhance transcription of most CYPs, but not CYP2D6 (29,80). Interestingly, clinical data indicate that CYP2D6-mediated drug metabolism is enhanced during pregnancy (Table VI). The apparent induction in CYP2D6 activity during pregnancy was first observed in a study back in 1985, where the systemic clearance of metoprolol (a CYP2D6 substrate) was increased 2-3-fold at term pregnancy as compared to postpartum (4). Subsequent studies showed that the rate of dextromethorphan O-demethylation (mediated by CYP2D6) was significantly higher (by 2-fold) in pregnant women than in postpartum controls, indicating increased CYP2D6-mediated drug metabolism during pregnancy (5,6). Clonidine, a CYP2D6 substrate and an antihypertensive used during pregnancy, exhibited 2-fold higher oral clearance in pregnant women than non-pregnant women (81,82). Similarly, the systemic clearance of antidepressant paroxetine, mainly metabolized by CYP2D6, increased 2-fold at third trimester pregnancy compared to first trimester of pregnancy (7). Increased dose of CYP2D6 substrate drugs (i.e., citalopram, escitalopram, and sertraline) were prescribed to patients at third trimester pregnancy to maintain optimal therapeutic effects (8).

Table VI. Examples of induced elimination of CYP2D6 substrate drugs during pregnancy.

CYP2D6 substrate	Parameter	Value		Reference
		During pregnancy	Postpartum (or non-pregnancy)	
Metoprolol	CL_{iv} (L/min•kg)	0.0017±0.003	0.009±0.001	[4]
Dextromethorphan	dextromethorphan/dextrorphan urinary ratio	EM & IM: 0.001-0.005 PM: 1.9-3.1	EM & IM: 0.002-0.014 PM: 1.3-2.0	[5]
Dextromethorphan	dextromethorphan/dextrorphan urinary ratio	0.003±0.002	0.006±0.002	[6]
Clonidine	CL_{oral} (mL/min)	440±168	245±72 (non-pregnancy)	[81]
Escitalopram	Plasma drug level to dose ratio	2.3±1.5 (36 weeks of pregnancy)	3.6±1.6 (2 weeks postpartum)	[8]
Sertraline	Plasma drug level to dose ratio	0.2-0.4 (36 weeks of pregnancy)	0.3-0.8 (4-6 weeks postpartum)	[8]

Despite the accumulating clinical evidence for CYP2D6 induction during pregnancy, the underlying mechanisms remained completely unknown. This is in part due to the lack of appropriate animal models to recapitulate CYP2D6 induction during pregnancy. Unlike the induction in CYP2D6-mediated drug metabolism shown in pregnant women, the expression of CYP2D6 functional homolog in rats or mice (i.e., *CYP2D2* in rats and *Cyp2d22* in mice) was decreased at term pregnancy as compared to pre-pregnancy or postpartum levels (83,84). Establishing an animal model of CYP2D6 induction and elucidating underlying mechanisms could potentially lead to identification of novel factors involved in the regulation of CYP2D6 expression.

1.4. Hypothesis and scope of the study

In our preliminary studies, we established CYP2D6-humanized transgenic (Tg-*CYP2D6*) mouse as a model to investigate regulation of CYP2D6 expression. Tg-*CYP2D6* mouse carries complete wild-type CYP2D6 gene with its 2.5-kb upstream regulatory region in the genome (9). In Tg-*CYP2D6* mice, pregnancy led to ~2-fold increases in the expression and activity of CYP2D6, consistent with the induction of CYP2D6 activity reported in pregnant women. Importantly, these results demonstrate that Tg-*CYP2D6* mouse model could be used to investigate molecular mechanisms underlying CYP2D6 induction during pregnancy.

<u>Aim 1: Elucidate mechanisms underlying CYP2D6 induction during pregnancy.</u>

To identify transcription factors involved in CYP2D6 induction during pregnancy, gene expression profiling in the livers of pregnant and non-pregnant Tg-*CYP2D6* mice was performed by using cDNA microarray. This led to the identification of small heterodimer partner (SHP) as a transcription factor whose expression was downregulated during

pregnancy in Tg-*CYP2D6* mice. The role of SHP as potential transcriptional regulator of CYP2D6 was investigated by using transient transfection, promoter reporter assays, and siRNA gene knockdown studies in Tg-*CYP2D6* mice. Potential upstream regulators of SHP that are responsible for the SHP downregulation during pregnancy were identified and characterized.

Aim 2: Determine whether SHP induced by FXR synthetic agonist (i.e., GW4064) alters CYP2D6 expression and activity.

SHP is a representative target gene of nuclear receptor farnesoid X receptor (FXR) (17,85,86). To verify the role of SHP in the regulation of CYP2D6 expression, we examined whether FXR activation leads to changes in CYP2D6 expression, by administering a synthetic agonist (GW4064) to Tg-*CYP2D6* mice.

Aim 3: Determine whether altered level of SHP expression in a pathological condition (i.e., cholestasis) affects CYP2D6 expression and activity.

Bile acids are endogenous ligands of FXR (19). To further characterize the effects of FXR activation on CYP2D6 expression, we examined how cholestasis (a condition accompanied by elevated bile acid level in the liver) can alter CYP2D6 expression. To this end, we examined the effects of cholestasis on CYP2D6 expression using two experimental animal models (17α-ethinylestradiol administration induced cholestasis model and cholic acid feeding induced cholestasis model).

2. ALTERED EXPRESSION OF SMALL HETERODIMER PARTNER GOVERNS CYP2D6 INDUCTION DURING PREGNANCY IN CYP2D6-HUMANIZED MICE

[Previously published as Koh KH, Pan X, Shen HW, Arnold SL, Yu AM, Gonzalez FJ, Isoherranen N, Jeong H. Altered expression of small heterodimer partner governs cytochrome P450 (CYP) 2D6 induction during pregnancy in CYP2D6-humanized mice, J Biol Chem. 2014 Feb 7;289(6):3105-13.]

2.1. Introduction

Over 50% of pregnant women take one or more prescription drugs, and the average number of prescriptions per patient during pregnancy ranges from 3 to 5 (87,88). Clinical evidence indicates that pregnancy alters hepatic drug disposition. However, our current understanding of the underlying mechanisms is limited due to a lack of appropriate study models recapitulating the complex physiological changes accompanying pregnancy (89). This is in turn manifested clinically by an overall paucity of drug safety and dosing guidelines for pregnancy.

Cytochrome P450 (CYP) enzymes play a key role in hepatic elimination of xenobiotics as well as in hormone homeostasis. CYP expression is largely regulated by the actions of multiple liver-enriched transcription factors. For example, expression of many CYP isoforms is transcriptionally activated by xenosensor nuclear receptors including pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (90). Drugs that bind and activate these transcription factors induce CYP expression, leading to clinically significant drug-drug interactions.

CYP2D6 is a major drug-metabolizing enzyme expressed in the liver and extrahepatic organs including the brain, kidney, and intestine. CYP2D6 mediates the

hepatic metabolism of the ~20% of marketed drugs, second-largest portion only after CYP3A4 (3,33). Also, CYP2D6 expressed in the brain is involved in the synthesis of neurotransmitters (38) and is implicated in Parkinson's disease (91). *CYP2D6* has been considered a non-inducible gene based on the finding that none of the previously reported CYP-inducing compounds (including PXR-activating rifampin and CAR-activating phenobarbital) affect CYP2D6 expression. Interestingly, however, pregnancy induces hepatic elimination of CYP2D6 substrates. Elimination of dextromethorphan and metoprolol, the prototypical CYP2D6 substrates, is increased in pregnant women as compared with postpartum controls (4-6). Higher doses of CYP2D6 substrates are required during pregnancy than before pregnancy (8). Despite the accumulating clinical evidence, the mechanisms underlying CYP2D6 induction during pregnancy remain unknown in part because appropriate experimental models have been lacking. For example, expression of the endogenous CYP2D6 homologs in rodents (i.e., CYP2D2 in rats and Cyp2d22 in mice) does not increase during pregnancy (83,84). This likely reflects the large divergence in the regulatory region sequences of genes encoding xenobiotic-metabolizing CYP enzymes between rodents and humans (92).

In this study, using *CYP2D6*-humanized (Tg-*CYP2D6*) mice (9) as an animal model, we investigated the molecular mechanisms underlying CYP2D6 induction during pregnancy and demonstrated a potential role for the interplay between retinoic acids and hepatic transcription factors in CYP2D6 induction.

2.2. Materials and methods

2.2.1. Animals

Tg-*CYP2D6*, *Hnf4*α/AlbCre, and *Shp*-null transgenic mice were previously described (9,60,93). Adult female (8 weeks old) mice were mated with male mice of the 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 that their estrous cycles were suppressed. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Institution's Animal Care and use Committee in the University of Illinois at Chicago.

2.2.2. Chemicals and reagents

Debrisoquine, (±)-4-hydroxydebrisoquin, and paraxanthine were purchased from Biomol (Plymouth Meeting, PA). Ammonium Acetate (HPLC grade), acetonitirile (Optima LCMS grade), and methanol (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). All-trans retinoic acid (atRA) and 13-cis retinoic acid (13cRA) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2.3. Plasmids

To construct the pGL3-CYP2D6 plasmid, the upstream region of *CYP2D6* (-2453 to +90) was PCR-amplified using the genomic DNA of Tg-*CYP2D6* mouse as a template and primers (forward: 3'- ATCGGGTACCCTTTCCGACATACACGCAAT -5', reverse: 3'-ATCGCCATGGACCTGCCTCACTACCAAATG-5'). The PCR product was digested by

*Kpn*I and *Nco*I restriction enzymes and cloned into promoterless pGL3-basic vectors (Promega, Madison, WI) digested by the same enzymes, yielding pGL3-CYP2D6. pcDNA3-HNF4α was received from Dr. Frances M. Sladek (University of California Riverside Human). pEBG-contol and pEBG-SHP expression vectors were received from Dr. Hueng-Sik Choi (Chonnam National University, Korea).

2.2.4. Western blot

Approximately 100mg of mouse liver samples were homogenized in PBS containing protease inhibitor cocktail (Sigma-Aldrich, Catalog#P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich, Catalog#P0044), and centrifuged at 4000rpm for 3 min at 4°C. The pellets were suspended in buffer containing 450 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% NP-40 (v/v), 0.25% sodium-deoxycholate, protease inhibitor cocktail, and phosphatase inhibitor cocktail. After incubation for 30 min on the ice, the suspension was centrifuged at 12,000g for 1 min and the pellets were discarded. CYP2D6, HNF4α, and SHP protein expression levels were determined by using the respective antibodies (CYP2D6, BD Gentest™, Catalog# 458246; HNF4α, Santa Cruz, sc-6556 & Aviva, ARP31946_P050; SHP, Santa Cruz, sc-30169; α-tubulin, EMD Millipore, Catalog#CP06; β-actin, Sigma-Aldrich, Catalog#A1978). Total liver lysates (approximately 50 μg protein) were loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, protein was transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk, followed by primary antibody and HRP-conjugated secondary antibody incubation. The immune complexes were detected with the enhanced chemiluminescence reagent kit and the signals were recorded by FluroChem E Imager (proteinsimple™, San Jose, CA).

2.2.5. CYP2D6 phenotyping

Liver microsomes or S9 fractions were prepared as described previously (94). Microsomes or S9 fractions were incubated with debrisoquine (at different concentrations ranging from 25 to 1000 μM) for 15 min, and the reaction was stopped by adding two volumes of ice-cold acetonitrile. The concentration of 4-hydroxydebrisoquine was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS, Agilent 1200 HPLC interfaced with Applied Biosystems Qtrap 3200) using an electrospray ion source. The mobile phase consisted of water (5 mM ammonium acetate, pH 4.7) and acetonitrile. Separation was performed with a Nova-Pak® C18 column (3.9 x 150mm) (Waters, Milford, MA) at a flow rate of 0.3 ml/min. 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 paraxanthine.

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

Total RNA was isolated from mouse liver tissues using Trizol (Life Technologies, Carlsbad, CA) and converted to cDNA using High Capacity cDNA Archive Kit (Life Technologies). Using the cDNA as template, qRT-PCR was performed using StepOnePlus Real-Time PCR System and primers listed in Table VII. The results are expressed as fold changes during pregnancy using the gene expression levels normalized to those of mouse Gapdh ($2^{-\Delta\Delta Ct}$ method).

Table VII. Primer sequences for qRT-PCR.

SYBR green probes			
Gene	Species	Forward primer	Reverse primer
ApoC2	Mouse	ACCTGTACCAGAAGACATACCC	CCTGCGTAAGTGCTCATGG
Hes6	Mouse	TTGGGCATTCTGAGGATCTA	CGCAACTGTGTTACAAACGA
Hnf4α	Mouse	CACGCGGAGGTCAAGCTAC	CCCAGAGATGGGAGAGGTGAT
Shp	Mouse	GGCCTCTACCCTCAAGAACAT	TGTCAACGTCTCCCATGATAGG
SHP	Human	TCAAGTCCATTCCGACCAGC	AAGAAGGCCAGCGATGTCAA
Smile	Mouse	GAGGAAAGGGGTAAACGC	AAGGTGTCGGTGGAGTTCTG
Gapdh	Mouse	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Commercial Taqman probes			
Gene	Species	Catalog#	Company
CYP2D6	Human	Hs.PT.49a.205234723/ Hs02576167_m1	IDT/ABI
Cyp2d22	Mouse	Mm00530542_m1	ABI
Fxr	Mouse	Mm00436425_m1	ABI
Shp	Mouse	Mm.PT.51.8920508	IDT
Gapdh	Mouse	Mm.PT.39a.1	IDT

2.2.7. Luciferase reporter assay

HepG2 or HEK293T cells were seeded in 12-well plates at a density of $4.5x10^5$ cells/ml or $1x10^5$ cells/ml, and on the next day, transfected with 0.3 μg of luciferase construct, 0.1 μg of expression plasmids (or empty vector as a control), and 0.006 μg of *Renilla* expression vector (Promega) using Fugene HD transfection reagent (Promega) according to the manufacturer's protocol. The transfected cells were grown for 48 hours and were harvested for determination of luciferase activity using a luciferase assay kit (Promega). At least two independent experiments were performed in triplicate.

2.2.8. Gene knock-down by small interfering RNA

Small interfering RNA (siRNA) targeting SHP (Thermo scientific; siGenome_NR0B2 siRNA, 50 μg in 1 ml PBS) or control siRNA (siGenome_non-targeting siRNA pool) was injected into Tg-*CYP2D6* mice through tail vein. The injection was repeated 8 hours later. Livers were collected 3 days after the last injection for the examination of gene expression.

2.2.9. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as previously described with minor modifications (95). 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, pH 7.9, 60 mM KCl, 2 mM EDTA, 0.5% BSA, 0.15 mM spermine, 0.5 mM spermidine, 0.32 M sucrose) and lysed by homogenization. Nuclei were pelleted and resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% SDS, and

protease inhibitor cocktail). The samples were sonicated to shear DNA to the length ranging from 100 to 500 bp by using Misonix S-4000 sonicator (Farmingdale, NY) with 80% amplitude for 12 min. After centrifugation, the chromatin sample was immunoprecipitated with 2 μg antibody (Santa Cruz; HNF4α, sc-6556x; RNA polymerase II, sc-899x; SHP, sc-30169; FXR, sc-13063x) or immunoglobulin G (normal rabbit IgG, sc-2027; normal goat IgG, sc-2028) at 4°C for overnight. The immune complex was collected, the magnetic beads were extensively washed, and the bound chromatin was eluted. Genomic DNA was purified by PCR Clean-up kit (Promega) and used as a template for qRT-PCR. Primer and probe sequences were listed in Table VIII.

Table VIII. Primer and probe sequences for ChIP assays.

Custom-made Taqman probe				
Gene	Species	Forward primer	Reverse primer	Taqman probe
CYP2D6	Human	CAACACAGCAGGTTCACT	CTACCAAATGGGCTCCTCT	AGGCCATCATCAGCTCCCTTTA
SYBR green probes				
Gene	Species	Forward primer	Reverse primer	
ApoC2	Mouse	CCATGCGTAGGGCATTAGAAGA	GGCCCATCCTGTAACAGAGCTT	
Hes6	Mouse	GGAGTCCTGCCCGGCCTAAGTGC	ATTGGCTGTGCGTGGTCGGAGAG	
Shp	Mouse	GCCTGAGACCTTGGTGCCCTG	CTGCCCACTGCCTGGATGC	

2.2.10. Retinoic acid measurement

The concentrations of all-trans retinoic acid (atRA) and 13-cis retinoic acid (13cRA) were analyzed by LC-MS/MS as previously described (96) with the following modifications: mouse livers (n=11, liver weight 36-208 mg) were homogenized with a 2 ml glass dounce homogenizer (Kimble Glass) in a 1:1 volume of 0.9% NaCl and the sample transferred into a 15 ml glass culture tube. A 2:1 volume of acetonitrile with 0.1% formic acid was added together with atRA-d_5 (internal standard). Retinoic acid was extracted with 10 ml of hexanes, the organic layer was transferred to a glass tube and dried under nitrogen at 37°C. The sample was reconstituted in 65 µl 60:40 ACN/H_2O for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. A 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 published chromatographic method (96) was modified to consist of mobile phase A of H_2O + 0.1% formic acid and B: ACN/ MeOH (60/40) + 0.1% formic acid with identical gradient as previously described.

2.2.11. Primary human hepatocytes

Freshly isolated human hepatocytes, derived from three donors, were obtained from Liver Tissue Cell Distribution System (Pittsburgh, PA; funded by NIH Contract # HHSN276201200017C). Briefly, hepatocytes were shipped overnight in cold preservation media. Upon receipt, the media was replaced with serum-free Williams' E media (without phenol red) containing 0.1 µM dexamethasone, 10 µg/mL gentamicin, 15 mM HEPES, 2 mM L-glutamine, and 1% insulin-transferrin-sodium selenite media supplement. Cells were allowed to recover from shipping for 10 hours at 37 °C in an

atmosphere containing 5% CO_2. After recovery, the hepatocytes were treated with vehicle control (DMSO) or atRA (10 μM) for 48 hours. Cell lysates were collected to prepare RNAs and S9 fractions.

2.2.12. Statistical analysis

The statistical difference between two groups was determined by the Student's t-test. For statistical testing among different treatment groups, one-way analysis of variance test was performed for multiple comparisons followed by posthoc Dunnett's test.

2.3. Results

2.3.1. Tg-*CYP2D6* mice serve as a model for CYP2D6 induction during pregnancy

The Tg-*CYP2D6* mice carrying the entire human CYP2D6 gene including ~2.5-kb of the upstream regulatory region was previously used as a model to characterize CYP2D6-mediated drug metabolism in an *in vivo* system (9). To determine the utility of Tg-*CYP2D6* mice as an animal model to study CYP2D6 induction during pregnancy, we first examined the hepatic expression and enzyme activity of CYP2D6 at different gestational time points [virgin, 7/14/21 days of pregnancy (G7/14/21), and 7 days postpartum (PP7)]. The mRNA and protein levels of CYP2D6 were elevated during pregnancy and returned to pre-pregnancy levels by the postpartum time point (Fig. 4A and 4B). In contrast, the expression of Cyp2d22 (a functional homolog of CYP2D6 in mice) remained unchanged during pregnancy (Fig. 4A), consistent with recent findings (84). Results from microsomal phenotyping (by using debrisoquine as a probe drug for CYP2D6) revealed a significant increase in CYP2D6 activity at term as compared with pre-pregnancy or postpartum (Fig. 4C), in accordance with the changes observed for CYP2D6 expression.

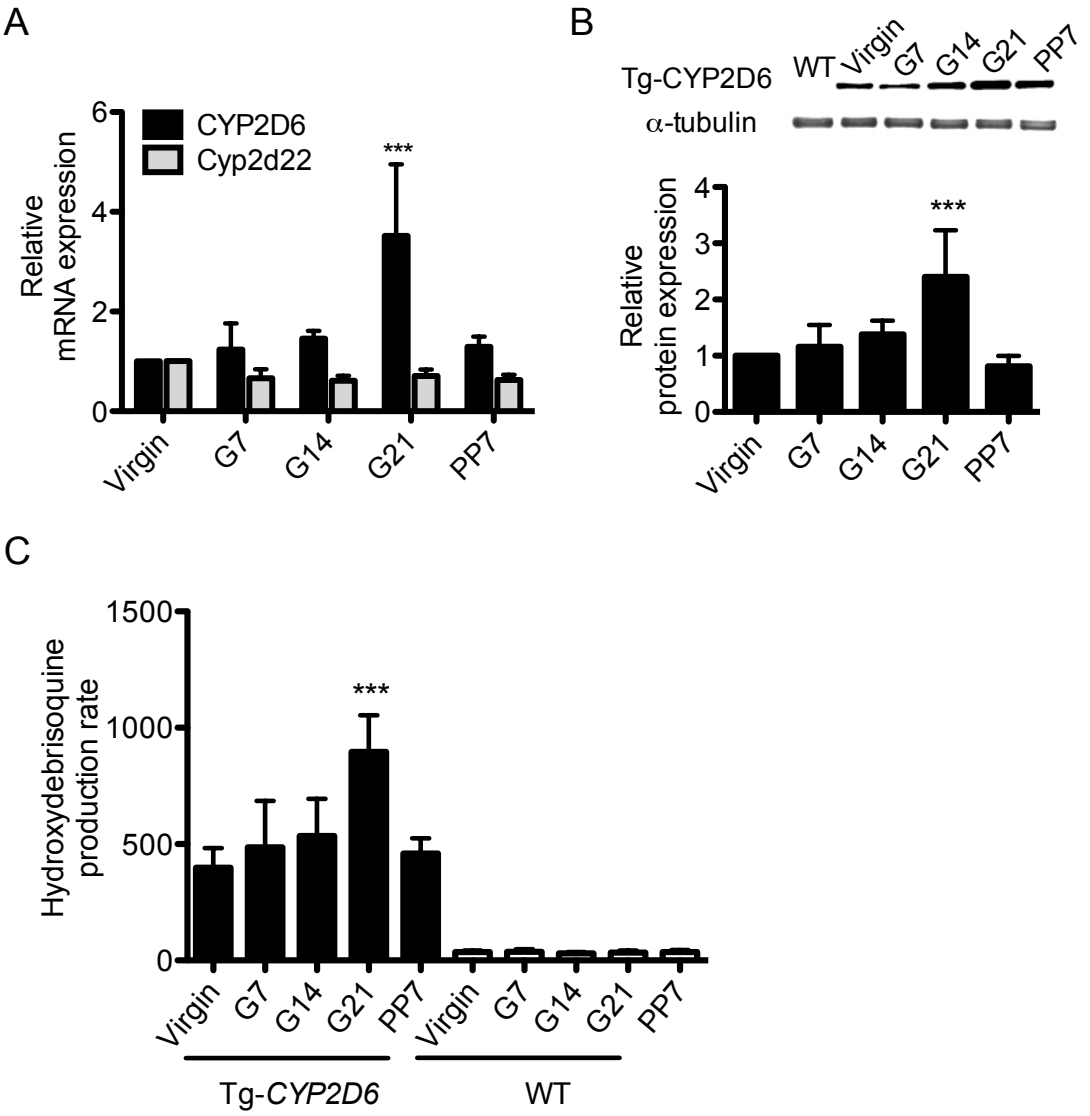

Figure 4. Hepatic CYP2D6 is induced in Tg-*CYP2D6* mice during pregnancy.
A, Liver tissues of Tg-*CYP2D6* mice were collected at pre-pregnancy (virgin), 7/14/21 days of pregnancy (G7, G14, and G21, respectively), or 7 days postpartum (PP7). mRNA expression levels of CYP2D6 and Cyp2d22 were determined by qRT-PCR. B, Protein levels of CYP2D6 in the liver tissues were determined by western blot. Representative image of western blot is shown including a liver tissue sample from wild-type (WT) mice (top), and the band intensities were quantified (CYP2D6/α-tubulin, bottom) after normalized by CYP2D6 expression in virgin mice. C, Microsomes were prepared from the liver tissues of Tg-*CYP2D6* or WT mice, and CYP2D6 phenotyping was performed using debrisoquine (200 μM). Values are presented as mean ± S.D. (n=4), ***p < 0.001 one-way ANOVA vs. virgin.

The increased CYP2D6 activity was accompanied by higher V_{max} values but no changes in K_m (Table IX), indicating that CYP2D6 activity is enhanced by increased protein amounts rather than changes in the catalytic activity of CYP2D6. The magnitude of CYP2D6 induction was similar to the clinically reported increases in elimination of a CYP2D6 substrate metoprolol (4), suggesting that Tg-*CYP2D6* mice may serve as a model system for identification and characterization of regulatory mechanisms for CYP2D6 induction during pregnancy.

Table IX. Kinetic parameters for debrisoquine hydroxylation activity in hepatic microsomes prepared from Tg-*CYP2D6* mice.

Parameter	Virgin	G7	G14	G21	PP7
V_{max} (pmol/min/mg protein)	749 ± 125	1130 ± 420	1240 ± 160*	2120 ± 60**	1080 ± 200
K_m (μM)	210 ± 77	291 ± 54	296 ± 87	278 ± 26	285 ± 19

n=4, mean ± S.D., V_{max}, maximum velocity; K_m, Michaelis–Menten constant.
* $p < 0.05$; ** $p < 0.01$ one way ANOVA vs. virgin.

2.3.2. SHP is downregulated during pregnancy and represses HNF4α-mediated transactivation of CYP2D6

Hepatocyte nuclear factor (HNF) 4α is a positive regulator of basal CYP2D6 expression under non-pregnant conditions via direct binding to the CYP2D6 promoter (9,63,97). To examine potential effects of pregnancy on HNF4α expression, mRNA and protein levels of HNF4α were examined in the livers of Tg-*CYP2D6* mice at different gestational time points. Pregnancy did not affect hepatic HNF4α expression at the levels of mRNA (Fig. 5A) or protein (Fig. 5B), consistent with results from a previous study (98).

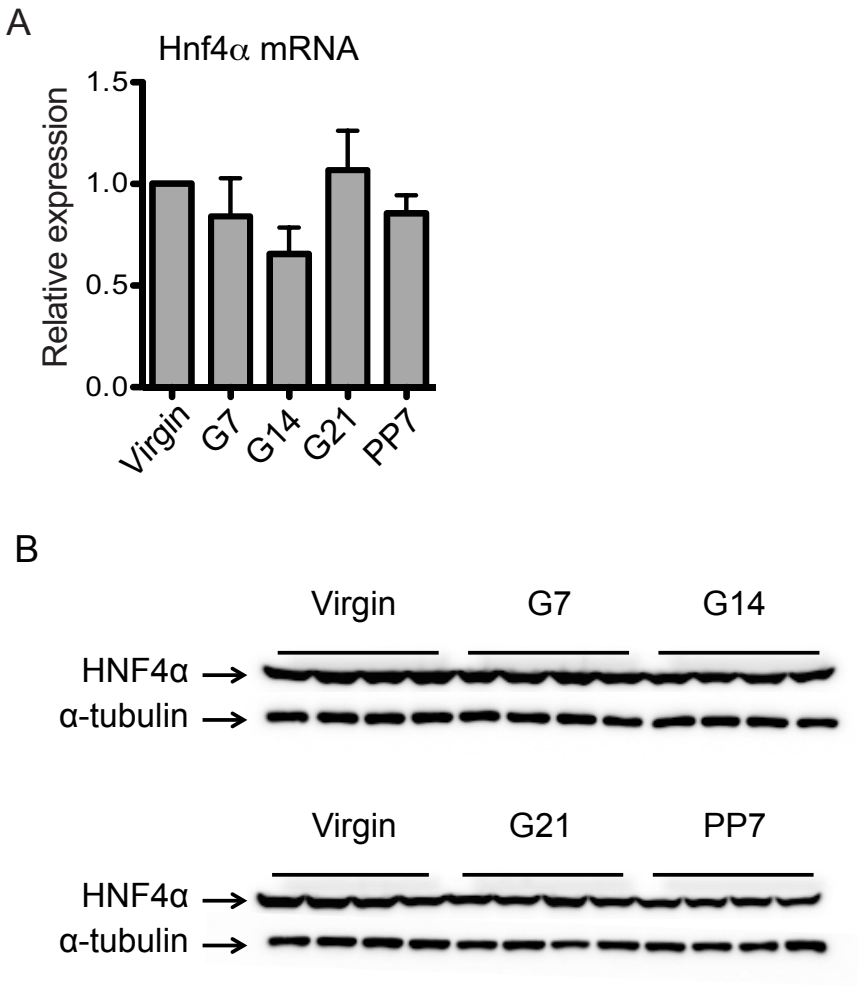

Figure 5. Pregnancy has no effect on HNF4α expression.
Liver tissues were collected from Tg-*CYP2D6* mice at different gestational time points, and total RNA and lysates were prepared from the tissues. A, mRNA levels of Hnf4α were determined by qRT-PCR. B, protein levels of HNF4α in liver tissues were determined by western blot. Values are presented as mean ± S.D. (*n*=4)

HNF4α activity can be functionally modulated by interaction with other transcription factors (99). To determine whether HNF4α activity on CYP2D6 is enhanced in mouse livers during pregnancy, ChIP assays were performed. To this end, liver tissues were collected from Tg-*CYP2D6* mice at different gestational time points and subjected to ChIP using antibodies against HNF4α or RNA polymerase II (Pol II; a positive control for CYP2D6 transcription). The protein-bound DNA was measured using a primer set that can detect the HNF4α response element at -55/-43bp region of CYP2D6 promoter (63). The results showed that HNF4α and Pol II recruitments to the CYP2D6 promoter significantly increased at term as compared with pre-pregnancy level (Fig. 6), indicating increased HNF4α activity during pregnancy.

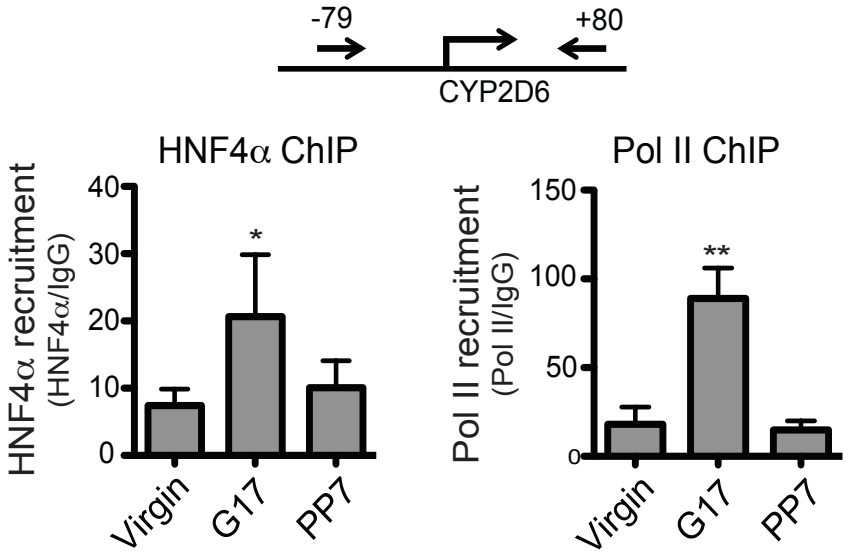

Figure 6. Pregnancy represses HNF4α transactivation of CYP2D6 promoter.
ChIP assays were performed using liver tissues of Tg-*CYP2D6* mice collected at different gestational time points. Values are presented as mean ± S.D. (*n*=4), * $p < 0.05$; ** $p < 0.01$, one-way ANOVA vs. virgin.

Transcriptional co-regulators acyl-CoA binding protein (100), CREB-binding protein (101), forkhead box protein o 1 (102), glutamate receptor interacting protein 1 (103), hepatocyte nuclear recetor 1 α (104), peroxisome proliferator-activated receptor γ coactivator 1 α (105), prospero-related homeodomain protein (106), p300 (107), silencing mediator for retinoid and thyroid receptors (107), small heterodimer partner (108), SMAD3/4 (109), SP1 (110), steroid receptor coactivator 1 (103), and sterol regulatory element-binding protein 2 (111) have been known to functionally modulate HNF4α through interacting with HNF4α, indicating altered expression of these proteins during pregnancy may attribute to the increased recruitment of HNF4α to CYP2D6 promoter. Data from our previous microarray experiments (GSE50166) showed that pregnancy only altered the expression of small heterodimer partner (SHP).

Nuclear receptor SHP functions as a corepressor of HNF4α transactivation of a HNF4α target gene (i.e., CYP8B1) (108). To examine whether SHP expression is altered during pregnancy, mRNA and protein levels of SHP were examined in liver tissues collected at different gestational time points. The results showed significantly decreased SHP expression at term as compared to pre-pregnancy or postpartum period (Fig. 7A and 7B).

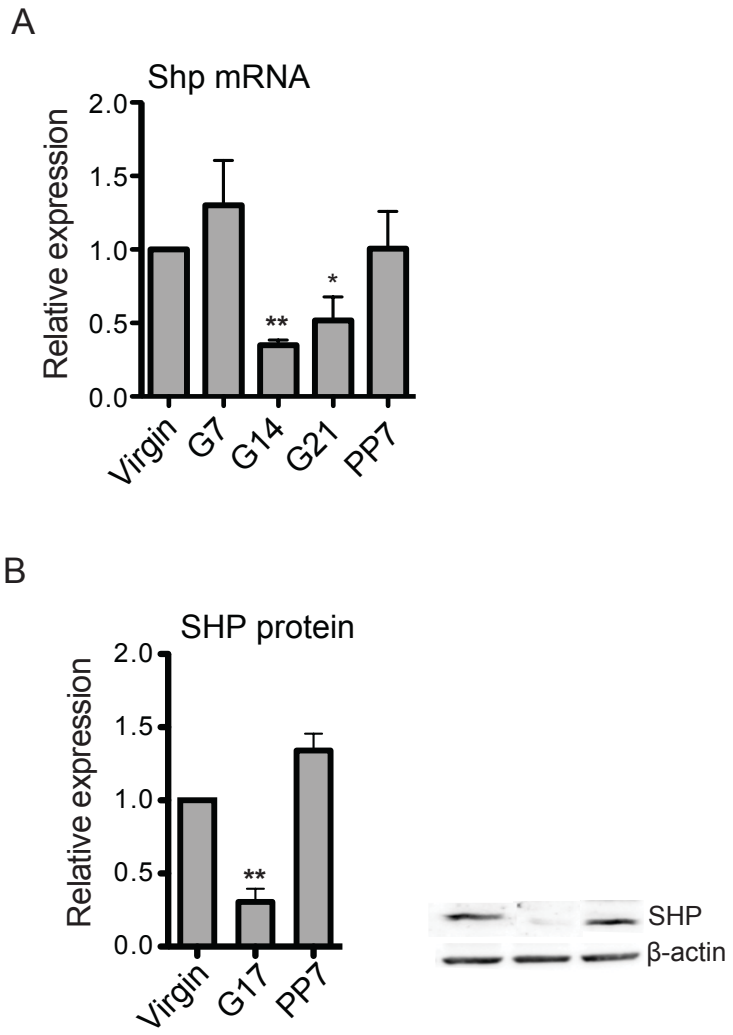

Figure 7. SHP is downregulated during pregnancy.
Liver tissues were collected from Tg-*CYP2D6* mice at different gestational time points. A, mRNA level of SHP in liver tissues was determined by qRT-PCR. B, protein level of SHP in liver tissues was determined by western blot. A representative image of western blot (right) is shown, and band intensities (SHP/β-actin, left) were quantified after normalized by the expression of SHP in virgin mice. Values are presented as mean ± S.D. (n=4), *p < 0.05; **p < 0.01, one-way ANOVA vs. virgin.

To determine the effects of SHP on HNF4α transactivation of CYP2D6 promoter, luciferase assays were performed in HEK293T cells where basal expression of HNF4α is minimal. Transient transfection of HNF4α led to a significant increase in CYP2D6 promoter activity as expected (Fig. 8A). SHP repressed HNF4α transactivation of the promoter in a SHP concentration dependent manner (Fig. 8A), indicating that SHP is a repressor of CYP2D6 promoter potentially by repressing HNF4α action.

To mimic the state of pregnancy, where SHP expression is decreased, we knocked down SHP expression by using siRNA and examined its effects on CYP2D6 expression. Tg-*CYP2D6* mice injected with SHP-targeting siRNA exhibited substantial knockdown of SHP and at the same time a marked increase in CYP2D6 expression, when compared to those injected with scrambled siRNA (Fig. 8B). These results suggest that repressed SHP expression plays a role in the upregulation of CYP2D6 promoter activity during pregnancy.

To determine whether the enhanced HNF4α activity on CYP2D6 promoter during pregnancy (Fig. 6) is accompanied by any changes in SHP recruitment to the promoter, ChIP assays were performed. Liver tissues collected at different gestational time points were subjected to ChIP using antibodies against SHP. The results showed that SHP recruitment to the CYP2D6 promoter significantly decreased at term (Fig. 8C). Together, these results suggest that downregulated SHP expression during pregnancy de-represses HNF4α activity on CYP2D6 promoter.

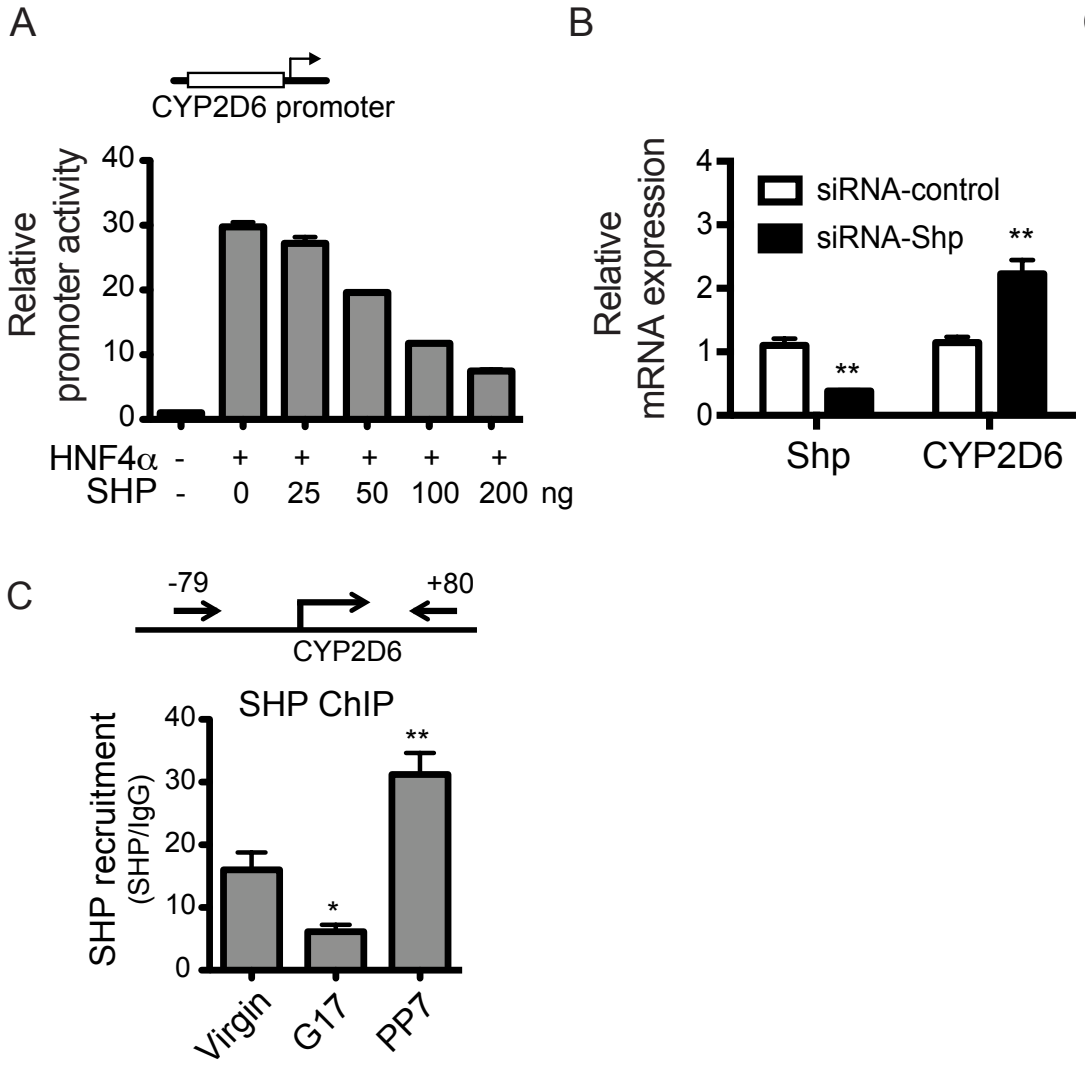

Figure 8. SHP represses HNF4α transactivation of CYP2D6 promoter.
A, CYP2D6 promoter activity was examined by dual luciferase assay in HEK293T cells Values are presented as mean ± S.D. (*n*=3). B, Tg-*CYP2D6* mice were injected with scrambled siRNA (siRNA-control) or siRNA targeting SHP (siRNA-SHP), and mRNA expression levels were determined by qRT-PCR. Values are presented as mean ± S.D. (*n*=3), $^{**}p < 0.01$, Student's t-test vs. siRNA-control. C, ChIP assays were performed using liver tissues of Tg-*CYP2D6* mice collected at different gestational time points. Values are presented as mean ± S.D. (*n*=4), $^{*}p < 0.05$; $^{**}p < 0.01$, one-way ANOVA vs. virgin.

2.3.3. CYP2D6 induction during pregnancy is abrogated in *Hnf4α(-/wt)* mice

To verify the importance of HNF4α for CYP2D6 induction during pregnancy, we examined whether reduced hepatic HNF4α expression influences the extent of CYP2D6 induction during pregnancy. Complete deletion of *Hnf4α,* even when localized to the liver, is fatal due to disruption of hepatic lipid homeostasis (60). Thus, Tg-*CYP2D6* mice were crossed with heterozygous *AlbCre;Hnf4α* $^{fl/wt}$ mice, generating pups carrying *CYP2D6* as well as either one or two copies of the *Hnf4α* allele in the liver [called *Hnf4α(-/wt);CYP2D6* and *Hnf4α(wt/wt);CYP2D6,* respectively], and the extent of CYP2D6 induction during pregnancy was compared. None of these mice exhibited any prominent phenotypes, and all grew normally. The hepatic protein level of HNF4α in *Hnf4α(-/wt)*;*CYP2D6* mice was about half that of the littermate control, as expected (Fig. 9A). In *Hnf4α(-/wt);CYP2D6* mice, CYP2D6 induction during pregnancy was completely abrogated (Fig. 9B), and the enhanced HNF4α recruitment to CYP2D6 promoter was disappeared (Fig. 10). On the other hand, pregnancy-mediated repression of Shp was maintained in *Hnf4α(-/wt);CYP2D6* mice (Fig. 9C) while decreased recruitment of SHP to the CYP2D6 promoter was abrogated in the mice (Fig. 10). Together, these results indicate that CYP2D6 induction during pregnancy is highly dependent on HNF4α expression level and suggest an essential role of HNF4α in mediating CYP2D6 induction by SHP during pregnancy.

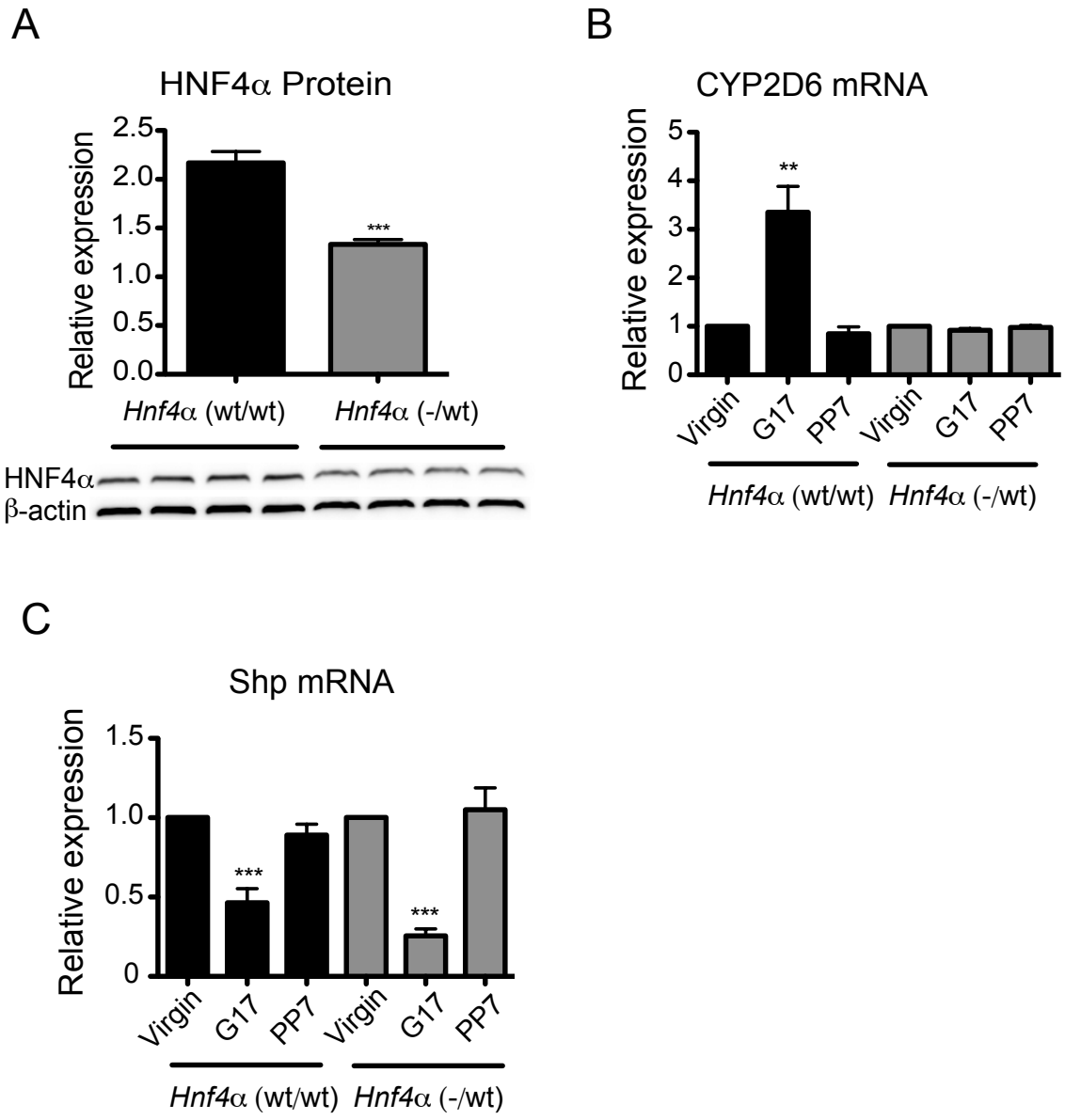

Figure 9. Pregnancy-mediated CYP2D6 induction is abrogated in *Hnf4α(-/wt);CYP2D6* mice.

A, Liver tissues were collected from *Hnf4α(wt/wt);CYP2D6* and *Hnf4α(-/wt);CYP2D6* mice at pre-pregnancy. HNF4α protein expression levels were determined by western blot (bottom). The image of western blot (bottom) and the quantified band intensities (HNF4α/β-actin, top) are shown. Values are presented as mean ± S.D. (n=4), ***, p < 0.001, Student's t-test vs. *Hnf4α(wt/wt)* mice. B and C, mRNA levels of CYP2D6 (B) and Shp (C) in the liver tissues were determined by qRT-PCR. Values are presented as mean ± S.D. (n=4), ** p < 0.01; ***p < 0.001, one-way ANOVA vs. virgin mice of respective genotype.

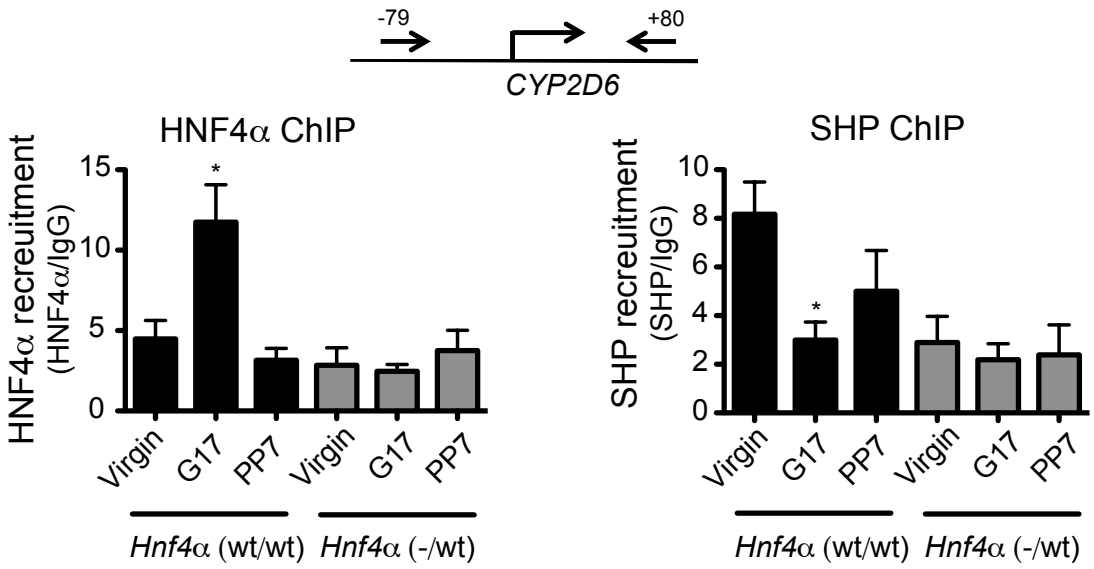

Figure 10. Pregnancy-induced HNF4α transactivation of CYP2D6 promoter is abrogated in Hnf4α *(-/wt);CYP2D6* mice.
Liver tissues were collected from *Hnf4α(wt/wt);CYP2D6* and *Hnf4α(-/wt);CYP2D6* mice at different gestational time points. Recruitment of HNF4α and SHP onto CYP2D6 promoter was analyzed by using ChIP assays. Values are presented as mean ± S.D. (*n*=4), * $p < 0.05$, one-way ANOVA vs. virgin mice of respective genotype.

To determine whether pregnancy enhances HNF4α activity on other target genes of HNF4α, HNF4α recruitment to promoters of two other known target genes [Hes6 (112) and apolipoprotein C-II (ApoC2) (113,114)] was examined in the mouse livers at different gestational time points. HNF4α recruitment to the Hes6 promoter was not affected by pregnancy (Fig. 11A), in agreement with insignificant changes in the mRNA level of Hes6 at term as compared to pre-pregnancy level (Fig. 11B). At postpartum, however, Hes6 mRNA level was significantly increased, potentially due to altered expression and/or activities of transcription factors known to regulate Hes6 expression, such as achaete-scute homolog 1, myogenic differentiation 1, and Clock/Bmal1 (115-117). On the other hand, HNF4α recruitment to the ApoC2 promoter was increased at term (Fig. 11C), but the extent of the increase (~2-fold) was much less than that observed for ApoC2 mRNA (>20-fold) (Fig. 11D), suggesting a relatively minor role of HNF4α in enhanced ApoC2 expression during pregnancy. Together, these results suggest that the regulatory effects of pregnancy on the expression of HNF4α target genes are likely gene-specific.

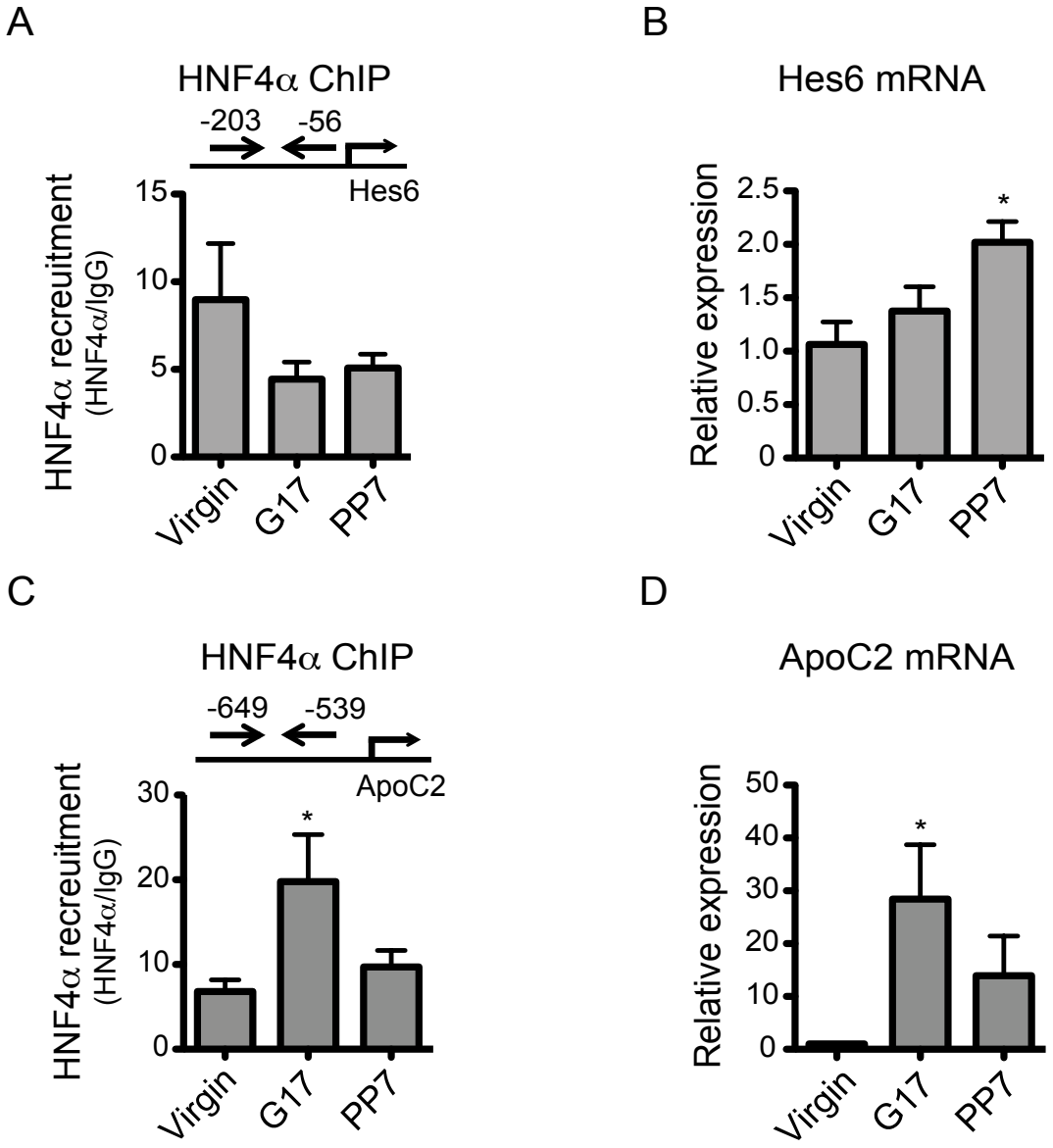

Figure 11. Effects of pregnancy on HNF4α activity are target gene-specific.
Liver tissues were collected from Tg-*CYP2D6* mice at different gestational time points. A and C, HNF4α recruitment to Hes6 promoter (A) and Apoc2 promoter (C) was analyzed by using ChIP assays. B and D, mRNA expression of Hes6 (B) and Apoc2 (D) was determined by qRT-PCR. Values are presented as mean ± S.D. (*n*=4), **p* < 0.05, one-way ANOVA vs. virgin.

2.3.4. CYP2D6 induction during pregnancy is abrogated in *Shp(-/-);CYP2D6* mice

To verify the importance of SHP for CYP2D6 induction during pregnancy, we examined whether complete deletion of SHP can influence the extent of CYP2D6 induction during pregnancy. Thus, Tg-*CYP2D6* mice were crossed with *Shp*-null mice, generating pups carrying *Shp(+/+);CYP2D6* or *Shp(+/+);CYP2D6* genotype. None of *Shp(+/+);CYP2D6* or *Shp(-/-);CYP2D6* mice exhibited any prominent phenotypes, and all grew normally. Western blot results showed that SHP protein expression was abolished in *Shp(-/-);CYP2D6* mice (Fig. 12A). In *Shp(-/-);CYP2D6* mice, CYP2D6 induction during pregnancy was completely abrogated (Fig. 12B-D). Data from ChIP assays showed that enhanced HNF4α recruitment and decreased SHP recruitment to CYP2D6 promoter were disappeared in *Shp(-/-);CYP2D6* mice (Fig. 13). Together, these results indicate that CYP2D6 induction during pregnancy is highly dependent on SHP expression level and suggest an essential role of SHP in mediating CYP2D6 induction during pregnancy.

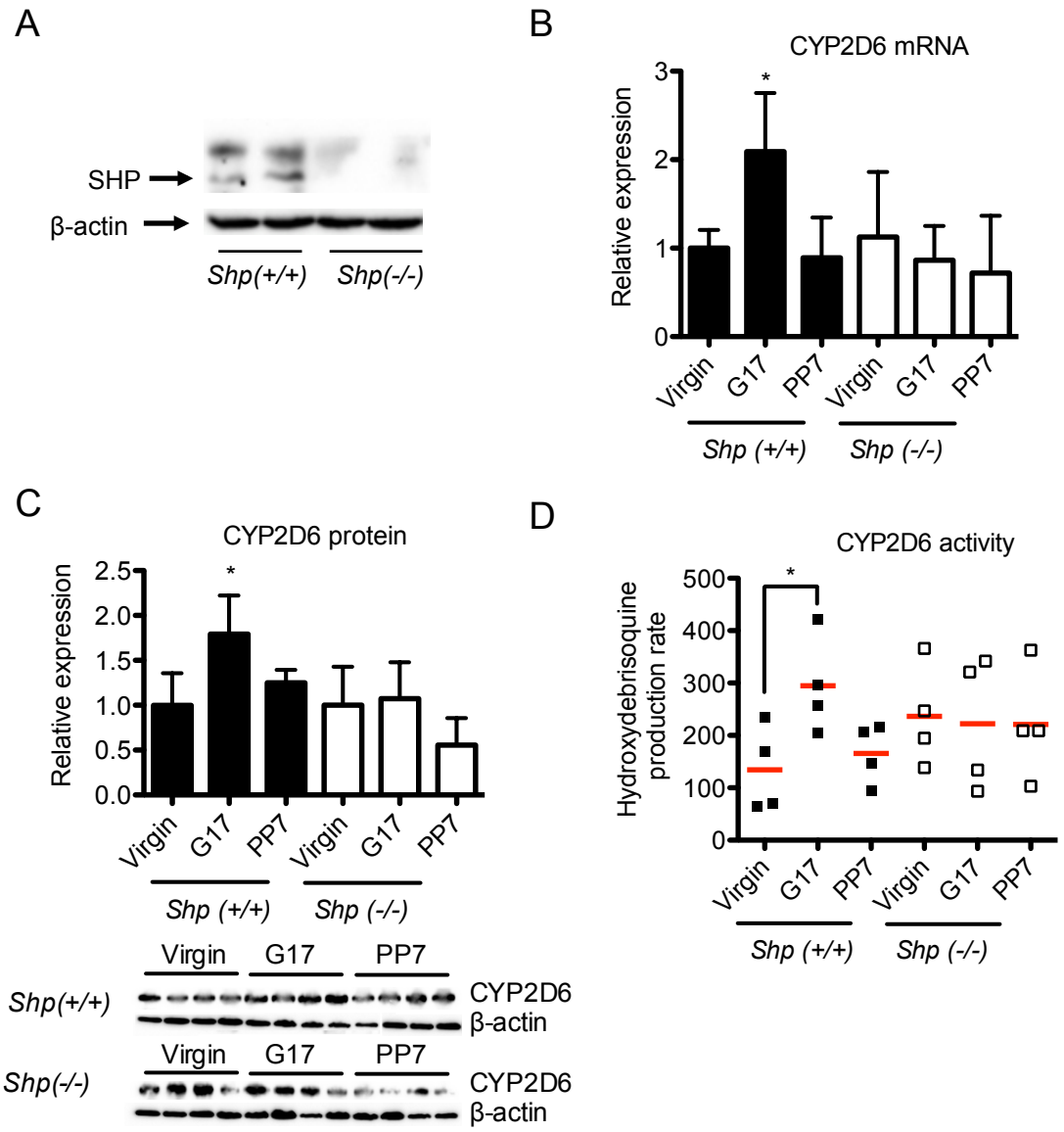

Figure 12. Pregnancy-mediated CYP2D6 induction is abrogated in *Shp(-/-);CYP2D6* mice.

Liver tissues were collected from *Shp(+/+);CYP2D6* and *Shp(-/-);CYP2D6* mice at different gestational time points. A, SHP protein expression levels were determined by western blot. B, CYP2D6 mRNA expression level was determined by qRT-PCR. C, CYP2D6 protein expression level was measured by western blot. The image of western blot (bottom) is shown, and band intensities (CYP2D6/β-actin, top) were quantified after normalization by CYP2D6 expression in virgin mice of respective genotype. D, S9 from the mice was incubated with debrisoquine (200 μM), and 4-hyrdoxydebrisoquine concentrations were measured by using LC-MS/MS. Data shown are 4-hydroxydebrisoquin production rates (in pmol/min/mg protein). Values are presented as mean ± S.D. (n=4), * $p < 0.05$, one-way ANOVA vs. virgin.

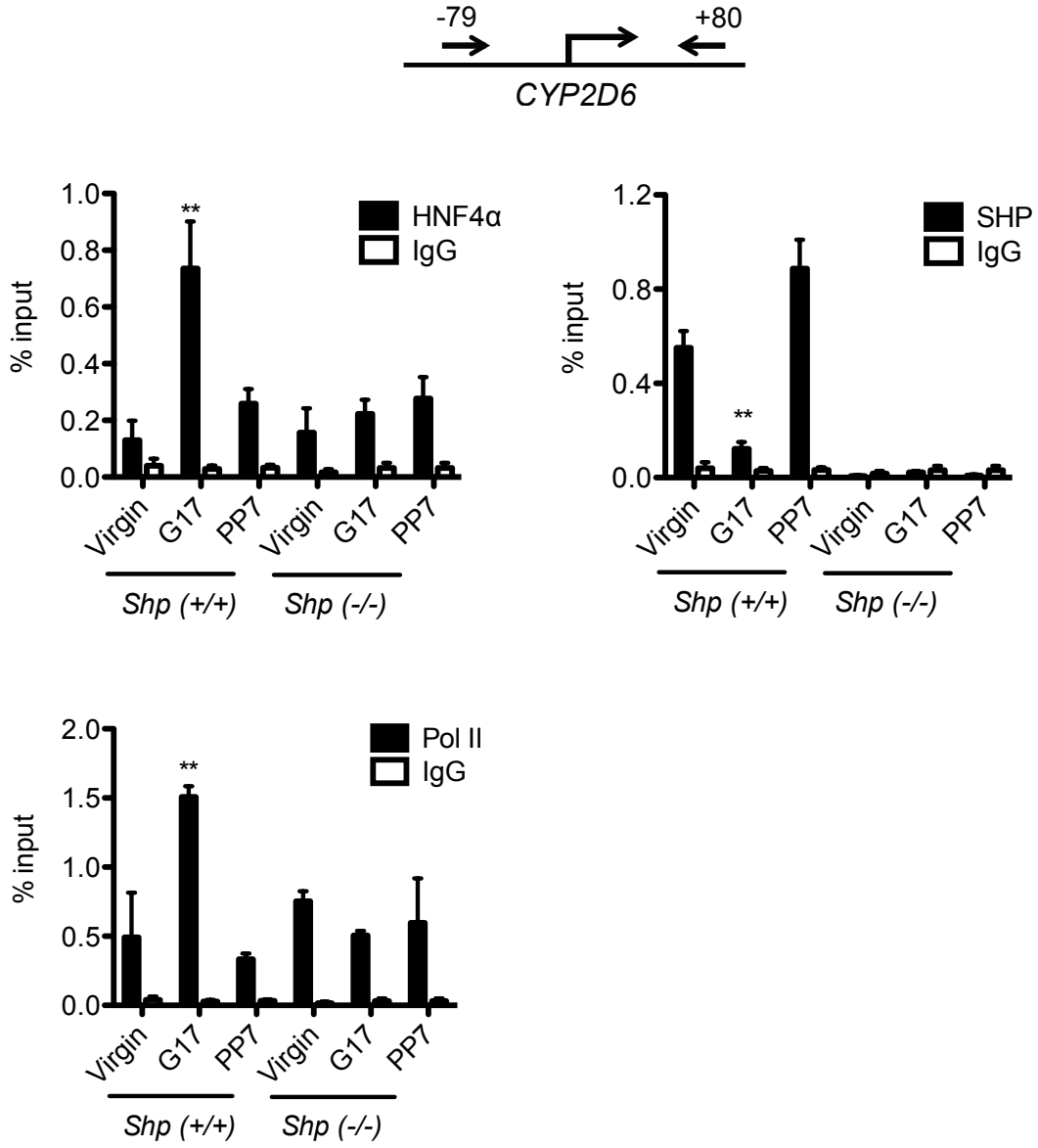

Figure 13. Pregnancy-induced HNF4α transactivation of CYP2D6 promoter is abrogated in *Shp(-/-);CYP2D6* mice.

Liver tissues were collected from *Shp(+/+);CYP2D6* and *Shp(-/-);CYP2D6* mice at different gestational time points. Recruitment of HNF4α, SHP, and RNA polymerase II (Pol II) onto CYP2D6 promoter was analyzed by using ChIP assays. Values are presented as mean ± S.D. (*n*=4), ** $p < 0.01$, one-way ANOVA vs. virgin mice of respective genotype.

2.3.5. Hepatic FXR activity is enhanced at postpartum

To identify potential upstream regulator(s) responsible for the changes in SHP expression during pregnancy, the effects of pregnancy on factors previously known to modulate SHP expression were examined.

SHP is a representative target gene of farnesoid X receptor (FXR), a nuclear receptor activated upon binding to bile acids (17). To determine whether altered expression and/or activity of FXR is responsible for SHP repression during pregnancy, the expression levels of FXR as well as FXR recruitment to the Shp promoter were examined in the livers of Tg-*CYP2D6* mice collected at different gestational time points. FXR expression showed insignificant changes during pregnancy (Fig. 14A), in agreement with a previous report (118). Also, ChIP results showed that FXR recruitment to Shp promoter did not decrease at term as compared to pre-pregnancy (Fig. 14B). Of note, FXR recruitment to Shp promoter increased at postpartum (Fig. 14B), suggesting a potential role for FXR in the return of decreased SHP expression to pre-pregnancy levels.

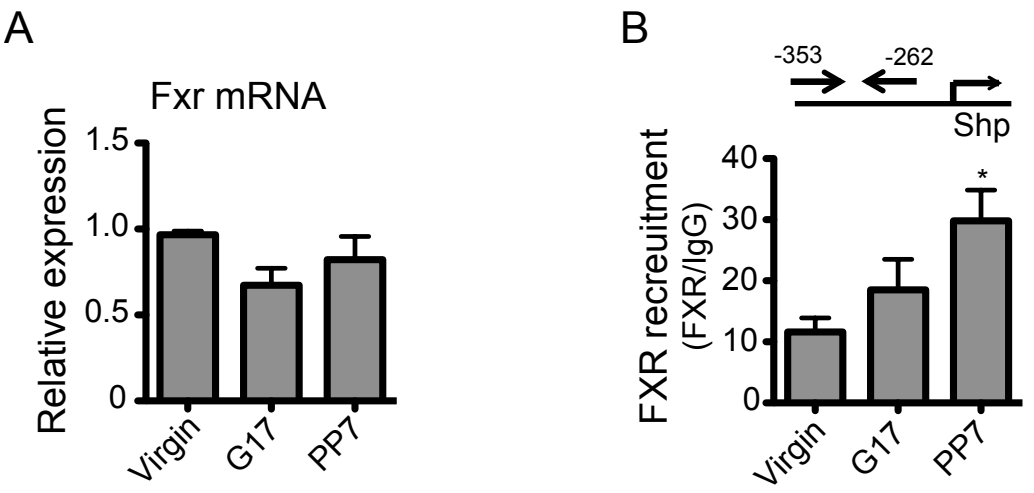

Figure 14. FXR expression and activity do not decrease during pregnancy.
Liver tissues of Tg-*CYP2D6* mice were collected at different gestational time points. A, mRNA expression was determined by qRT-PCR. B, FXR recruitment to Shp promoter was analyzed by using ChIP assays. Values are presented as mean ± S.D. (*n*=4), **p* < 0.05, one-way ANOVA vs. virgin.

2.3.6. Retinoic acid modulates CYP2D6 expression

A previous study reported that all-trans retinoic acid (atRA; the bioactive form of vitamin A) induces SHP expression in HepG2 cells (119). To examine whether changes in RA levels are potentially responsible for CYP2D6 induction during pregnancy, hepatic levels of the two predominant RA isomers atRA and 13-cis retinoic acid (13cRA) were measured by using LC-MS/MS. The results revealed significantly decreased hepatic levels of atRA at term as compared with those at pre-pregnancy (Fig. 15). A similar finding was observed for 13cRA (Fig. 15). This suggests that the decreased hepatic RA content may be in part responsible for the reduced SHP expression observed during pregnancy.

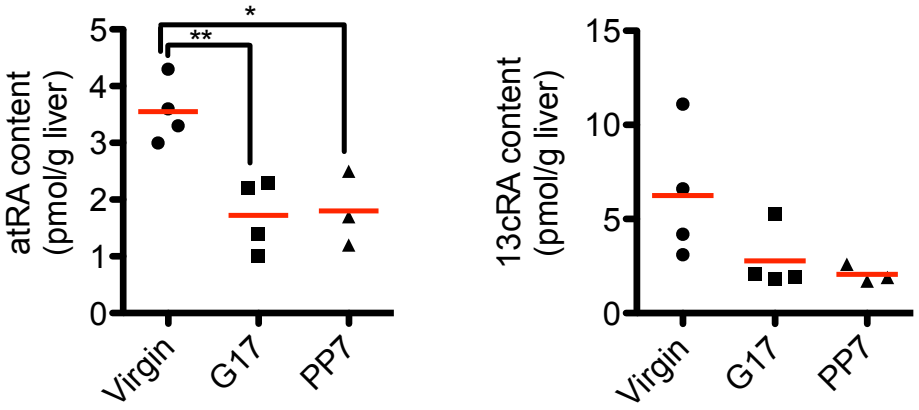

Figure 15. RAs are decreased during pregnancy.
The levels of all-trans retinoic acid (atRA) and 13-cis retinoic acid (13cRA) in mouse liver tissues collected at different gestational time points were measured by LC-MS/MS. (*n*=3-4), * $p < 0.05$; ** $p < 0.01$, one-way ANOVA vs. virgin.

To determine whether atRA alters CYP2D6 expression and activity *in vivo*, atRA was intraperitoneally administered to Tg-*CYP2D6* mice, and livers were collected to measure expression and activity of hepatic CYP2D6. atRA significantly decreased CYP2D6 in mRNA and protein levels, and this was accompanied by enhanced SHP expression (Fig. 16A and 16B). Results from CYP2D6 phenotyping (by using debrisoquine as a probe drug for CYP2D6) revealed a significant decrease in CYP2D6 activity upon atRA treatment as compared with vehicle treatment (Fig. 16C).

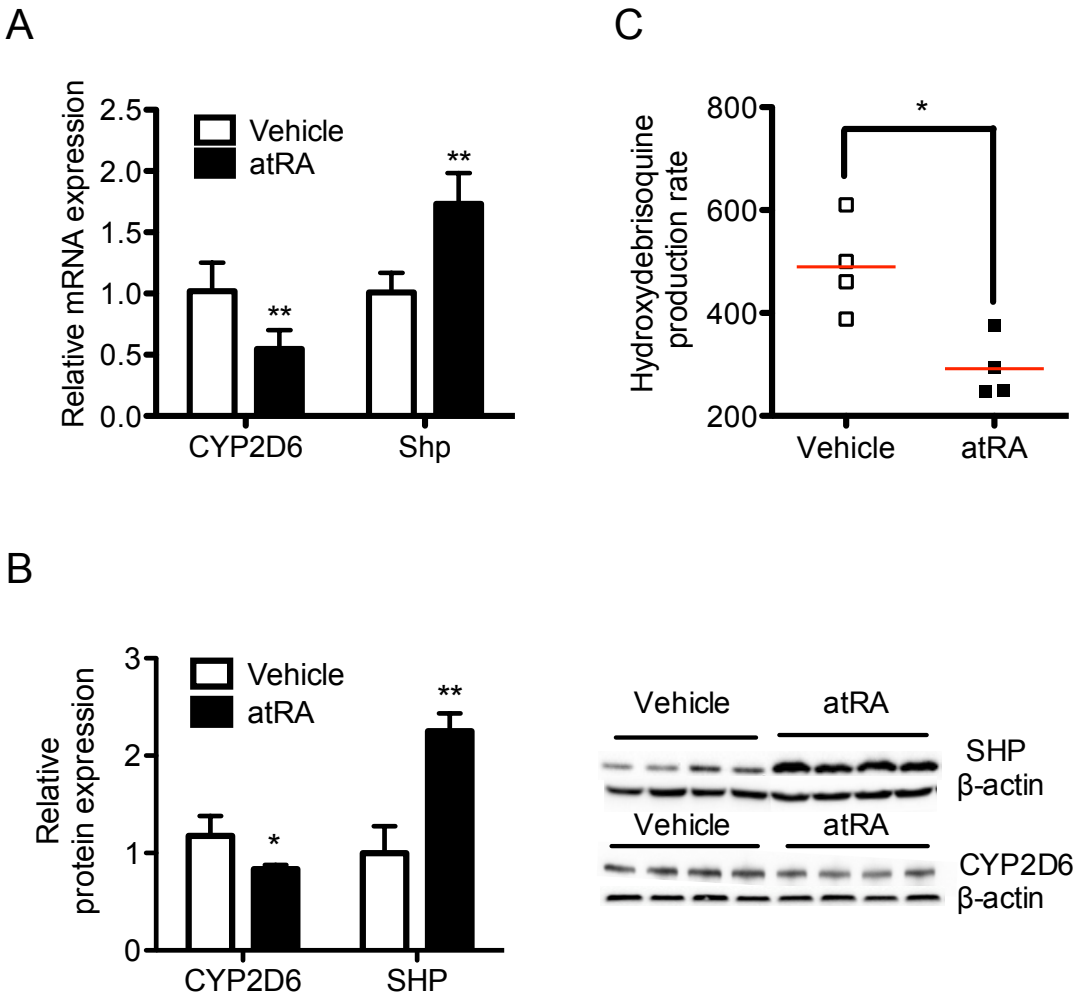

Figure 16. atRA represses CYP2D6 expression and activity in Tg-*CYP2D6* mice. Tg-*CYP2D6* mice were injected intraperitoneally with atRA (5 mg/kg) or vehicle (olive oil) daily for 5 days. A, CYP2D6 and Shp mRNA expression was determined by qRT-PCR. B, CYP2D6 and SHP protein expression levels were measured by western blot. The image of western blot (right) is shown, and band intensities (divided by β-actin, left) were quantified after normalization by CYP2D6 or SHP expression in the vehicle-treated mice, respectively. C, Liver S9 fractions from the liver tissues of Tg-*CYP2D6* mice were incubated with debrisoquine (200 μM), and 4-hyrdoxydebrisoquine concentrations were measured by using LC-MS/MS. Data shown are 4-hydroxydebrisoquin production rates (in pmol/min/mg protein). Values are presented as mean ± S.D. (n=4), *p < 0.05; **p < 0.01, Student's t-test vs. vehicle treatment.

To determine whether repressive effect of atRA on CYP2D6 is mediated by changes in HNF4α activity on CYP2D6 promoter, ChIP assays were performed using liver tissues of Tg-*CYP2D6* mice administered with atRA. The results showed that atRA decreased the recruitment of HNF4α as well as Pol II to CYP2D6 promoter as compared with the vehicle treated control, while increasing SHP recruitment to the promoter (Fig. 17).

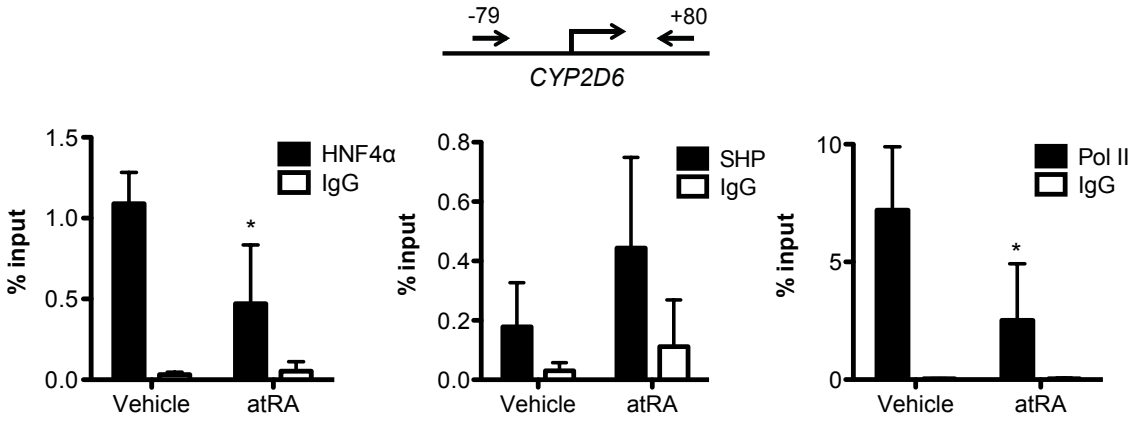

Figure 17. atRA represses HNF4α transactivation of CYP2D6 promoter.
Tg-*CYP2D6* mice were injected intraperitoneally with atRA (5 mg/kg) or vehicle (olive oil) daily for 5 days. Recruitment of HNF4α, SHP, and Pol II to CYP2D6 promoter was analyzed by ChIP assays using mouse liver tissues. Values are presented as mean ± S.D. (*n*=4), **p* < 0.05, Student's t-test vs. vehicle treatment.

To determine whether atRA effects on CYP2D6 expression obtained in mice can be extrapolated to humans, primary human hepatocytes were treated with atRA or vehicle for 48 hours, and CYP2D6 expression and activity were examined. The results showed that atRA treatment decreased CYP2D6 mRNA expression by ~3-fold (Fig. 18A) while enhancing the SHP expression by ~3-fold (Fig. 18A) in human hepatocytes. In one batch of human hepatocytes (i.e., HH2), CYP2D6 activity levels were measured by using debrisoquine as a probe drug for CYP2D6. CYP2D6 activity in atRA-treated cells was

significantly lower (Fig. 18B), but to a small extent (~25%) as expected from the long degradation half-life of CYP2D6 protein (i.e., 51 hours) (120). Together, these results indicate that as in Tg-*CYP2D6* mice, atRA represses CYP2D6 expression in human hepatocytes.

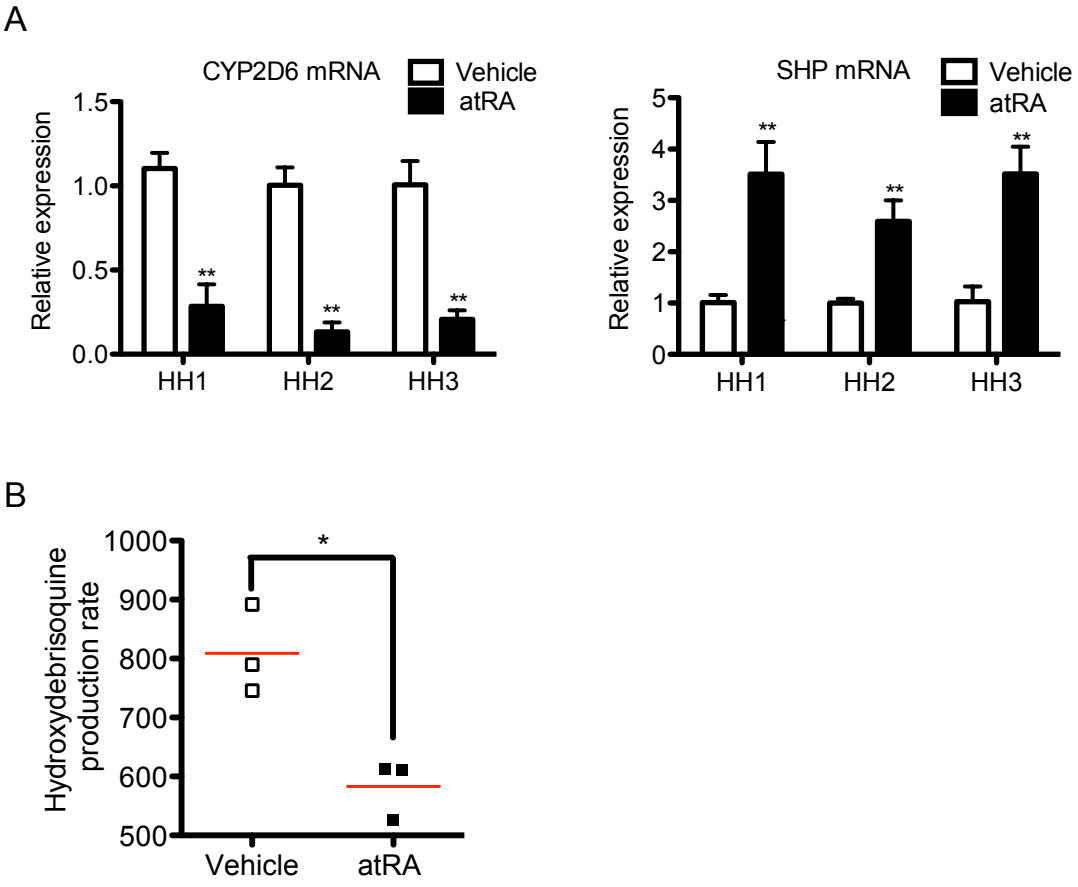

Figure 18. atRA represses CYP2D6 expression and activity in primary human hepatocytes.
Primary human hepatocytes (HHs) from three different donors were treated with atRA (10 μM) or vehicle control (DMSO) for 48 hours. A, CYP2D6 and SHP mRNA expression was determined by qRT-PCR. B, Liver S9 fractions prepared from HH2 were incubated with debrisoquine (200 μM), and 4-hyrdoxydebrisoquine concentrations were measured by LC-MS/MS. Data shown are 4-hydroxydebrisoquin production rates (in pmol/min/mg protein). Values are presented as mean ± S.D. (n=3), * $p < 0.05$; ** $p < 0.01$, Student's t-test vs. vehicle treatment.

A model depicting the proposed mechanisms underlying CYP2D6 induction during pregnancy is shown in Fig. 19. We identified SHP as a novel transcriptional regulator of CYP2D6. SHP represses the transactivation of CYP2D6 promoter by HNF4α. During pregnancy, SHP plays an essential role in enhanced CYP2D6 expression. Decreased hepatic atRA content is in part responsible for decreased SHP expression and subsequently enhanced CYP2D6 expression during pregnancy.

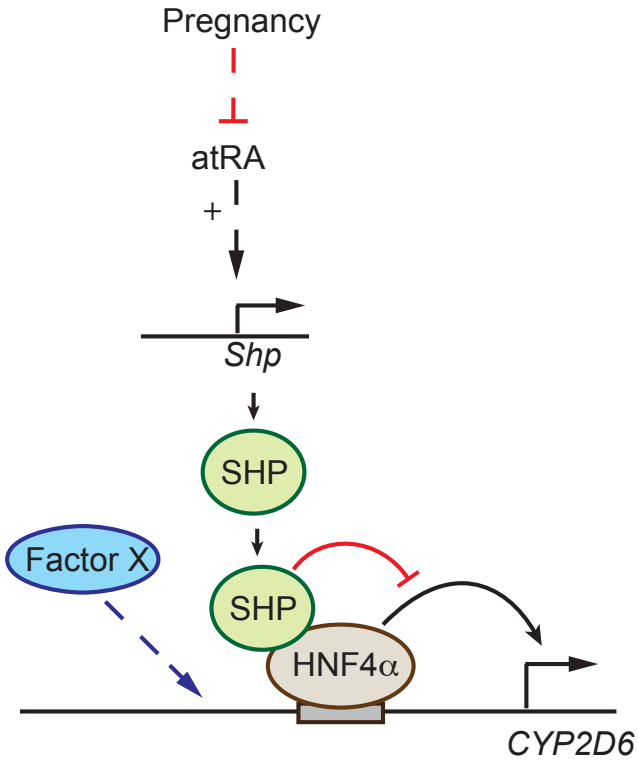

Figure 19. Proposed model for CYP2D6 induction during pregnancy.
This working model illustrates a mechanism underlying CYP2D6 induction during pregnancy. SHP is a novel transcriptional regulator of CYP2D6; SHP represses HNF4α transactivation of CYP2D6 promoter. During pregnancy, decreased SHP expression may be due to low retinoic acid content in the liver. Decreased SHP expression then de-represses the transactivation of CYP2D6 promoter by HNF4α, leading to CYP2D6 induction in expression and activity levels. As-yet-unknown factor (factor x) may also play a role in the regulation of CYP2D6 basal expression, but play a minimal role in up-regulating CYP2D6 expression during pregnancy.

2.4. Discussion

CYP2D6-mediated drug metabolism is increased during pregnancy, but the underlying mechanisms remained unknown. Results from a previous study in human hepatocytes indicate that estrogen and progesterone (whose concentrations increase >100-fold during human pregnancy) do not affect CYP2D6 expression (121), suggesting that female hormones play minor roles (if any) in CYP2D6 induction during pregnancy. In this study, using Tg-*CYP2D6* mice as an *in vivo* model, we provide evidence that CYP2D6 induction during pregnancy is coordinated by liver-enriched transcription factors SHP and HNF4α and that this is in part triggered by the altered hepatic RA contents. This study represents the first report on the mechanisms underlying CYP2D6 induction during pregnancy.

The identification of mechanisms underlying altered drug metabolism during pregnancy has been challenging due to the lack of appropriate experimental models. In this study, we demonstrate that Tg-*CYP2D6* mice can serve as an *in vivo* model for CYP2D6 induction during pregnancy, achieving a major breakthrough for the mechanistic studies of pregnancy-mediated changes in drug metabolism. An improved understanding of these mechanisms may provide a basis to develop physiologically based pharmacokinetic models (122) that can be used to predict dosage adjustments required for pregnant women. This underscores the necessity and utility of appropriate *in vivo* mouse models for CYP2D6 regulation during pregnancy.

Our mechanistic studies reveal that repressed SHP expression during pregnancy leads to enhanced HNF4α activity on CYP2D6 promoter. SHP is a nuclear receptor lacking the DNA binding domain and represses the activity of multiple transcription

factors (85). A previous study has shown that SHP binds to N-terminal region of HNF4α, thereby blocking HNF4α binding to the promoter of a target gene (123). Consistent with the reported mechanism, our ChIP results from Tg-*CYP2D6* mice showed increased HNF4α recruitment to CYP2D6 promoter at term pregnancy, accompanied by decreased the recruitment of SHP to the promoter. The reciprocal relationship between HNF4α and SHP recruitment to CYP2D6 promoter is likely due to the rapid and dynamic interaction between DNA and transcription factors (124). Of note, in the repression of HNF4α transactivation of CYP2D6 by SHP, an appropriate expression level of HNF4α appears to be critical; the pregnancy-mediated changes in CYP2D6 expression or in SHP recruitment to CYP2D6 promoter were abrogated in *Hnf4α(wt/-);CYP2D6* mice. HNF4α expression is known to decrease during hyperinsulinemia accompanying diabetes (125). Whether diabetes during pregnancy (affecting ~18% of pregnancies) impacts the extent of CYP2D6 induction remains to be determined.

The pregnancy-mediated changes in HNF4α activity apparently affect only a subset of HNF4α target genes (including CYP2D6) because mRNA levels of other target genes (ApoC2 and Hes6) as well as HNF4α recruitment to their promoters exhibited patterns different from those of CYP2D6. These findings are in part consistent with the previous ChIP results that SHP associates with only a subset of the promoters occupied by HNF4α (126). The promoter context may provide an additional specificity of SHP-mediated repression of HNF4α action.

The essential role of SHP in CYP2D6 induction during pregnancy was further verified in *Shp(-/-);CYP2D6* mice in that CYP2D6 induction at term was abrogated in the mice. Interestingly, however, the basal expression levels of CYP2D6 in *Shp(-/-);CYP2D6*

mice did not differ from those in *Shp(+/+);CYP2D6* mice (Fig. 12A). This appears inconsistent with the results obtained in Tg-*CYP2D6* mice that SHP knockdown (by using siRNA) led to increased CYP2D6 expression (Fig. 8B). Compensational changes in hepatic gene expression (or activity) in *Shp(-/-);CYP2D6* mice may explain the results. For example, DAX-1 (dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X chromosome, gene 1, NR0B1) is a nuclear receptor that lacks the DNA-binding domain similarly to SHP (127), and it is known to repress HNF4α transactivation of a hepatic gene (128). Our qRT-PCR experiment, however, revealed that Dax-1 mRNA expression is undetectable in mouse liver tissues, suggesting that DAX-1 is not a transcription factor that may compensate for the loss of SHP in the liver. Another candidate is SMILE (SHP-interacting leucine zipper protein, initially identified as a SHP-interacting protein (129)) has been shown to repress HNF4α transactivation of target genes in the absence of SHP (130). Our result showed, however, the mRNA expression level of Smile was lower in *Shp(-/-);CYP2D6* female mice as compared to the *Shp(+/+);CYP2D6* female mice (Fig. 20). Whether the repressive transcriptional *activity* of SMILE is enhanced in *Shp(-/-);CYP2D6* mice remains to be determined. While basal CYP2D6 expression appears to be governed by as-yet-unknown factors in *Shp(-/-);CYP2D6* mice, the abrogation of CYP2D6 induction at term pregnancy in *Shp(-/-);CYP2D6* mice indicates that SHP plays a critical role in CYP2D6 induction during pregnancy. The as-yet-unknown factors that maintain the basal level of CYP2D6 expression in *Shp(-/-);CYP2D6* mice play a minimal role (if any) in the regulation of CYP2D6 expression during pregnancy.

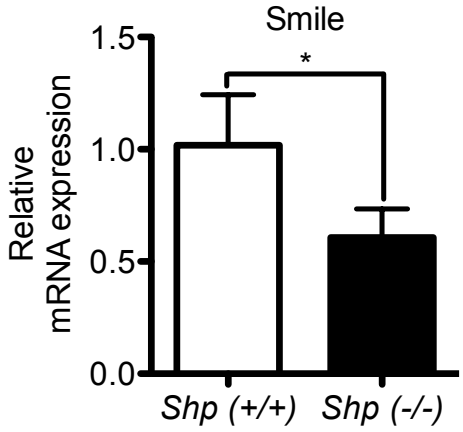

Figure 20. Smile expression is decreased in *Shp(-/-);CYP2D6* female mice.
Liver tissues were collected from *Shp(-/-);CYP2D6* and *Shp(+/+);CYP2D6* female mice (virgin, *n*=4). Smile mRNA expression was determined by qRT-PCR. Values are presented as mean ± S.D. **p* < 0.05, Student's t-test.

FXR is a key mediator in maintenance of cholesterol/bile acid homeostasis; during cholestasis, bile acids bind to FXR, which transactivates SHP promoter. SHP in turn represses the expression of genes involved in bile acid synthesis (126). Hepatic Shp mRNA levels in *Fxr*-null mice was only ~80% of those in wild type (17), suggesting that FXR plays a key role in modulating SHP expression. Our data indicate, however, that pregnancy affects neither FXR expression nor FXR recruitment to Shp promoter. Furthermore, our microarray data (GSE50166) showed that pregnancy did not repress the expression of known transcriptional coactivators of FXR, namely arginine-methyl transferases (131), coactivator-associated arginine methyltransferase 1 (132), peroxisome proliferator-activated receptor γ coactivator 1 α (133), and vitamin D-interacting protein 205 (134). Together, these results indicate that FXR plays a minimal role, if any, in the downregulation of SHP during pregnancy.

Retinoids are derivatives of vitamin A (retinol) that provides support to many crucial physiological processes including embryogenesis, epithelial differentiation, vision, energy balance, and reproduction (135,136). Liver is an essential site for retinoids

uptake, storage, and metabolism. In the liver, stellate cells account for 80% of total liver retinoids, while hepatocytes that account for 67% of all cells present in the liver plays a key role in forming the bioactive metabolite atRA (137,138). Our data revealed that repressed SHP expression at term pregnancy was associated with decreased hepatic levels of atRA. The decrease in hepatic contents of atRA may be attributable to enhanced hepatic expression of CYP26A1 during pregnancy (139). CYP26A1 expressed in livers is a major RA-metabolizing enzyme and a key contributor to endogenous RA clearance (140). How pregnancy increases Cyp26a1 expression remains unknown. atRA was shown to induce SHP expression in HepG2 cells (119), and our results further showed that exogenously administered atRA decreases CYP2D6 expression. Together, these results suggest that decreased hepatic RA contents and subsequent decreases in SHP expression may be in part responsible for enhanced CYP2D6 expression during pregnancy.

The repressed SHP expression levels at term pregnancy returned to pre-pregnancy levels after delivery, while hepatic levels of RA did not. This suggests the presence of other regulators of SHP expression whose expression and/or activity may be altered during the postpartum period. Our ChIP study demonstrated a significant increase in FXR recruitment to the Shp promoter at postpartum, suggesting that activation of FXR may be responsible for the rebound in SHP expression after delivery. Of note, expression levels of other target genes of FXR (e.g., bile salt export pump) did not differ between the term pregnancy and postpartum period (GSE50166), indicating that enhanced FXR activity after delivery may be specific for Shp promoter. Shp promoter harbors an ERα-binding site overlapping with the FXR binding site (141) such that ligand-activated ERα can repress FXR transactivation (118). After delivery, plasma

concentrations of both estrogens and bile acids change in the direction of promoting FXR transactivation; concentrations of estrogen decrease while those of bile acid increase (142). These changes may lead to increased FXR transactivation of Shp promoter in an additive or synergistic manner. Together, SHP expression level during postpartum period may be coordinated by multiple endogenous substances, including estrogens and bile acids.

CYP2D6 shows the largest phenotypic variability among drug-metabolizing CYPs. This is in part due to genetic polymorphisms of *CYP2D6*; over 100 different variants have been reported to date, and some of the gene products exhibit no or reduced enzyme activity (http://www.cypalleles.ki.se). On the other hand, CYP2D6 activity in human liver tissues correlates well with CYP2D6 mRNA levels (the correlation coefficient ranging from 0.71 to 0.91) (14,15,143), suggesting that transcriptional regulation of CYP2D6 may play a major role in governing the extent of CYP2D6-mediated drug metabolism. The finding of interplay among retinoic acid and hepatic transcription factors HNF4α and SHP during pregnancy may provide a basis to better understand the factors contributing to large phenotypic variability in CYP2D6 activity. For example, one can speculate that vitamin A deficiency or overload may impact CYP2D6-mediated drug metabolism through modulating SHP expression.

In conclusion, we present the first case of utilizing CYP-humanized mice for the *in vivo* functional and mechanistic investigation of altered drug metabolism during pregnancy. Our results will likely provide a basis to improve drug therapy during pregnancy as well as to better understand factors governing variability in CYP2D6-mediated drug metabolism.

3. FARNESOID X RECEPTOR AGONIST REPRESSES CYP2D6 EXPRESSION BY UPREGULATING SMALL HETERODIMER PARTNER

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3.1. Introduction

CYP2D6-mediated drug metabolism is known to exhibit significant interindividual variability (10,11); the populations can be divided into four phenotype categories ranging from poor metabolizer (PM) to ultra-rapid metabolizer (UM) (10-13,49). This interindividual variability is in part explained by genetic polymorphisms in CYP2D6 gene; polymorphisms associated with low or minimal expression of CYP2D6 protein (e.g., due to frame-shift mutation) or the expression of nonfunctional CYP2D6 proteins lead to the PM phenotype. On the other hand, individuals with multiple copies of CYP2D6 gene present UM phenotype. Of note, these individuals comprise only a small portion (~10%) of populations, and the sources of CYP2D6 variability in the remaining populations remain unclear. For example, CYP2D6 activity (as determined by urinary ratio of dextrorphan/dextromethorphan) exhibits significant overlap among non-PM individuals (53) such that CYP2D6 genotypes do not fully explain the inter-individual variability.

Previous studies have shown that the mRNA expression and activity levels of CYP2D6 are well correlated (14,15), suggesting that differential transcriptional regulation of CYP2D6 may contribute to the large interindividual variability in CYP2D6 activity (14). Yet, transcriptional regulation of CYP2D6 is poorly understood. For example, hepatocyte nuclear receptor 4α (HNF4α) has been the only well-characterized transcription factor for the transcriptional regulation of CYP2D6 until recently (9). In Chapter 2, we have

demonstrated a novel transcriptional regulator of CYP2D6 expression, SHP, through studies of regulatory mechanisms underlying CYP2D6 induction during pregnancy (144). SHP is a nuclear receptor that lacks the DNA binding domain. SHP functions as a co-repressor; SHP binds to other transcription factors (e.g., HNF4α) and inhibits their transcriptional activity. This leads to altered expression of many genes involved in bile acid homeostasis, glucose metabolism, and testosterone synthesis (85,145). We also demonstrated that SHP represses HNF4α transactivation of CYP2D6 promoter and that SHP knockdown (by using siRNA targeting SHP) led to increased CYP2D6 expression in CYP2D6-humanized transgenic (Tg-*CYP2D6*) mice (Fig. 8B) (144). What remains unknown is whether upstream regulators of SHP expression alter CYP2D6 expression such that differential hepatic levels of SHP modulators may contribute to interindividual variability in CYP2D6-mediated drug metabolism.

SHP is a representative target gene of farnesoid X receptor (FXR). FXR is a ligand-activated nuclear receptor, and bile acids are its biological ligands (16). When hepatic concentrations of bile acids are high (e.g., in cholestasis), the ligand-activated FXR induces SHP transcription. SHP in turn inhibits bile acid synthesis and represses hepatic bile acid uptake, thereby protecting the liver from the toxicity of excess bile acids (145-149). Recent studies also indicate FXR as a modulator of nutrient homeostasis, and many synthetic FXR agonists (e.g., GW4064) are currently developed as novel therapeutics to treat metabolic diseases including primary biliary cirrhosis, nonalcoholic steatohepatitis, diabetes and insulin resistance (150).

In this proof-of-concept study, we aimed to verify the role of SHP in the regulation of CYP2D6 expression by examining how a known regulator of SHP expression (FXR agonist GW4064 (18)) alters CYP2D6 expression. To this end, we investigated whether

a, alters CYP2D6 expression by inducing SHP expression. We provide evidence that GW4064 indeed represses CYP2D6 expression in a SHP-dependent manner.

3.2. Materials and methods

3.2.1. Animals

CYP2D6-humanized transgenic (Tg-*CYP2D6*) and *Shp*-null mice were previously described (9,93). Both Tg-*CYP2D6* and *Shp*-null mice were on the C57BL/6 background. Adult male mice (8 weeks of age; 20-25 g body weight) were used for the experiments. GW4064 (10 mg/kg) or vehicle (olive oil) was injected intraperitoneally into the mice daily for 5 days (*n*=4-5 mice/group). Mice were sacrificed on the 6th day, and liver tissues were collected. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Institution's Animal Care and use Committee in the University of Illinois at Chicago.

3.2.2. Chemicals and reagents

Debrisoquine, (±)-4-hydroxydebrisoquin, and paraxanthine were purchased from Biomol (Plymouth Meeting, PA). GW4064, (3-[2-[2-Chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid) was purchased from Sigma-Aldrich (St. Louis, MO).

3.2.3. Primary human hepatocytes

Freshly isolated human hepatocytes, derived from three donors, were obtained from Liver Tissue Cell Distribution System (Pittsburgh, PA; funded by NIH Contract #

HHSN276201200017C). Hepatocytes were cultured as previously described in Chapter 2. After recovery from delivery, hepatocytes were treated with vehicle control (DMSO) or GW4064 (1 μM) for 48 hours. Cell lysates were collected to prepare RNAs and S9 fractions.

3.2.4. Western blot

Western blot was performed as described previously in Chapter 2. CYP2D6 and SHP protein expression levels were determined by using the respective antibodies (CYP2D6, Catalog#458246, BD Gentest™, Franklin Lakes, NJ; SHP, sc-30169, Santa Cruz, Dallas, TX; β-actin, Catalog#A1978, Sigma-Aldrich).

3.2.5. Determination of CYP2D6 activity

Hepatic S9 fractions were prepared as described previously (94). S9 fractions were incubated with debrisoquine (a CYP2D6 probe substrate, 200 μM) based on the report that mouse endogenous CYP2Ds play minor roles in debrisoquine hydroxylation (144). The concentration of 4-hydroxydebrisoquine was measured by LC-MS/MS using paraxanthine as the internal standard (144).

3.2.6. RNA isolation and qRT-PCR

Total RNA isolation and qRT-PCR were performed as described previously in Chapter 2. The PrimeTime® qRT-PCR assay for mouse and human CYP8B1 genes were purchased from Integrated DNA Technologies (Coralville, IA; Mm.PT.58.12268653.g and Hs.PT.58.40608207.g, respectively). Primer sequences for all other target genes were described in Table VII. Results are expressed as fold

changes by drug treatment by using the gene expression levels normalized to those of Gapdh ($2^{-\Delta\Delta Ct}$ method).

3.2.7. ChIP assays

Liver samples were subjected to ChIP as described previously in Chapter 2. qRT-PCR was performed using the following probes for Cyp8b1: 5′-AAGGCAGGCAAACATGGAGA-3′ (forward) and 5′- CAATGCAAAGGTTCCTGCCC-3′ (reverse). Primer sequences for all other genes were described in Table VIII.

3.2.8. Statistical analysis

Values were reported as mean ± standard deviation (S.D.). Statistical differences were determined by using Student's t-test.

3.3. Results

3.3.1. FXR agonist GW4064 represses CYP2D6 expression and activity in Tg-*CYP2D6* mice

To determine whether GW4064 alters CYP2D6 expression and activity *in vivo*, GW4064 or vehicle control was intraperitoneally administered to Tg-*CYP2D6* mice for 5 days, and hepatic CYP2D6 mRNA and protein levels were measured by qRT-PCR and western blot, respectively. Cyp8b1, a gene known to be down-regulated by SHP (151), was used as a positive control. The results showed that GW4064 significantly decreased both mRNA and protein expression levels of CYP2D6 by ~2-fold (Fig. 21A and 21B). CYP2D6 activity, determined by measuring debrisoquine hydroxylation rate in hepatic

S9 fraction (144), was also found decreased by ~2-fold upon GW4064 treatment (Fig. 21C). GW4064 increased SHP expression ~2-fold (Fig. 21A and 21B), consistent with the previous results (86,152).

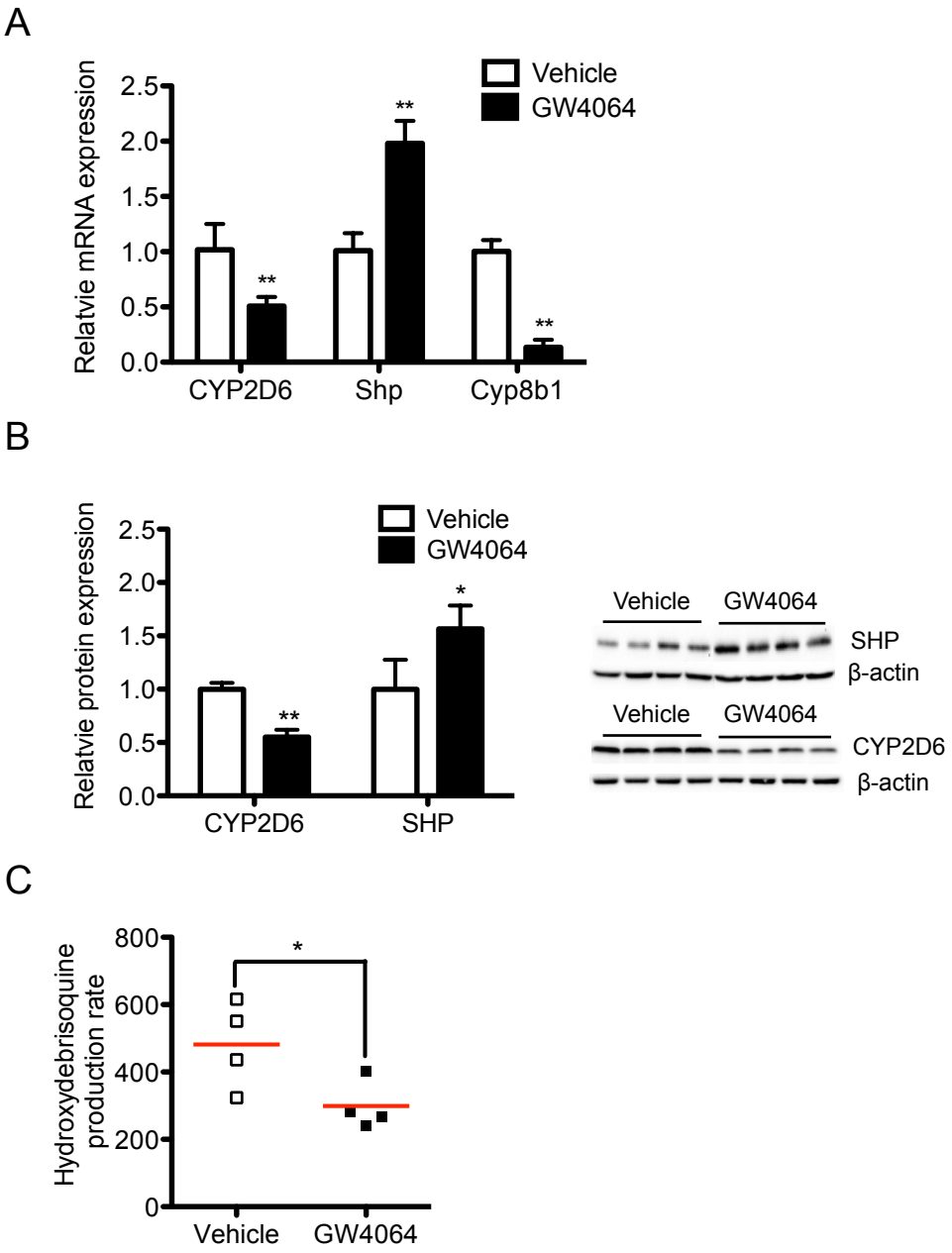

Figure 21. GW4064 represses CYP2D6 expression in Tg-*CYP2D6* mice.
Tg-*CYP2D6* mice were administered with GW4064 (10 mg/kg) or vehicle (olive oil) intraperitoneally daily for 5 days (*n*=4). A, CYP2D6, Shp, and Cyp8b1 mRNA expression was determined by qRT-PCR. B, CYP2D6 and SHP protein expression levels were determined by western blot. The image of western blot (right) is shown, and the band intensities (after divided by β-actin, left) were quantified after normalization by CYP2D6 or SHP expression in the vehicle-treated mice, respectively. C, S9 fractions were prepared from the liver tissues of Tg-*CYP2D6* mice treated with GW4064 or vehicle control, and CYP2D6 phenotyping was performed using debrisoquine (200 μM). Data shown are 4-hydroxydebrisoquin production rates (in pmol/min/mg protein). Values are presented as mean ± S.D. *p < 0.05; **p < 0.01, versus vehicle treatment.

Previously, we showed that SHP represses HNF4α transactivation of CYP2D6 promoter (144). To determine whether GW4064 alters CYP2D6 promoter activity in mice, ChIP assays were performed. Liver tissues were collected from GW4064 (or vehicle)-treated mice and subjected to ChIP using antibodies against SHP, HNF4α or RNA polymerase II (Pol II, a marker of transcription initiation). The protein-bound DNA was analyzed by using a primer set that can detect the HNF4α response element at -55/-43 of CYP2D6 (63). As a positive and a negative control, recruitment of the transcription factors to Cyp8b1 promoter or a downstream region of CYP2D6 (+3913/+4368), respectively, was examined. The results demonstrated increased recruitment of SHP and decreased recruitments of HNF4α and Pol II to CYP2D6 promoter (Fig. 22A). The similar trends were observed for SHP, HNF4α, and Pol II recruitments to Cyp8b1 promoter region (that harbors HNF4α response element (151)) (Fig. 22B). Recruitment of the transcription factors to the downstream region of CYP2D6 was minimal and not affected by GW4064 (Fig. 22C). Together, these results suggest that the repressive effect of GW4064 on CYP2D6 is potentially mediated by enhanced SHP expression.

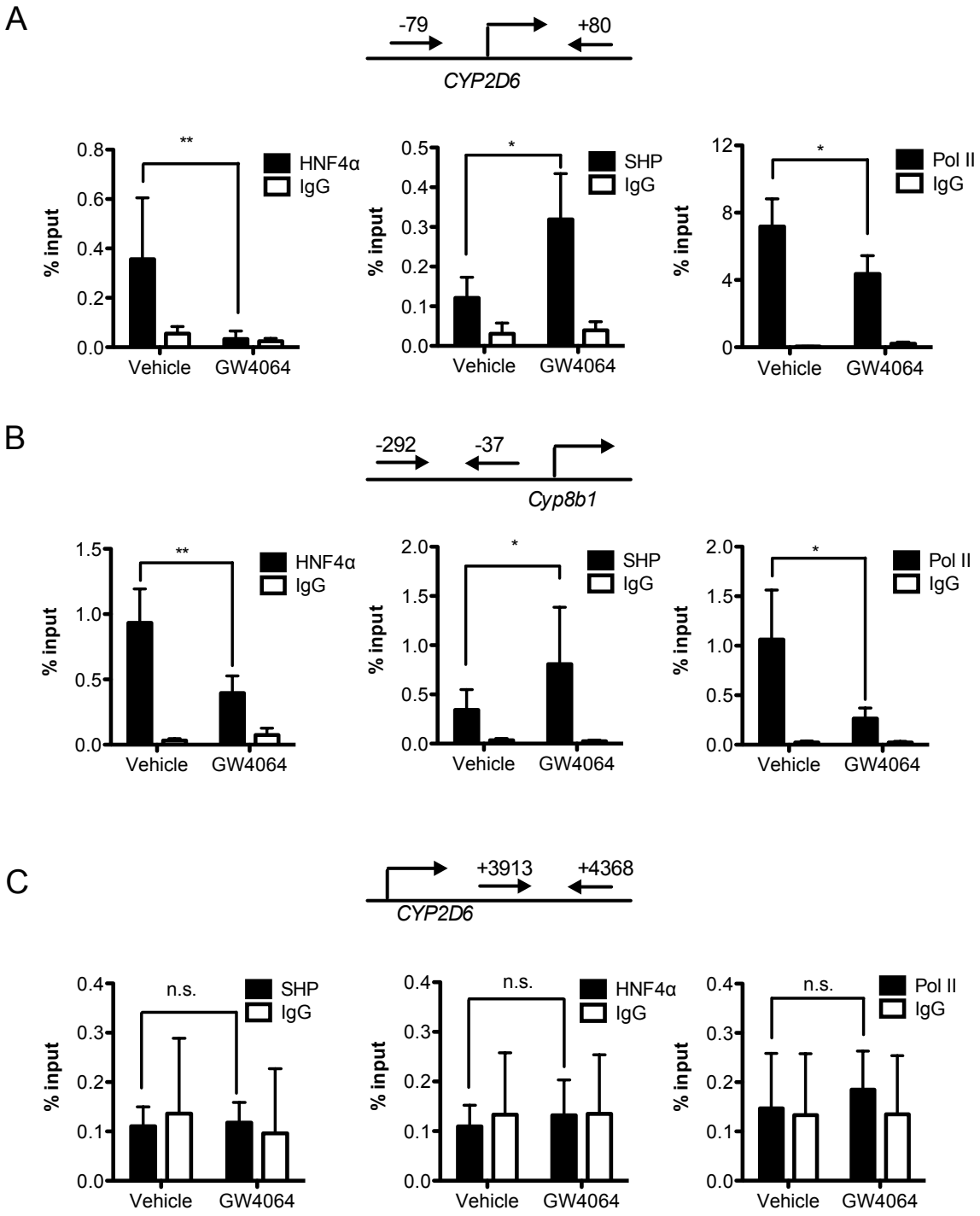

Figure 22. GW4064 represses HNF4α transactivation of CYP2D6 promoter.
Tg-*CYP2D6* mice were administered with GW4064 (10 mg/kg) or vehicle (olive oil) intraperitoneally daily for 5 days (*n*=4). Recruitment of HNF4α, SHP, and RNA polymerase II onto (A) CYP2D6 promoter, (B) Cyp8b1 promoter, and (C) CYP2D6 distal downstream region were analyzed by ChIP assay using mouse liver tissues. Values are presented as mean ± S.D. **p* < 0.05; ***p* < 0.01, n.s. = not statistically significant.

3.3.2. CYP2D6 repression by GW4064 is abrogated in *Shp(-/-);CYP2D6* mice

To examine the essentiality of SHP in CYP2D6 repression by GW4064, Tg-*CYP2D6* mice were crossed with *Shp*-null mice, and mice carrying *Shp(+/+);CYP2D6* or *Shp(-/-);CYP2D6* genotype were generated. None of *Shp(+/+);CYP2D6* or *Shp(-/-);CYP2D6* mice exhibited any prominent phenotypes, and all grew normally. Western blot results showed that SHP protein expression was abolished in *Shp(-/-);CYP2D6* mice (Fig. 12A). CYP2D6 repression by GW4064 or vehicle was compared between the mice of different genotypes. In the vehicle-treated mice, the basal mRNA expression levels of Cyp8b1 were higher in *Shp(-/-);CYP2D6* as compared to *Shp(+/+);CYP2D6* mice (Fig. 23A; $p = 0.0004$) while the basal CYP2D6 expression did not differ between the mice of different genotypes (Fig. 23A; $p = 0.16$). GW4064 treatment led to decreased expression of CYP2D6 and Cyp8b1 in *Shp(+/+);CYP2D6* mice (Fig. 23A), similar to the results from Tg-*CYP2D6* mice (Fig. 21A). These repressive effects of GW4064 on CYP2D6 and Cyp8b1 expression were abrogated in *Shp(-/-);CYP2D6* mice (Fig. 23A), suggesting that GW4064 represses CYP2D6 transcription through SHP. The protein expression as well as activity level of CYP2D6 was consistent with the decreased mRNA levels of CYP2D6 by GW4064 (Fig. 23B and 23C).

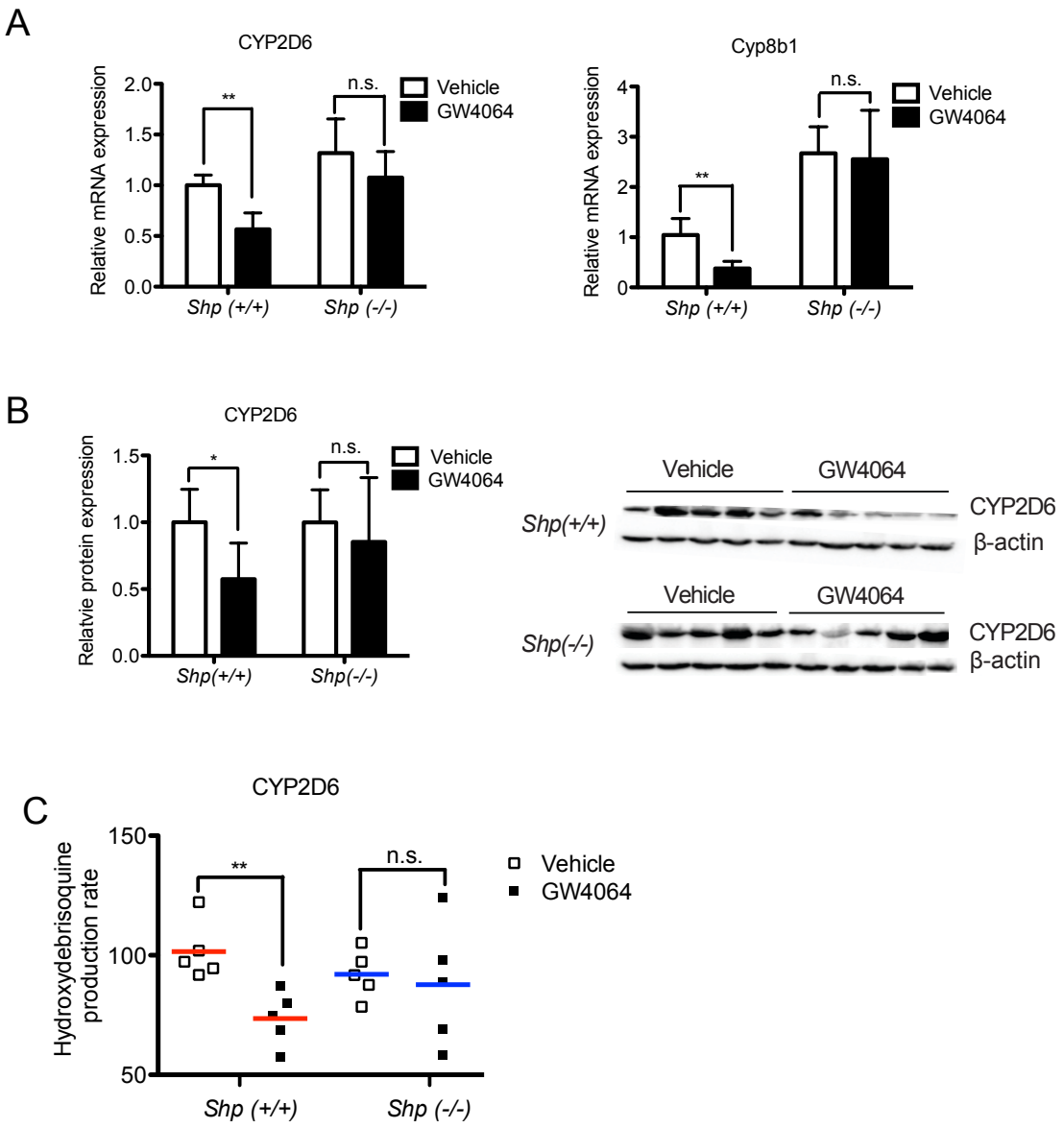

Figure 23. CYP2D6 repression by GW4064 is abrogated in *Shp(-/-);CYP2D6* mice. *Shp(+/+);CYP2D6* or *Shp(-/-);CYP2D6* mice were injected with GW4064 (10 mg/kg) or vehicle (olive oil) intraperitoneally daily for 5 days (*n*=5). A, CYP2D6 and Cyp8b1 mRNA expression levels were measured by using qRT-PCR and normalized by those in vehicle-treated *Shp(+/+);CYP2D6* mice. B, CYP2D6 protein expression level was measured by western blot. The image of western blot (right) is shown, and the band intensities (CYP2D6/β-actin, left) were quantified after normalization by CYP2D6 expression in the vehicle-treated mice of respective genotype. C, S9 from the mice was incubated with debrisoquine (200 μM), and 4-hyrdoxydebrisoquine concentrations were measured by using LC-MS/MS. Data shown are 4-hydroxydebrisoquin production rates (in pmol/min/mg protein). Values are presented as mean ± S.D. *$p < 0.05$; **$p < 0.01$; n.s. = not statistically significant.

To determine whether Shp deletion leads to altered GW4064 effects on the HNF4α transactivation of CYP2D6 promoter, ChIP assays were performed. In *Shp(+/+);CYP2D6* mice, GW4064 decreased the recruitments of HNF4α and Pol II while increasing SHP recruitment to CYP2D6 promoter (Fig. 24A) as in Tg-*CYP2D6* mice (Fig. 22). These changes in transcription factors recruitment disappeared in *Shp(-/-);CYP2D6* mice (Fig. 24A). Similar results were observed in the transcription factor recruitment to Cyp8b1 promoter (Fig. 24B). Together, these results suggest an essential role of SHP in CYP2D6 repression by GW4064.

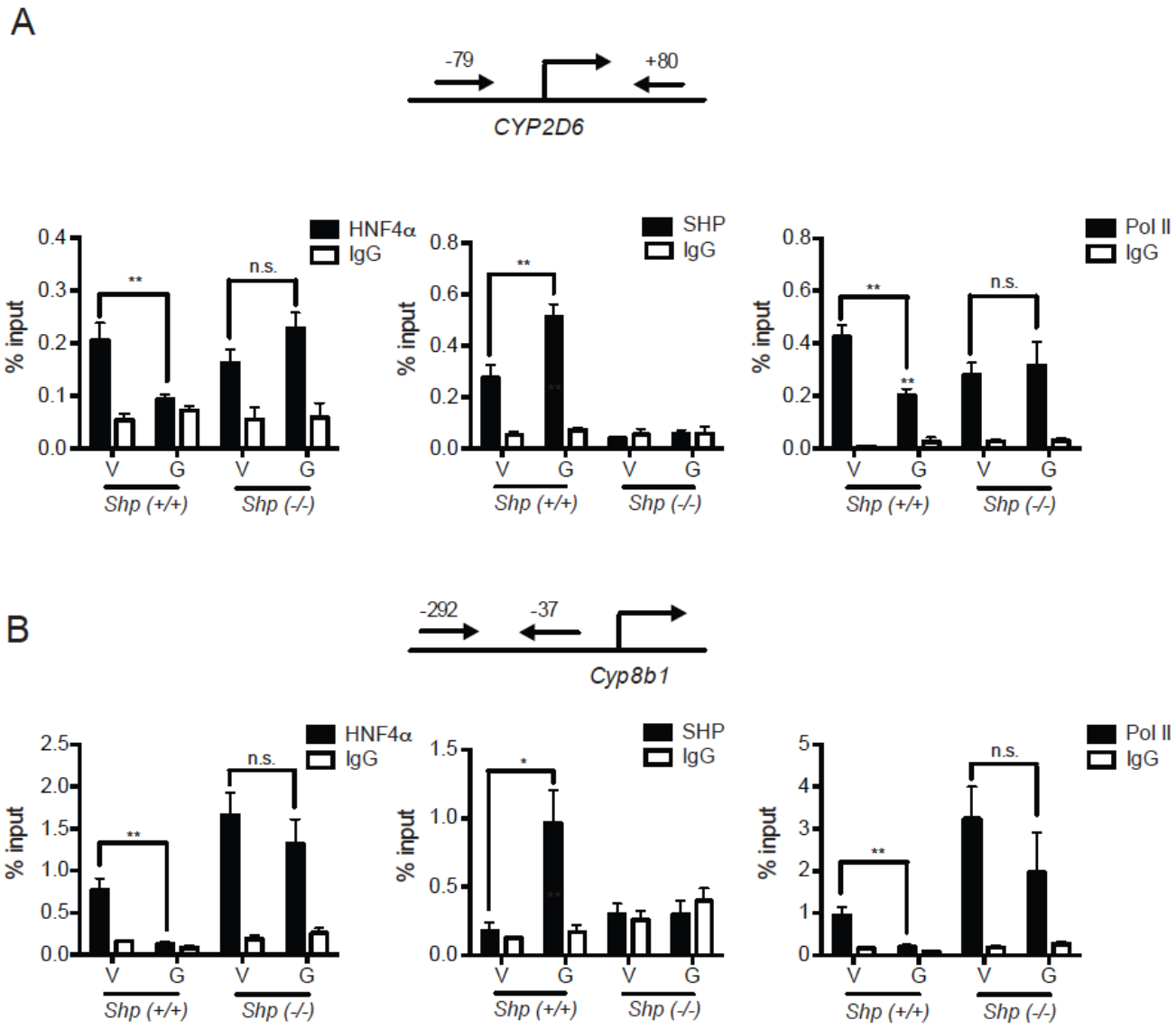

Figure 24. Repressed HNF4α transactivation of CYP2D6 promoter is abrogated in *Shp(-/-);CYP2D6* mice.
Shp(+/+);CYP2D6 or *Shp(-/-);CYP2D6* mice were injected with GW4064 (G, 10 mg/kg) or vehicle (V, olive oil) intraperitoneally daily for 5 days (*n*=5). Recruitment of HNF4α, SHP, and RNA polymerase II (Pol II) onto (A) CYP2D6 promoter and (B) Cyp8b1 promoter were analyzed by ChIP assay using mouse liver tissues. Values are presented as mean ± S.D. **p* < 0.05; ***p* < 0.01; n.s. = not statistically significant.

3.3.3. SHP represses CYP2D6 expression in primary human hepatocytes

To determine whether GW4064 effects on CYP2D6 expression obtained in mice can be extrapolated to humans, primary human hepatocytes were treated with GW4064 or vehicle for 48 hours, and CYP2D6 expression and activity were examined. The results showed that GW4064 treatment decreased CYP2D6 mRNA expression by ~2-fold (Fig. 25A) while enhancing the SHP expression (Fig. 25B) in human hepatocytes. The mRNA expression levels of positive control gene CYP8B1 were also increased in GW4064-treated hepatocytes (Fig. 25C). In one batch of human hepatocytes (i.e., HH2), CYP2D6 activity levels were measured by using debrisoquine as a probe drug for CYP2D6. CYP2D6 activity in GW4064-treated cells was significantly lower (Fig. 25D), but to a small extent (~20%) as expected from the long degradation half-life of CYP2D6 protein (i.e., 51 hours) (120). Together, these results indicate that as in Tg-*CYP2D6* mice, GW4064 represses CYP2D6 expression in human hepatocytes.

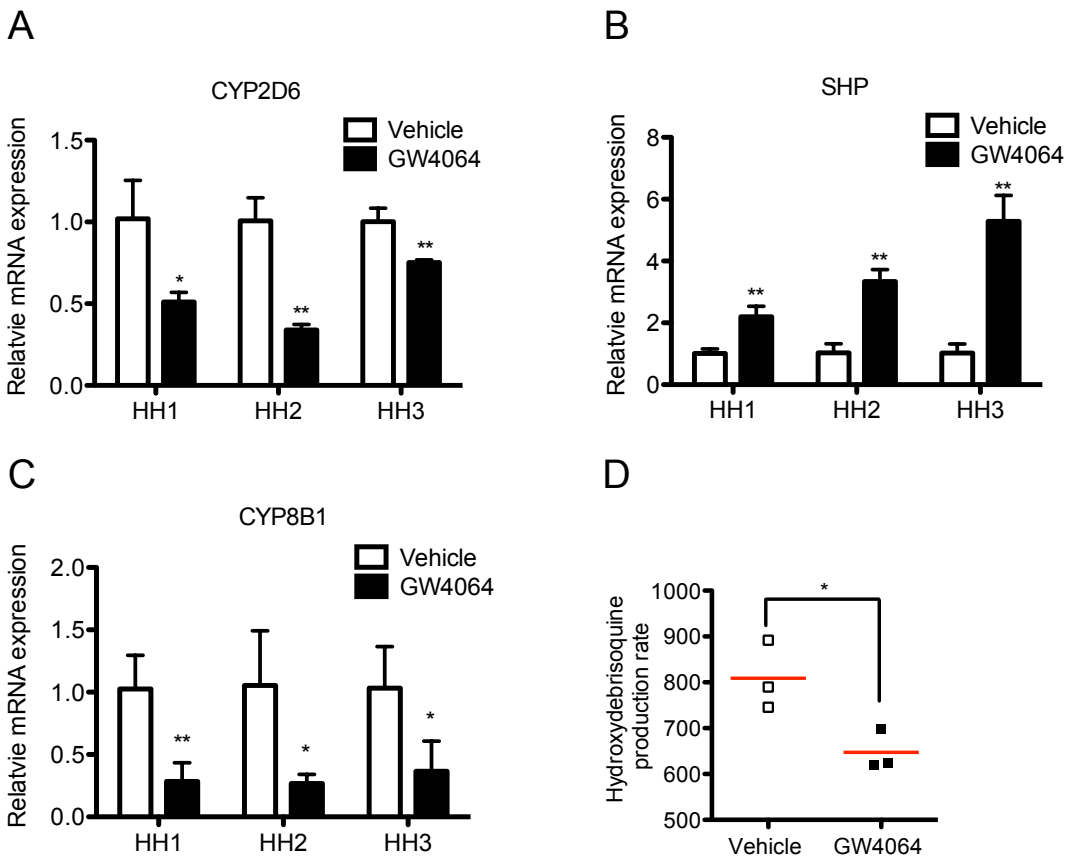

Figure 25. GW4064 represses CYP2D6 expression and activity in primary human hepatocytes.

Primary human hepatocytes (HHs) from three different donors were treated with GW4064 (1 μM) or vehicle control (DMSO) for 48 hours. A-C, CYP2D6, SHP, and CYP8B1 mRNA expression was determined by using qRT-PCR. D, HH2 was incubated with debrisoquine (200 μM), and 4-hyrdoxydebrisoquine concentrations in the culture media were measured by using LC-MS/MS. Data shown are 4-hydroxydebrisoquin production rates (in pmol/min/mg protein). Values are presented as mean ± S.D. *p < 0.05; **p < 0.01, versus vehicle treatment.

3.4. Discussion

Previously, we have identified SHP as a novel regulator of CYP2D6 transcription; SHP represses HNF4α transactivation of CYP2D6 promoter (144). SHP being a representative target gene of FXR, in this study we examined whether a synthetic FXR agonist (GW4064) alters CYP2D6 expression and showed that GW4064 represses CYP2D6 expression in Tg-*CYP2D6* mice as well as in human hepatocytes.

Results from this study demonstrated a key role of SHP in CYP2D6 repression by GW4064. The decrease in CYP2D6 expression in GW4064-treated mice was accompanied by the increases in SHP expression as well as in SHP recruitment to CYP2D6 promoter. The essential role of SHP in GW4064 action on CYP2D6 expression was further verified in *Shp(-/-);CYP2D6* mice in that CYP2D6 repression by GW4064 was abrogated in the mice. Considering that SHP induction is a class action of FXR agonists, these results suggest that FXR activation by other drugs or diseases (e.g., cholestasis) is also expected to repress CYP2D6 expression. Indeed, we found that treatment of human hepatocytes with cholic acid (a major bile acid elevated in cholestasis) led to a significantly decrease in CYP2D6 expression (Fig. 26).

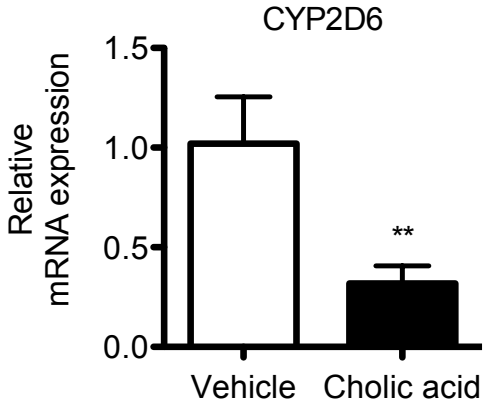

Figure 26. Cholic acid represses CYP2D6 expression in primary human hepatocytes.
Primary human hepatocytes were treated with cholic acid (100 μM) or vehicle control (DMSO) for 48 hours (*n*=3). CYP2D6 mRNA expression was determined by qRT-PCR. Values are presented as mean ± S.D. ***p* < 0.01, versus vehicle treatment.

Cholestasis is often triggered by drugs such as rifampicin, erythromycin, ethinylestradiol, and oxypenicilins (153). While it remains to be determined whether cholestasis represses CYP2D6-mediated drug metabolism in humans, results from this study provide a mechanistic basis for the possibility. Additionally, this study provides evidence that supports important roles of FXR/SHP pathway in the regulation of CYP2D6 expression and that differential SHP expression and/or activity may potentially contribute to interindividual variability in CYP2D6-mediated drug metabolism in humans. Whether or to what extent different SHP modulators affect CYP2D6 expression and activity in humans remains to be examined.

Many FXR agonists are currently under development for different hepatic or metabolic diseases including primary biliary cirrhosis, nonalcoholic steatohepatitis, and diabetes (150). For example, obeticholic acid (i.e., INT-747), a potent synthetic FXR agonist, is in phase III trials for primary biliary cirrhosis (154). Also, the hepatoprotective effects of GW4064 and its analogs have been shown in cholestatic rats and in mice with gallstones (155-159). Our results suggest that drug-drug interactions between CYP2D6 substrates and FXR agonists may occur if these FXR agonists are approved and clinically used. It remains difficult to quantitatively predict the clinical outcome of these potential interactions based on the results from human hepatocytes. This is in part due to the long degradation half-lives of CYP2D6 protein (i.e., 51 hours [28]) such that CYP2D6 protein levels in human hepatocytes do not reach the steady state after the typical time of drug treatment (e.g., 48 hour) passes. In accordance, CYP2D6 mRNA expression decreased ~2-fold in GW4064-treated human hepatocytes whereas the decrease in catalytic activity of CYP2D6 (as determined in the S9 fraction of hepatocytes) was only ~20% (Fig. 25D). The clinical consequences of FXR agonists repressing CYP2D6 expression thus remain to be examined.

Results from our previous study in Tg-*CYP2D6* mice showed that SHP knockdown (by using siRNA) led to increased CYP2D6 expression (Fig. 8B), indicating that decreased SHP expression triggers CYP2D6 induction. Interestingly, however, results from this study showed that the basal expression levels of CYP2D6 in *Shp(-/-);CYP2D6* mice did not differ from those in *Shp(+/+);CYP2D6* mice (Fig. 23A). Compensational changes in hepatic gene expression (or activity) in *Shp(-/-);CYP2D6* mice may explain the results. For example, DAX-1 (dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X chromosome, gene 1, NR0B1) is a nuclear receptor that lacks the DNA-binding domain similarly to SHP (127), and it is known to repress HNF4α transactivation of a hepatic gene (128). Our qRT-PCR experiment, however, revealed that Dax-1 mRNA expression is undetectable in mouse liver tissues, suggesting that DAX-1 may not compensate for the loss of SHP in the liver. Also, SMILE (SHP-interacting leucine zipper protein; initially identified as a SHP-interacting protein (129)) has been shown to repress HNF4α transactivation of target genes in the absence of SHP (130), suggesting that increased SMILE expression/activity may compensate for the loss of SHP. Our results showed, however, the mRNA expression levels of Smile did not differ between *Shp(-/-);CYP2D6* and *Shp(+/+);CYP2D6* male mice (Fig. 27). Whether the transcriptional *activity* of SMILE is enhanced in *Shp(-/-);CYP2D6* male mice remains to be determined. Together, these results suggest that basal CYP2D6 expression in *Shp(-/-);CYP2D6* mice may be governed by as-yet-unknown factors.

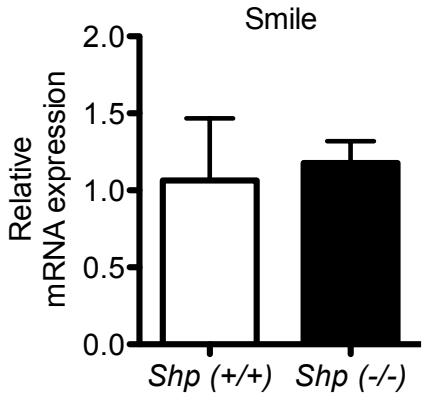

Figure 27. Smile expression does not change in *Shp(-/-);CYP2D6* male mice.
Liver tissues were collected from *Shp(-/-);CYP2D6* and *Shp(-/-);CYP2D6* male mice (*n*=5). Smile mRNA expression was examined by qRT-PCR. Values are presented as mean ± S.D.

In conclusion, we showed that FXR agonist GW4064 represses CYP2D6 expression through inducing SHP expression (Fig. 28). This suggests that potential drug-drug interactions may occur between CYP2D6 substrates and FXR agonists that are currently under development for hepatic and metabolic disorders. Our results also provide a mechanistic basis to identify potential factors (e.g., bile acids) that may contribute to the interindividual variability in CYP2D6 activity.

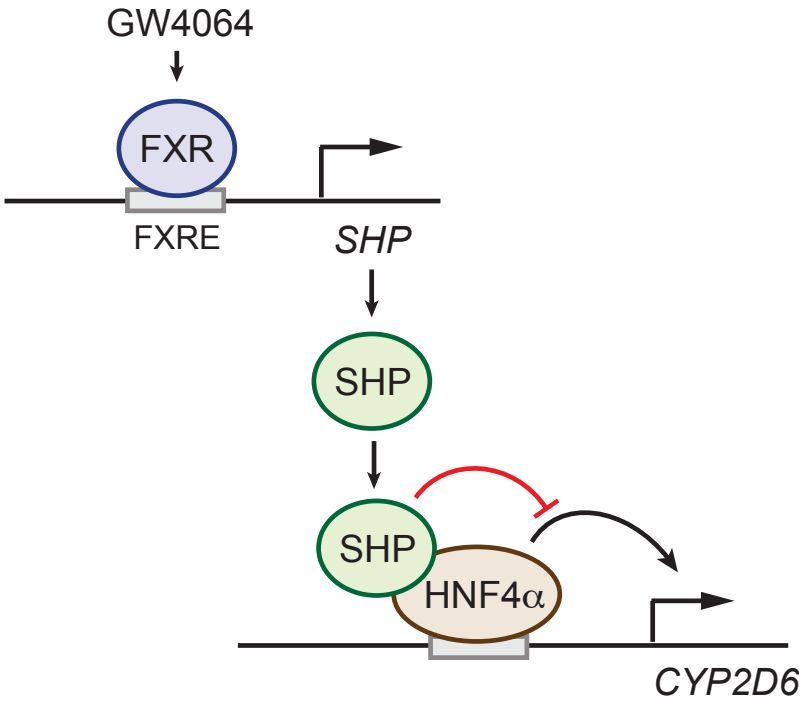

Figure 28. Working model for CYP2D6 repression upon GW4064 treatment.
GW4064 treatment induces FXR transactivation of SHP promoter and subsequently upregulates SHP expression. Increased level of SHP in turn suppresses HNF4α transactivation of CYP2D6 promoter, leading to repressed CYP2D6 expression and activity.

4. ESTROGEN-INDUCED CHOLESTASIS LEADS TO REPRESSED CYP2D6 EXPRESSION IN CYP2D6-HUMANIZED MICE

[Previously published as Pan X, Jeong H. Estrogen-Induced Cholestasis Leads to Repressed CYP2D6 Expression in CYP2D6-Humanized Mice, Mol Pharmacol. 2015 Jul;88(1):106-12.]

4.1. Introduction

Cholestasis is a disease caused by impaired bile formation in liver cells (i.e., intrahepatic cholestasis) or obstruction of bile flow via bile ducts (i.e., extrahepatic cholestasis) (22,160,161). Intrahepatic cholestasis can be caused by medications (e.g., cyclosporine A) or pregnancy, and extrahepatic cholestasis by bile duct blockade from gallstones or tumors. Regardless of the underlying causes, cholestasis results in hepatic and systemic accumulation of cytotoxic bile acids. This induces liver damage accompanied by pruritus and indigestion, ultimately leading to biliary fibrosis and cirrhosis (23,162). Treatment of cholestasis involves surgical removal of underlying causes, if possible, and drug therapy using ursodeoxycholic acid and immunosuppressive drugs (163). The management of symptoms accompanying cholestasis may also require the use of drug therapy (e.g., serotonin reuptake inhibitors) (164).

CYP2D6 is a major drug-metabolizing enzyme and responsible for eliminating ~20% of marketed drugs (Table II). Despite its importance as a drug-metabolizing enzyme, regulation of CYP2D6 expression remains poorly understood as compared to that of other drug-metabolizing enzymes such as CYP3A4. Recently, we have identified small heterodimer partner (SHP) as a novel transcriptional regulator of CYP2D6 expression. Previously, we showed that SHP represses hepatocyte nuclear factor (HNF)

4α transactivation of CYP2D6 promoter (144). Also, in CYP2D6-humanized transgenic (Tg-*CYP2D6*) mice, SHP knockdown led to a significant increase in CYP2D6 expression (144). SHP expression is a representative target gene of the bile acid-sensor, farnesoid X receptor (FXR). Upon binding to bile acids, FXR transactivates SHP promoter and upregulates SHP expression (86). SHP in turn represses the transcription of genes involved in bile acid synthesis such as CYP7A1 and CYP8B1 (86). Whether enhanced expression of SHP in cholestasis also leads to decreased CYP2D6 expression remains unknown.

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 its cognate receptor, estrogen receptor (ER) α and ERβ. In the liver, expression of ERβ is localized to the cholangiocytes (165), and ERα is the major isoform expressed in the parenchymal cells (166). Of note, estrogens can cause intrahepatic cholestasis in premenopausal women receiving oral contraceptives, in postmenopausal women on hormone replacement therapy (167), or in men who receive estrogens therapy for prostate cancer (168). Estrogen-induced cholestasis can also occur during pregnancy; intrahepatic cholestasis of pregnancy is the most common liver disease in pregnant women (169,170). An experimental intrahepatic cholestasis model established by 17α-ethinylestradiol (EE2) administration in rodents has been commonly used to study the pathogenesis of estrogen-induced intrahepatic cholestasis (20-23).

In this study, we examined the effects of estrogen-induced intrahepatic cholestasis on CYP2D6-mediated drug metabolism. Our results indicate EE2-induced cholestasis increases SHP and represses CYP2D6 expression in Tg-*CYP2D6* mice.

Studies of the underlying mechanisms revealed a role of ERα in CYP2D6 regulation in estrogen-induced cholestasis.

4.2. Materials and methods

4.2.1. Animals

CYP2D6-humanized transgenic (Tg-*CYP2D6*) mice were previously described (9). Adult male (8 weeks of age and weighing 20-25 g) were used for the experiments. For estrogen-induced intrahepatic cholestasis, mice received subcutaneous injections of 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (20). For the activation of ERα alone (without cholestasis), mice received intraperitoneal injections of EE2 (5 μg/kg) or vehicle (olive oil) daily for 5 days (171). Mice were sacrificed on the 6th day, blood and liver tissues were collected. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Institution's Animal Care and use Committee in the University of Illinois at Chicago.

4.2.2. Chemicals and reagents

Debrisoquine, (±)-4-hydroxydebrisoquin, and paraxanthine were purchased from Biomol (Plymouth Meeting, PA). 17α-ethinylestradiol was purchased from Sigma-Aldrich (St. Louis, MO).

4.2.3. Western blot

Western blot was performed as described previously in Chapter 2 using SHP antibody from Santa Cruz (sc-30169).

4.2.4. Determination of CYP2D6 activity

Liver S9 fractions were prepared as described previously (144). CYP2D6 activity was determined by incubating S9 fractions with debrisoquine (200 μM) as described previously in Chapter 3.

4.2.5. RNA isolation and qRT-PCR

Total RNA isolation was described previously in Chapter 2. qRT-PCR was performed by using probes for Cyp7a1 (Mm.PT.58.41588826, IDT); Ostβ: 3'-CCAGGACCAGGATGGAATAA-5' (forward); 3'- AGAGAAAGCTGCAGCCAATG -5' (reverse); Stat5a: 3'- GTCTCCAGGGACACTTGCTT -5' (forward); 3'-CTGCAGCAGACCCAAGAGTA -5' (reverse). Primer sequences for all other target genes were described in Table VII. Results were expressed as fold changes upon drug treatment by using the gene expression levels normalized to those of Gapdh ($2^{-\Delta\Delta Ct}$ method).

4.2.6. ChIP assays

ChIP assays were performed as described previously in Chapter 2 using antibodies from Santa Cruz (ERα, sc-543x; FXR, sc-13063x; HNF4α, sc-6556x; RNA polymerase II, sc-899x; SHP, sc-30169). qRT-PCR was performed using the following probes for Ostβ_FXRE: 5′- TGCCCTGATCGCACAATCTC -3′ (forward) and 5′-GTGAATGACCCCACGAATGC -3′ (reverse); Shp_FXRE/ERE: 5′-GCCTGAGACCTTGGTGCCCTG -3′ (forward) and 5′- CTGCCCACTGCCTGGATGC -3′ (reverse). Stat5a_ERE: 5′- CCCCCCCCTGTTTTCAAT -3′ (forward) and 5′-

CCCCGCCCTCTGTGTGT -3′ (reverse). CYP2D6 probes were listed in Table VIII and Cyp8b1 probes were described in Chapter 3.

4.2.7. Alkaline phosphatase and alanine transaminase measurement

Plasma Alkaline phosphatase (ALP) and alanine transaminase (ALT) levels were measured by chemistry analyzer (Olympus AU 680, Center Valley, PA) following the manufacturer's protocol.

4.2.8. Statistical analysis

Values were reported as mean ± standard deviation (S.D.). Statistical differences were determined by using Student's t-test.

4.3. Results

4.3.1. EE2-induced cholestasis leads to decreased HNF4α transactivation of CYP2D6 promoter

Previous studies have shown that administration of high dose estrogen triggers intrahepatic cholestasis (20-23). To investigate the effects of EE2-induced cholestasis on CYP2D6 expression and activity *in vivo*, Tg-*CYP2D6* mice were treated with EE2 or vehicle control. As expected from EE2-induced cholestasis, the plasma levels of ALP (a marker for cholestasis (172)) and ALT (a marker for liver injury) were significantly increased in EE2-treated mice (Fig. 29).

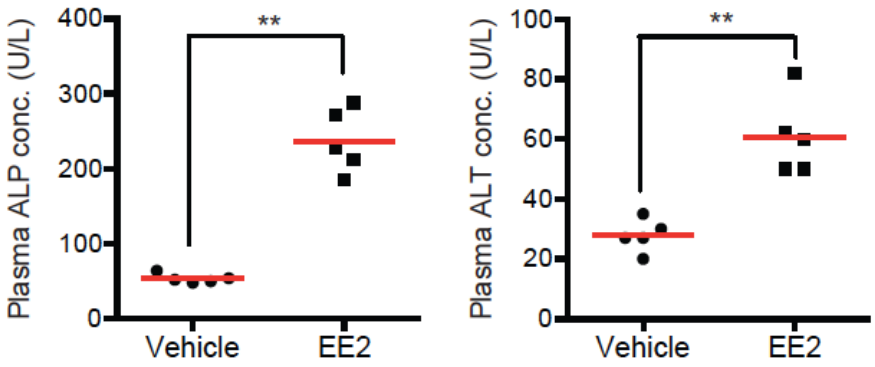

Figure 29. EE2 induces cholestasis in Tg-*CYP2D6* mice.
Tg-*CYP2D6* mice were injected subcutaneously with 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (*n*=5). Alkaline phosphatase (ALP) level and alanine aminotransferase (ALT) level were measured in mouse plasma. ** $p < 0.01$, versus vehicle treatment.

To examine potential effects of EE2-induced cholestasis on hepatic CYP2D6 expression, CYP2D6 mRNA and protein levels were determined in the mice by using qRT-PCR and western blot. The mRNA expression levels of Cyp7a1 and Cyp8b1 are known to be repressed in cholestasis (20,173) and were examined as controls. The results showed that EE2-induced cholestasis led to significant decreases in Cyp7a1 and Cyp8b1 expression as expected (Fig. 30A). EE2 also decreased mRNA level of CYP2D6 by 2-fold (Fig. 30A). Results from S9 phenotyping (by using debrisoquine as a probe drug for CYP2D6) revealed a 3-fold decrease in CYP2D6 activity (Fig. 30B). The decreased CYP2D6 expression was accompanied by increased expression of SHP at mRNA and protein levels (Fig. 30C and 30D). EE2 did not alter mRNA expression levels of Hnf4α (Fig. 30A).

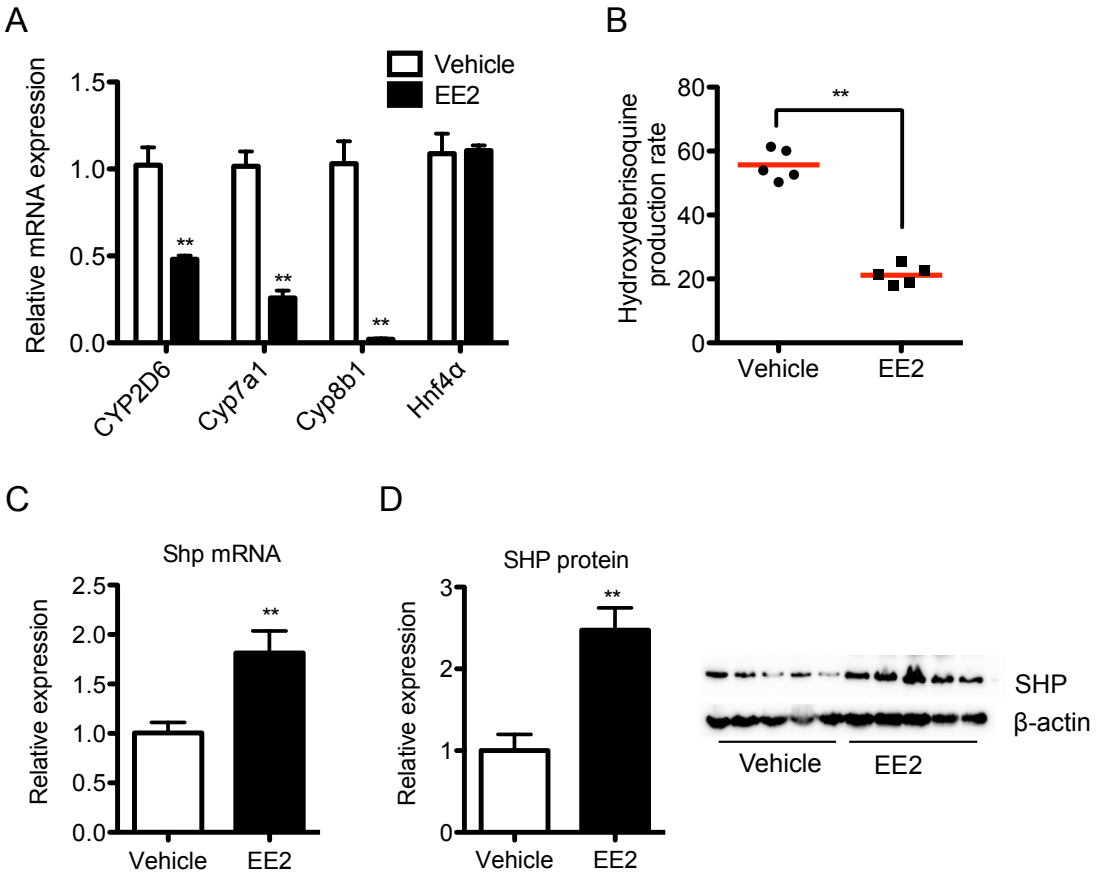

Figure 30. EE2-induced cholestasis represses CYP2D6 expression in Tg-*CYP2D6* mice.

Tg-*CYP2D6* mice were injected subcutaneously with 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (*n*=5). A and C, Hepatic CYP2D6, Cyp7a1, Cyp8b1, Hnf4α, and Shp mRNA expression was determined by qRT-PCR. B, S9 fractions were prepared from the liver tissues of Tg-*CYP2D6* mice treated with EE2 or vehicle, and CYP2D6 activity in S9 fractions was measured using debrisoquine (200 μM) as a probe drug. Data shown are metabolite production rates in pmol/min/mg protein. D, SHP protein expression level was determined by western blot. The image of western blot (right) is shown, and the band intensities (SHP/β-actin, left) were quantified after normalization by SHP expression in the vehicle-treated mice. Values are presented as mean ± S.D. ** $p < 0.01$, versus vehicle treatment.

To determine whether the altered SHP expression upon EE2 administration leads to changes in HNF4α transactivation of CYP2D6 promoter, ChIP assays were performed using mouse liver tissues. Livers from vehicle- or EE2-treated mice were collected and subjected to ChIP using antibodies against HNF4α, SHP, or RNA polymerase II (Pol II, a marker of transcription initiation). Based on previous reports that HNF4α binds to -53/-41 bp of *CYP2D6* (63) and SHP suppresses HNF4α transactivation of CYP2D6 promoter (144), recruitment of the transcription factors to the proximal promoter region of *CYP2D6* was examined. The results showed a significant increase in the recruitment of SHP to CYP2D6 promoter, accompanied by decreased recruitments of HNF4α and Pol II to CYP2D6 promoter (Fig. 31A). A similar pattern of changes in SHP, HNF4α, and Pol II recruitments was observed for Cyp7a1 and Cyp8b1, the known target genes of SHP (151) (Fig. 31B and Fig. 31C).

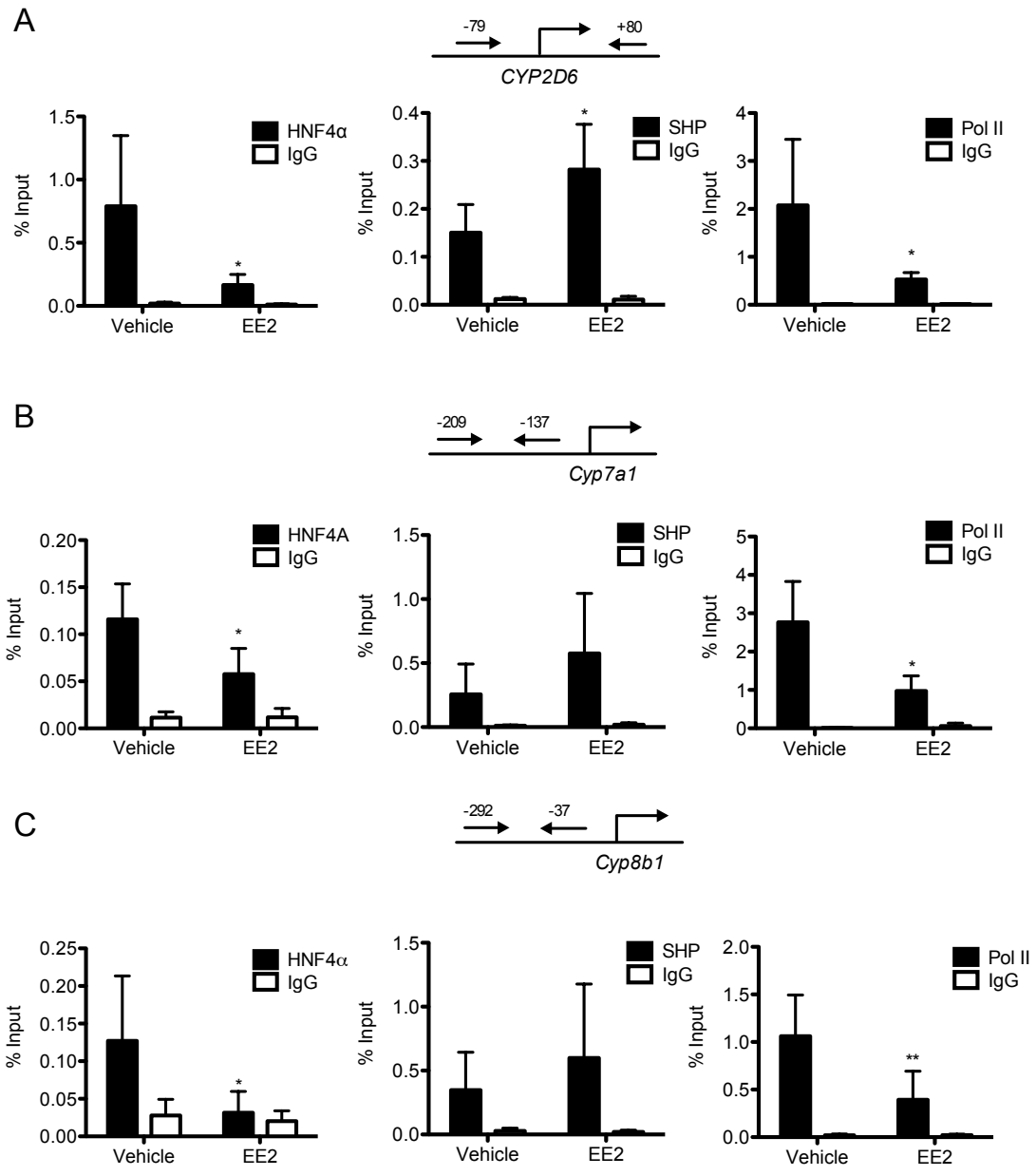

Figure 31. EE2-induced cholestasis represses HNF4α transactivation of CYP2D6 promoter.

Tg-*CYP2D6* mice were injected subcutaneously with 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (*n*=5). Recruitment of HNF4α, SHP, and RNA polymerase II (Pol II) onto (A) CYP2D6 promoter, (B) Cyp7a1 promoter, and (C) Cyp8b1 promoter were analyzed by ChIP assays using mouse liver tissues. Values are presented as mean ± S.D. * $p < 0.05$; ** $p < 0.01$, versus vehicle treatment.

Such changes in the transcriptional factor recruitment were absent when a PCR primer set detecting a downstream region (+3913/+4112 bp) of CYP2D6 gene was used as a negative control (Fig. 32).

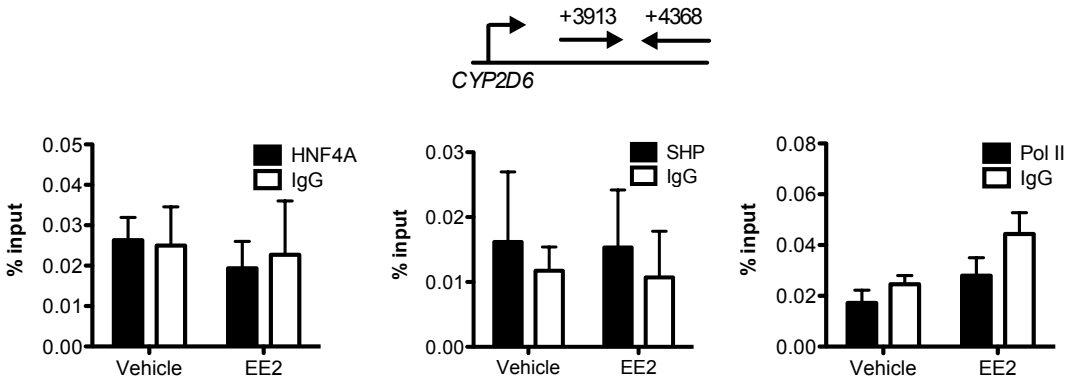

Figure 32. HNF4α, SHP and RNA polymerase II are not recruited to CYP2D6 downstream region.
Tg-*CYP2D6* mice were injected subcutaneously with 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (*n*=5). Recruitment of HNF4α, SHP, and RNA polymerase II (Pol II) onto CYP2D6 distal downstream region was analyzed by ChIP assays using mouse liver tissues. Values are presented as mean ± S.D.

Also, the decrease in HNF4α recruitment to the promoter region was not observed for other target genes of HNF4α (i.e., ApoC2 and Hes6 (112,114)) (Fig. 33), suggesting that the repressive action of SHP on HNF4α transactivation may be target gene-specific. Together, these results suggest that EE2-induced cholestasis leads to decreased HNF4α transactivation of CYP2D6 promoter, potentially through enhanced SHP expression.

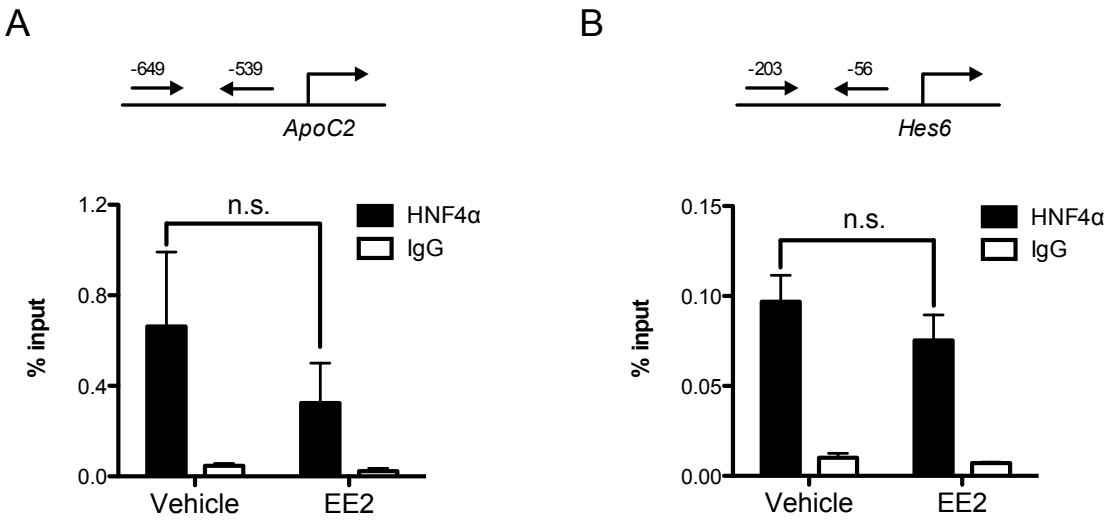

Figure 33. Effects of EE2-induced cholestasis on HNF4α activity are target gene-specific.
Tg-*CYP2D6* mice were injected subcutaneously with 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (*n*=5). HNF4α recruitment to the previously known HNF4α response elements in Apoc2 promoter (A) and Hes6 promoter (B) were analyzed by ChIP assays using mouse liver tissues. Values are presented as mean ± S.D. n.s.= not statistically significant.

4.3.2. ERα plays a predominant role in SHP upregulation in EE2-induced cholestasis

FXR transactivates SHP promoter by binding to –294/–281 bp of *SHP* in humans (86). Interestingly, results from a previous study indicate that ERα also transactivates SHP promoter by binding to an overlapping promoter region (141). As the *SHP* promoter sequence is highly conserved between humans and mice (141), we examined the comparative roles of ERα and FXR in the regulation of SHP expression in mice with EE2-induced cholestasis. To this end, recruitments of ERα and FXR to *Shp* promoter were compared between the livers of EE2- vs. vehicle-treated mice by ChIP assays. Previously known target genes of ERα and FXR (signal transducer and activator of transcription 5a, Stat5a and organic solute transporter β, Ostβ, respectively (174,175)), were used as positive controls. The results showed that ERα recruitment to *Shp* promoter was increased significantly upon EE2 treatment (Fig. 34A). Such increase in ERα recruitment was not observed when a PCR primer set detecting a downstream region (+3968/+4158 bp) of *Shp* gene was used as a negative control (Fig. 35). EE2 treatment also led to a significant increase in ERα recruitment to Stat5a promoter (Fig. 34B) and increased mRNA expression of Stat5a (Fig. 34C). In contrast to the increased ERα recruitment to Shp promoter, the recruitment of FXR to the promoter region did not differ between the EE2-treated and the control groups (Fig. 34D). On the other hand, FXR recruitment to Ostβ promoter and mRNA expression of Ostβ was significantly increased (Fig. 34E and 34F), indicating a robust FXR activation by EE2-induced cholestasis. Together, these results suggest that when both ERα and FXR are activated in EE2-induced cholestasis, ERα may play a predominant role in the regulation of SHP expression.

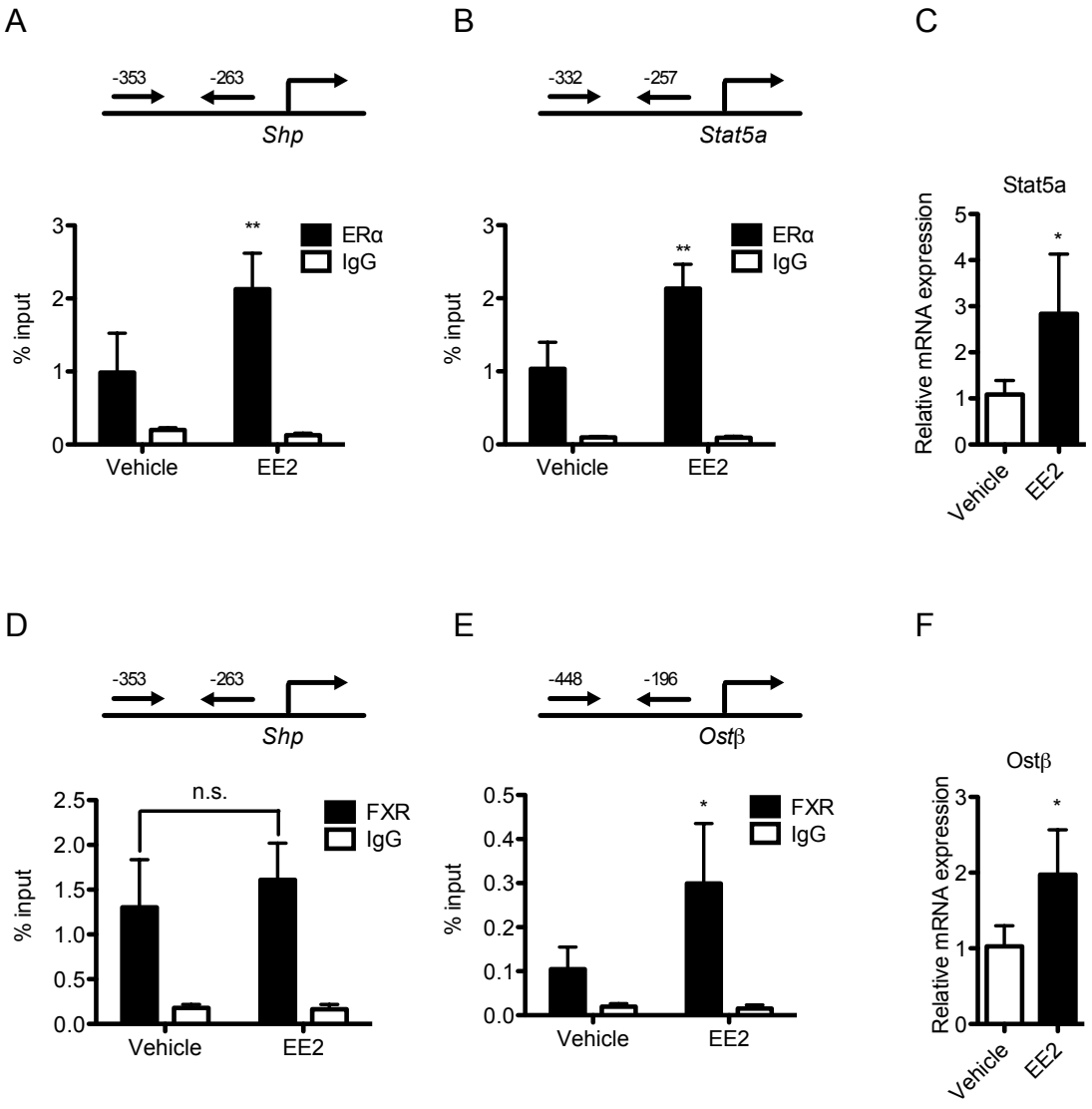

Figure 34. ERα plays a major role in SHP up-regulation in EE2-induced cholestasis.

Tg-*CYP2D6* mice were injected subcutaneously with 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (*n*=5). Recruitment of ERα onto (A) Shp promoter and (B) Stat5a promoter; recruitment of FXR onto (D) Shp promoter and (E) Ostβ promoter were analyzed by ChIP assays using mouse liver tissues. C and F, Stat5a and Ostβ mRNA expression was determined by qRT-PCR. Values are presented as mean ± S.D. * $p < 0.05$; ** $p < 0.01$; n.s.=not statistically significant, versus vehicle treatment.

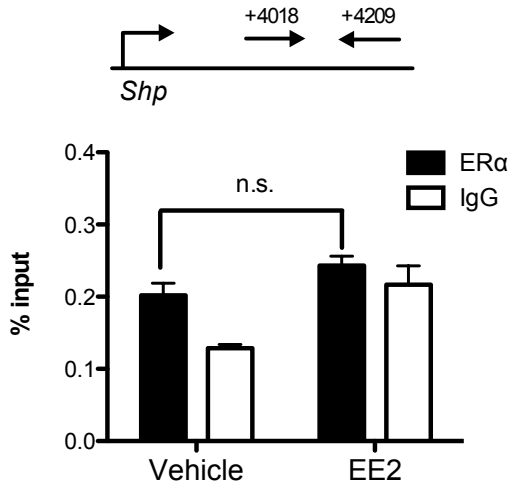

Figure 35. ERα is not recruited to Shp distal downstream region.
Tg-*CYP2D6* mice were injected subcutaneously with 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (*n*=5). Recruitment of ERα onto Shp distal downstream region was analyzed by using ChIP assays. Values are presented as mean ± S.D. n.s.= not statistically significant.

4.3.3. ERα activation leads to CYP2D6 repression

To determine whether ERα activation alone (without accompanying cholestasis) can alter CYP2D6 expression via upregulating SHP, mice were administered with EE2 at a low dose (5 μg/kg) for 5 days, and CYP2D6 expression was examined. Plasma ALP levels were slightly higher in mice treated with low-dose EE2 (Fig. 36A) but within the normal range (i.e., 113 ± 49 U/L) for 8 week-old mice (172). ALT levels did not increase in EE2-treated mice (Fig. 36A). Also, the dramatic decreases in Cyp7a1 and Cyp8b1 expression or increased Ostβ expression (i.e., hallmarks of cholestasis and FXR activation) were not observed in these mice (Fig. 36B). Together, the data indicate a lack of cholestasis in mice treated with low-dose EE2.

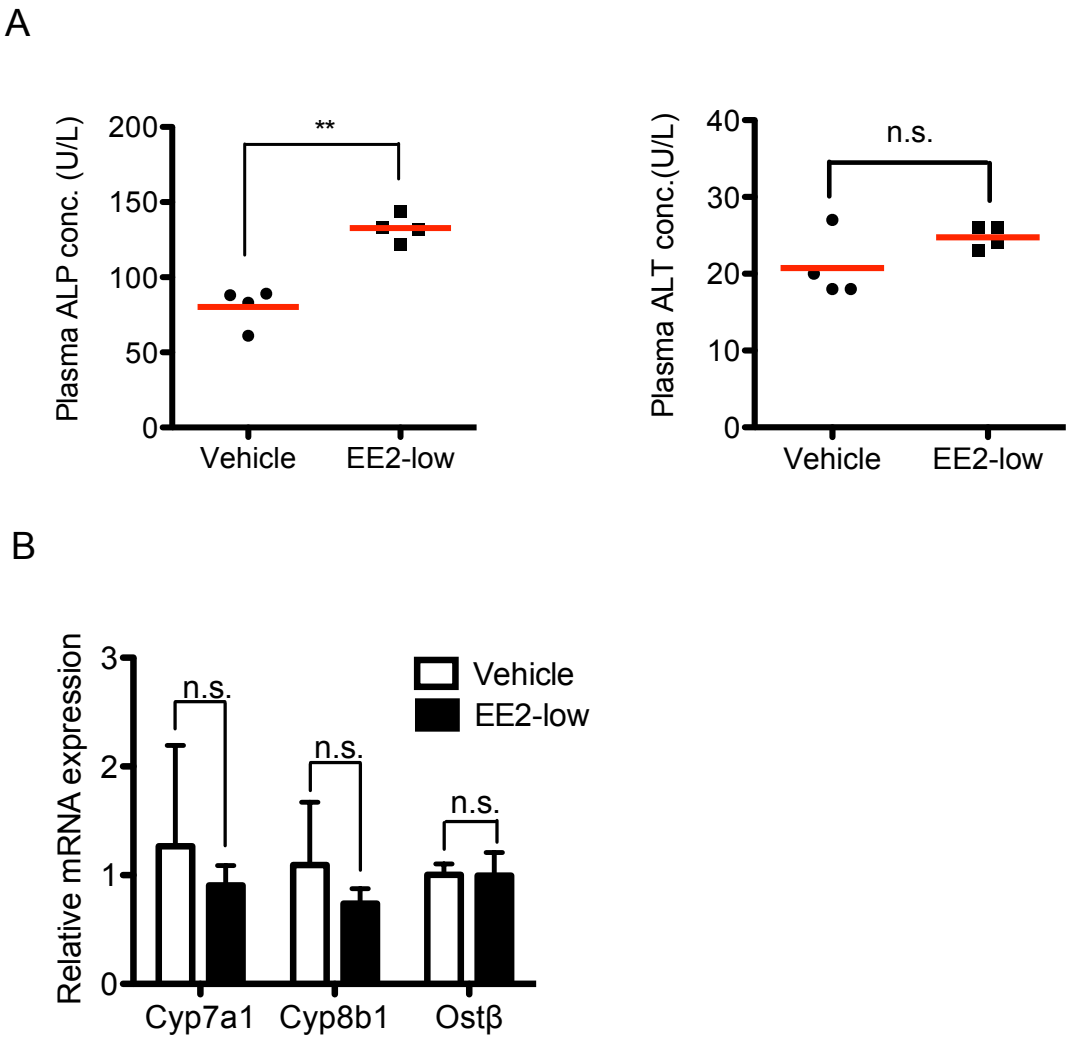

Figure 36. Low dose EE2 dose not induce cholestasis in Tg-*CYP2D6* mice.
Tg-*CYP2D6* mice were injected intraperitoneally with EE2 (5 μg/kg) or vehicle control (olive oil) daily for 5 days (*n*=4). A, Measurement of plasma ALP and ALT levels. B, Hepatic Cyp7a1, Cyp8b1, and Ostβ mRNA expression were determined by qRT-PCR. Values are presented as mean ± S.D. ** $p < 0.01$; n.s.=not statistically significant, versus vehicle treatment.

The treatment with EE2 at the low dose led to increased Shp and decreased CYP2D6 mRNA levels (Fig. 37A). The mRNA expression of Stat5a (an ERα target gene) was also increased in EE2-treated mice as expected (Fig. 37A). In accordance with increased Shp mRNA levels, SHP protein level was increased upon EE2 treatment, but to an extent smaller than that by EE2 at the high dose (1.5-fold vs. 2.5-fold for low- and high-dose EE2-treated groups, respectively) (Fig. 30D and 37B). CYP2D6 activity was decreased in the EE2-treated group only by ~20% as compared to the vehicle control group (Fig. 37C).

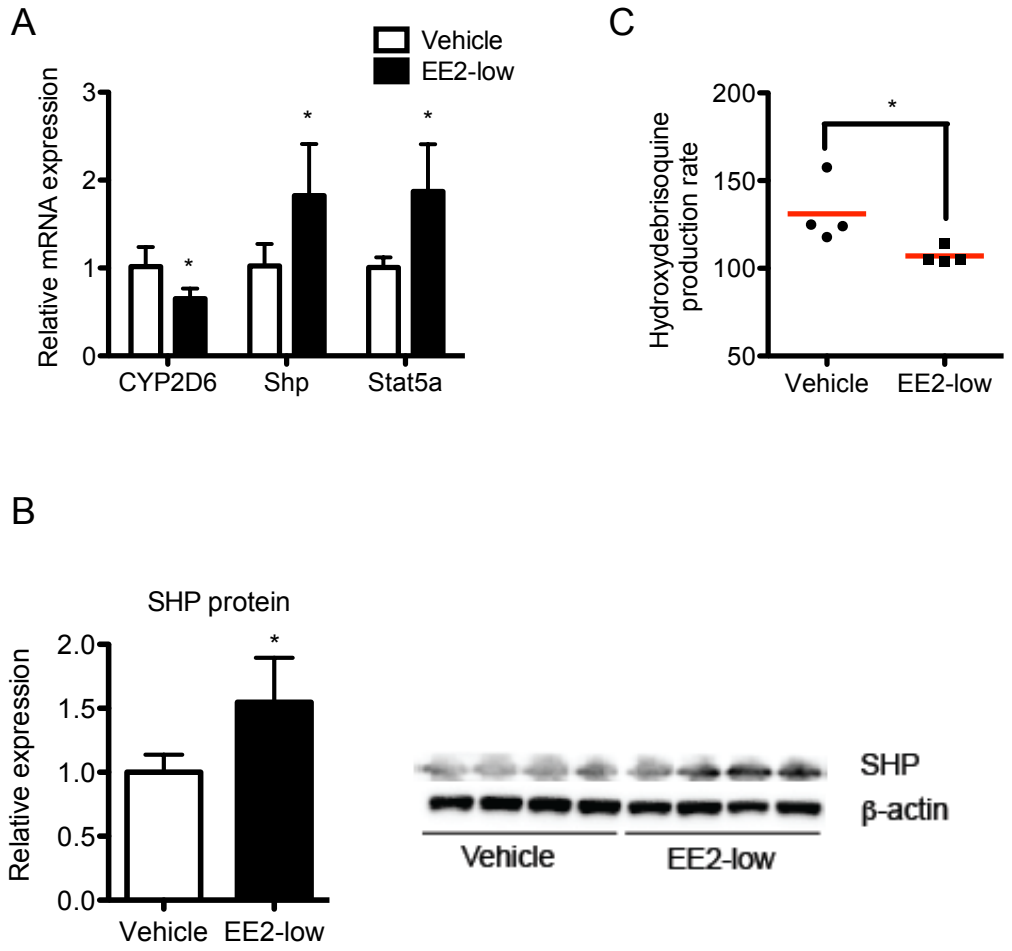

Figure 37. Low dose EE2 represses CYP2D6 expression in Tg-*CYP2D6* mice.
Tg-*CYP2D6* mice were injected intraperitoneally with EE2 (5 μg/kg) or vehicle control (olive oil) daily for 5 days (*n*=4). A, Hepatic mRNA expression levels of CYP2D6, Shp, and Stat5a were measured by qRT-PCR. B, SHP protein expression level was determined by western blot. The image of western blot (right) is shown, and the band intensities (SHP/β-actin, left) were quantified after normalized by SHP expression in vehicle-treat mice. C, CYP2D6 activity in hepatic S9 fractions was measured using debrisoquine (200 μM) as a probe drug. Data shown are metabolite production rates (in pmol/min/mg protein). Values are presented as mean ± S.D. * $p < 0.05$, versus vehicle treatment.

Consistent with the changes in SHP and CYP2D6 expression, ChIP results showed increased recruitment of SHP to CYP2D6 promoter, and decreased recruitments of HNF4α and Pol II to CYP2D6 promoter (Fig. 38A). Such changes in transcription factor recruitment were not observed for Cyp8b1 (Fig. 38B).

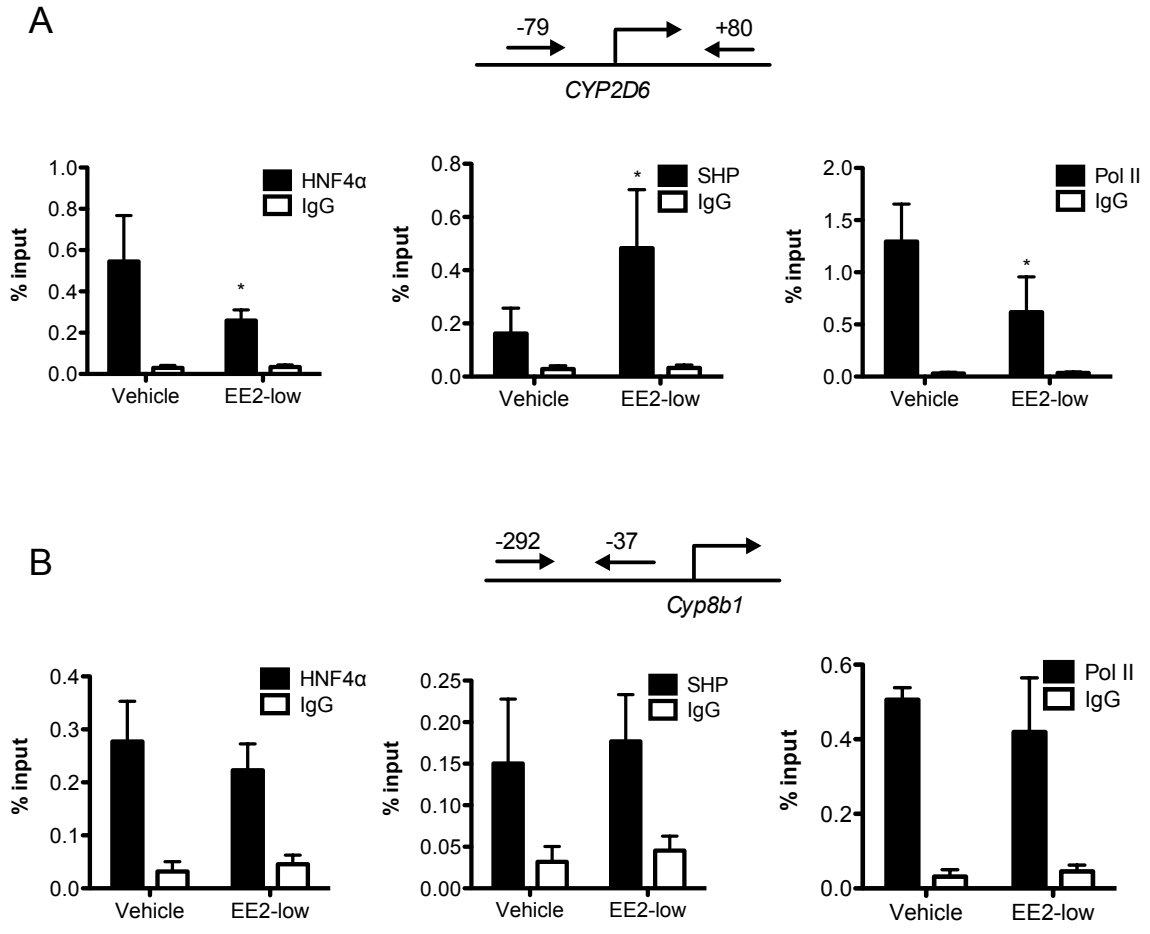

Figure 38. Low dose EE2 represses HNF4α transactivation of CYP2D6 promoter. Tg-*CYP2D6* mice were injected intraperitoneally with EE2 (5 μg/kg) or vehicle control (olive oil) daily for 5 days (*n*=4). Recruitment of HNF4α, SHP, and RNA polymerase II (Pol II) onto CYP2D6 promoter and Cyp8b1 promoter were analyzed by ChIP assays using mouse liver tissues. Values are presented as mean ± S.D. * $p < 0.05$, n.s.= not statistically significant.

ERα recruitment to Shp promoter was increased upon low dose EE2 treatment, indicating enhanced ERα transactivation of Shp promoter (Fig. 39). A similar pattern was observed for ERα recruitment to the promoter of Stat5a (Fig. 39). Together, these results suggest that ERα activation leads to CYP2D6 repression through enhanced SHP expression although the magnitude of CYP2D6 repression was smaller than that in EE2-induced cholestasis.

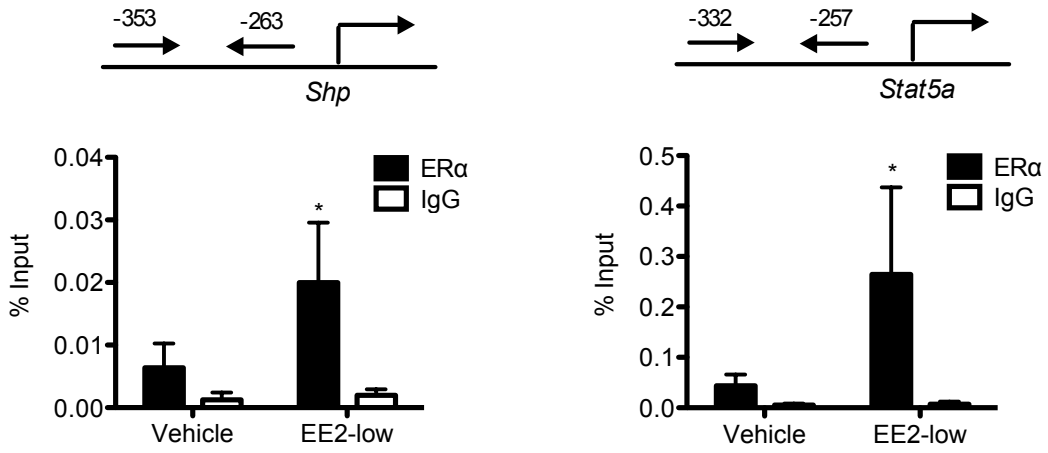

Figure 39. Low dose EE2 induces ERα transactivation of its target gene promoters.
Tg-*CYP2D6* mice were injected intraperitoneally with EE2 (5 μg/kg) or vehicle control (olive oil) daily for 5 days (*n*=4). Recruitment of ERα to Shp promoter and Stat5a promoter were analyzed by ChIP assays using mouse liver tissues. Values are presented as mean ± S.D. * $p < 0.05$, versus vehicle treatment.

4.4. Discussion

Previously, we have shown that SHP is a transcriptional repressor of CYP2D6 expression (144) and that FXR activation (by using a FXR agonist GW4064) leads to decreased CYP2D6 expression via enhancing SHP expression (176). In this study, we examined whether cholestasis (a condition known to enhance FXR activity) also alters CYP2D6 expression by up-regulating SHP expression. To this end, we employed the estrogen-induced cholestasis model that is widely used to examine the mechanisms involved in intrahepatic cholestasis (20-23).

In Tg-*CYP2D6* mice, EE2 at a dose of 10 mg/kg/day led to increased plasma concentrations of liver enzymes and decreased expression of Cyp7a1 and Cyp8b1, a hallmark of cholestasis. In the EE2-treated mice, CYP2D6 expression and activity were significantly repressed, suggesting that CYP2D6-mediated drug metabolism may be decreased in cholestasis. Cholestasis is caused by factors that impair bile formation or bile flow. For example, in 0.4-1% of pregnant women in North American and up to 15-20% of pregnant women in some areas of Europe, intrahepatic cholestasis occurs likely due to high plasma concentrations of estrogen during pregnancy (169,170,177,178). Based on our results, it appears possible that CYP2D6-mediated drug metabolism is lower in pregnant women with increased hepatic bile acid levels (than in pregnant women whose bile acid levels are within the normal range), and this may lead to greater inter-individual variability in CYP2D6 activity in pregnant women as compared to the nonpregnant subjects. Indeed, it was previously shown that CYP2D6 activity (i.e., clearance of metoprolol) exhibited greater variability during pregnancy than after delivery (4). Whether altered hepatic bile acid levels are indeed responsible for the increased inter-individual variability in CYP2D6 activity during pregnancy by controlling hepatic CYP2D6 expression remains to be determined.

CYP2D6 repression in EE2-induced cholestasis was accompanied by increased SHP expression and subsequent changes in transcription factor recruitment to CYP2D6 promoter, i.e., increased SHP and decreased HNF4α recruitment to the promoter. These results suggest increased SHP expression may be responsible for CYP2D6 repression in cholestasis. SHP promoter is transactivated by FXR and ERα (86,141), both of which are likely activated in EE2-induced cholestasis. Of note, FXR and ERα bind to overlapping promoter regions (141), suggesting that the roles of ERα and FXR in SHP up-regulation could be mutually exclusive in EE2-induced cholestasis. To determine which one of two transcription factors (ERα or FXR) plays a major role in SHP induction in EE2-induced cholestasis, the extent of ERα and FXR recruitment to Shp promoter was examined. Our results showed that ERα recruitment (but not that of FXR) to Shp promoter was increased in mice administered with EE2 at a high dose. The results suggest that ERα transactivation of Shp promoter is potentially responsible for CYP2D6 repression in EE2-induced cholestasis.

Based on the results indicating an important role of ERα in the regulation of SHP expression, we further examined the effects of ERα activation alone (without cholestasis and subsequent FXR activation) on CYP2D6 expression. To this end, Tg-*CYP2D6* mice were administered with EE2 at a low dose that does not cause cholestasis or liver damage. At the low dose, EE2 increased SHP and decreased CYP2D6 expression; however, the extent of CYP2D6 repression in these mice was much smaller than that in mice with EE2-induced cholestasis (2.6 vs. 1.2-fold decrease in CYP2D6 activity for high- and low-dose EE2 groups, respectively). The greater magnitude of CYP2D6 repression in cholestatic mice could be in part due to bile acid-induced stabilization of SHP protein; bile acids increases stability of hepatic SHP protein by inhibiting its proteasomal degradation in an extracellular signal-regulated kinase (ERK)-dependent

manner (179). Indeed, the extent of increases in SHP protein level was greater in mice administered with high-dose EE2 than in those with low-dose EE2 (Fig. 40).

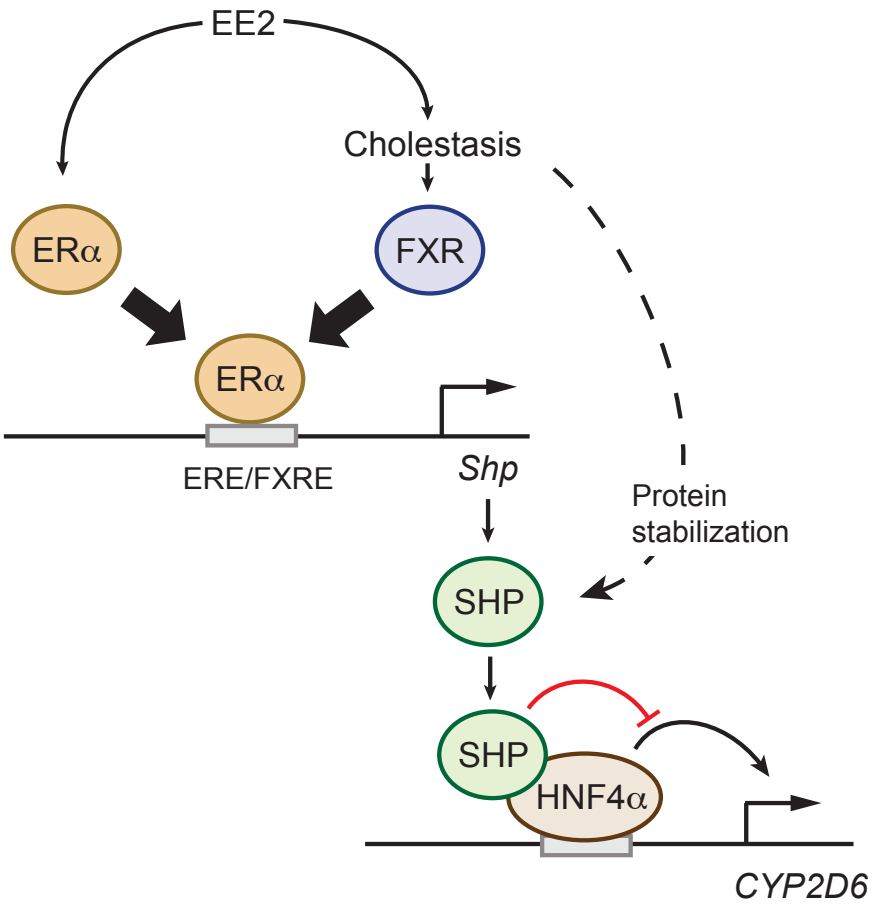

Figure 40. Working model for CYP2D6 regulation in EE2-induced cholestasis.
The working model illustrates the overlapping estrogen response element (ERE) and FXR response element (FXRE) in SHP promoter. In EE2-induced cholestasis, ERα recruitment to SHP promoter increases, leading to higher SHP expression. Also, cholestasis stabilizes SHP protein, further enhancing SHP expression. SHP in turn suppresses HNF4α transactivation of CYP2D6 promoter, leading to CYP2D6 repression in EE2-induced cholestasis.

Cholestasis triggers hepatic inflammation that is known to down-regulate CYP2D6 expression (180), and this may have also contributed to CYP2D6 repression in EE2-induced cholestasis. Our qRT-PCR results showed that expression levels of inflammatory cytokines (i.e., TNFα, IL-1β, and IL-6) were significantly higher in the livers of mice treated with high-dose EE2 as compared to the vehicle-treated mice, and such increase was not observed in mice treated with low-dose EE2 (Fig. 41A and 41B).

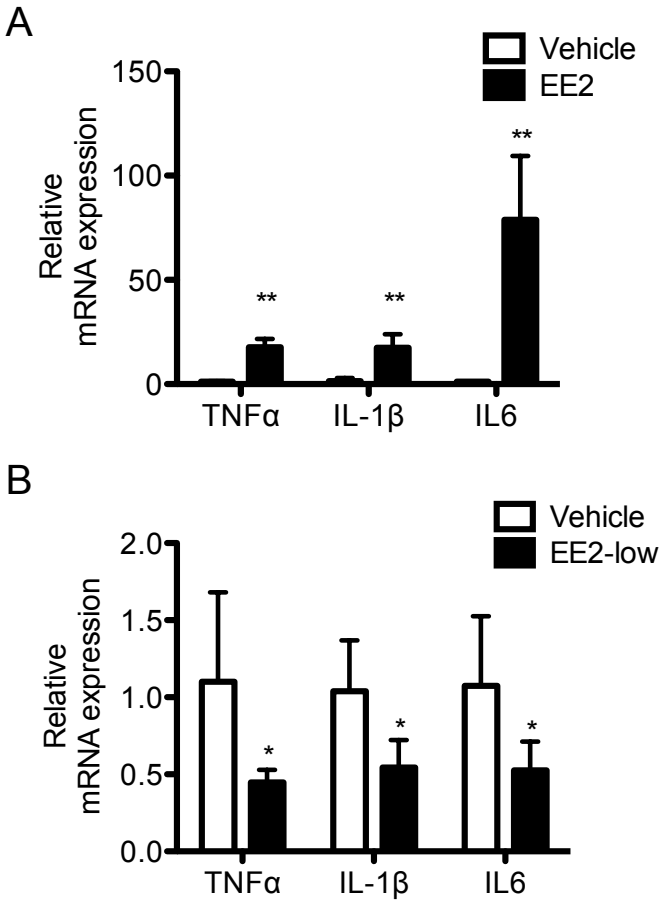

Figure 41. TNFα, IL-1β, and IL-6 mRNA expression is increased in mice treated with high-dose EE2.
A, Tg-*CYP2D6* mice were injected subcutaneously with 17α-ethinylestradiol (EE2, 10 mg/kg) or vehicle (olive oil) daily for 5 days (*n*=5). B, Tg-*CYP2D6* mice were injected intraperitoneally with EE2 (5 μg/kg) or vehicle control (olive oil) daily for 5 days (*n*=4). The mRNA expression of TNFα, IL-1β, and IL-6 was measured by qRT-PCR. Values are presented as mean ± S.D. ** $p < 0.01$; * $p < 0.05$, versus vehicle treatment.

Consistent with the relatively small changes in CYP2D6 activity upon administration of EE2 at the low dose, clinical data suggest minor roles of estrogen (if any) in the regulation of CYP2D6 expression/activity in humans. For example, the use of oral contraceptive steroids had no influence on the urinary metabolic ratios of CYP2D6 substrates (sparteine or dextromethorphan) (181,182). Also, sex did not affect the extent of CYP2D6-mediated sparteine oxidation in 194 subjects (181) or the CYP2D6 expression levels in 300 human liver tissues (183). Overall, these results suggest that CYP2D6 repression in estrogen-induced cholestasis is triggered in part by a combination of (1) ERα activation and (2) biological changes accompanying cholestasis (e.g., SHP protein stabilization and inflammation).

We showed that EE2-induced cholestasis increases SHP and represses CYP2D6 expression in Tg-*CYP2D6* mice. Importantly, this study presents estrogen and bile acids as potential contributors to the differential regulation of CYP2D6 expression. This potentially provides a mechanistic basis to identify the sources of interindividual variability in CYP2D6-mediated drug metabolism.

5. CHOLIC ACID FEEDING LEADS TO INCREASED CYP2D6 EXPRESSION IN CYP2D6-HUMANIZED MICE

5.1. Introduction

Cytochrome P450 2D6 (CYP2D6) is a major drug-metabolizing enzyme, responsible for eliminating ~20% of marketed drugs (Table II). Despite its importance as a drug-metabolizing enzyme, regulation of CYP2D6 expression remains poorly understood as compared to other drug-metabolizing enzymes such as CYP3A4. Recently, we have identified a transcriptional repressor small heterodimer partner (SHP) as a novel regulator of CYP2D6 expression using CYP2D6-humanized transgenic (Tg-*CYP2D6*) mice; SHP represses hepatocyte nuclear factor (HNF) 4α transactivation of CYP2D6 promoter (144).

SHP is a representative target gene of bile acid sensor farnesoid X receptor (FXR) (17). Bile acids are endogenous ligands of FXR (19). Upon bile acid binding, activated FXR transactivates SHP promoter via a FXR response element in the promoter (85). Upregulated SHP in turn represses transcription of genes involved in bile acid synthesis (e.g., CYP7A1 and CYP8B1) (86). Cholestasis is a condition that interrupts bile flow, accompanied by increased hepatic bile acid. The increased hepatic bile acid levels lead to FXR activation and subsequently enhanced SHP expression. In mice, Shp mRNA level was increased by ~2-fold within 3-24 hours feeding of cholic acid (CA) (95,148). However, the effects of cholestasis for longer duration of time (e.g., days or weeks) on SHP expression remain unknown. Also unknown is whether and how cholestasis alters CYP2D6 expression.

Cholestasis is accompanied by enlarged bile acid pool size and also a remarkable change in bile acid composition. In cholestasis, the composition of CA can increase from 40% (184) to ~80% in humans (24). Accordingly, CA feeding is commonly used to mimic cholestatic conditions, especially the altered bile acid pool size and composition, in animal models (25-28); in mice, feeding of 1% CA-supplemented diet generates cholestatic liver conditions and a 2-fold increase in the size of bile acid pool, composed predominantly of CA (25).

In this study, we examined how cholestasis alters SHP and CYP2D6 expression in Tg-*CYP2D6* mice by feeding the mice with CA for 2 weeks. Our results revealed unexpected directional changes in SHP protein levels, accompanied by increased and CYP2D6 activity in these mice.

5.2. Materials and methods

5.2.1.Animals

Tg-*CYP2D6* mice were previously described (9). Adult male mice (8 weeks of age and weighing 20-25 g) were used for the experiments. Mice were fed with normal chow (TD.00588, Teklad Diets, Harlan Laboratories, Indianapolis, IN) or 1% (w/w) cholic acid-supplemented diet (TD.10056) for 14 days to mimic cholestasis conditions. Mice were sacrificed on the 15th day; blood and liver tissues were collected. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Institution's Animal Care and use Committee in the University of Illinois at Chicago.

5.2.2. Chemicals and reagents

Debrisoquine, (±)-4-hydroxydebrisoquin, and paraxanthine were purchased from Biomol (Plymouth Meeting, PA).

5.2.3 Western blot

Western blot was performed as described previously in Chapter 2-4.

5.2.4. Determination of CYP2D6 activity

Liver S9 fractions were prepared as described previously (144). CYP2D6 activity was determined by incubating S9 fractions with debrisoquine as described previously in Chapter 3.

5.2.5. RNA isolation and qRT-PCR

Total RNA isolation and qRT-PCR were described previously in Chapter 2-4. For analysis of miR-142-3p, total RNA was isolated and converted to cDNA using TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Specific primers and probes were used for miR-142-3p (Catalog#000464, Life Technologies) and snoRNA202 (Catalog#001232, Life Technologies). The results were expressed as fold changes under treatment using the gene expression levels normalized to those of snoRNA202 ($2^{-\Delta\Delta Ct}$ method).

5.2.6. ChIP assays

ChIP assays were performed as described previously in Chapter 2 using antibodies from Santa Cruz or EMD Millipore (C/EBPα, sc-61x; HNF4α, sc-6556x; FXR,

sc-13063x; RNA polymerase II, sc-899x; SHP, sc-30169; HDAC, sc-7872, Santa Cruz; dimethyl-H3K9, Catalog#07-521, EMD Millipore, Billerica, MA).

5.2.7. Alkaline phosphatase and alanine aminotransferase measurement

Plasma alkaline phosphatase (ALP) and alanine aminotransferase (ALT) levels were measured by chemistry analyzer (Olympus AU 680, Center Valley, PA) following the manufacturer's protocol.

5.2.8. Cytochrome P450 reductase activity

Cytochrome P450 reductase (CPR) activity in the mouse livers was measured by using Cytochrome c Reductase (NADPH) Assay Kit following manufacture's protocol (Catalog#CY0100, Sigma-Aldrich).

5.2.9. Cytochrome b_5 concentration

The differential absorbance between β-NADH-reduced and –oxidized Cytochrome b_5 (Cyb5) in liver S9 factions were measured using spectrometry. The concentration of Cyb5 was calculated using Beer-Lambert law (185,186). β-NADH was purchased from Sigma-Aldrich (Catalog#N4505).

5.2.10. Statistical analysis

Values were reported as mean ± standard deviation (S.D.). Statistical differences were determined by using Student's t-test.

5.3. Results

5.3.1. Cholic acid feeding leads to decreased SHP expression in mice

To determine the effects of long-term cholestasis on SHP expression, Tg-*CYP2D6* mice were fed on CA-diet (or normal chow) for 2 weeks and hepatic SHP expression was examined. Serum ALP (a marker for cholestasis (172)) and ALT (a marker for liver injury) levels were significantly increased in CA-fed mice (Fig. 42), consistent with previous findings (25-28).

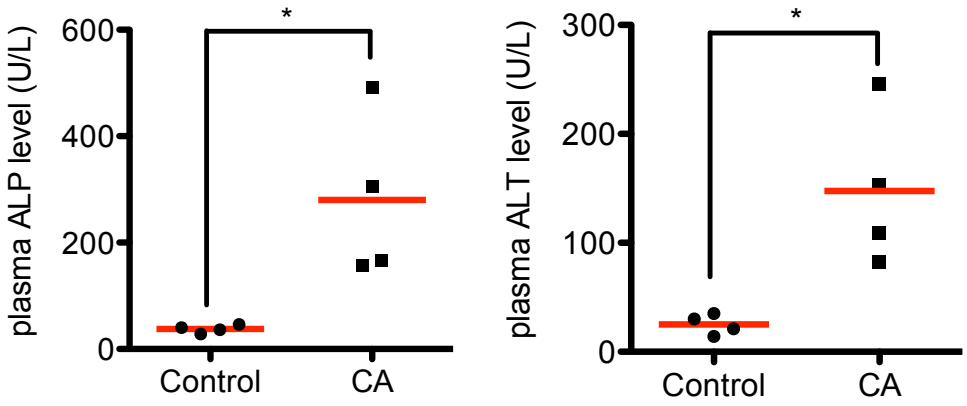

Figure 42. Cholic acid feeding increases plasma ALP and ALT levels.
Tg-*CYP2D6* mice were fed with normal chow (Control) or 1% (w/w) cholic acid-supplemented diet (CA) for 14 days (n=4). ALP level and ALT level were measured in mice plasma. * $p < 0.05$.

In CA-fed mice, hepatic mRNA levels of Shp did not increase (Fig. 43A), contrary to the previous finding that Shp mRNA level was increased by 2-fold at 3~24 hours post-CA feeding (148). While FXR transactivates SHP promoter (86), CCAAT-enhancer-binding protein (C/EBP) α has been recently shown to transrepress SHP promoter via binding to C/EBPα response element (GTTAGGCAAA) of *SHP* promoter (-476/-466 bp) (187). *In silico* anaylsis of mouse Shp promoter revealed an identical C/EBPα putative binding site located at -503/-493 bp region of Shp promoter. To examine the role of FXR and C/EBPα in the transcriptional regulation of Shp upon CA feeding, their recruitments to Shp promoter was examined by using ChIP assays using liver tissues from CA-fed (or control diet-fed) mice. The results showed that recruitments of both FXR and C/EBPα to Shp promoter were increased in CA-fed mice as compared to the control mice (Fig. 43B), suggesting that transrepression of Shp promoter by C/EBPα may offset the transactivation of the promoter by FXR upon long-duration CA feeding.

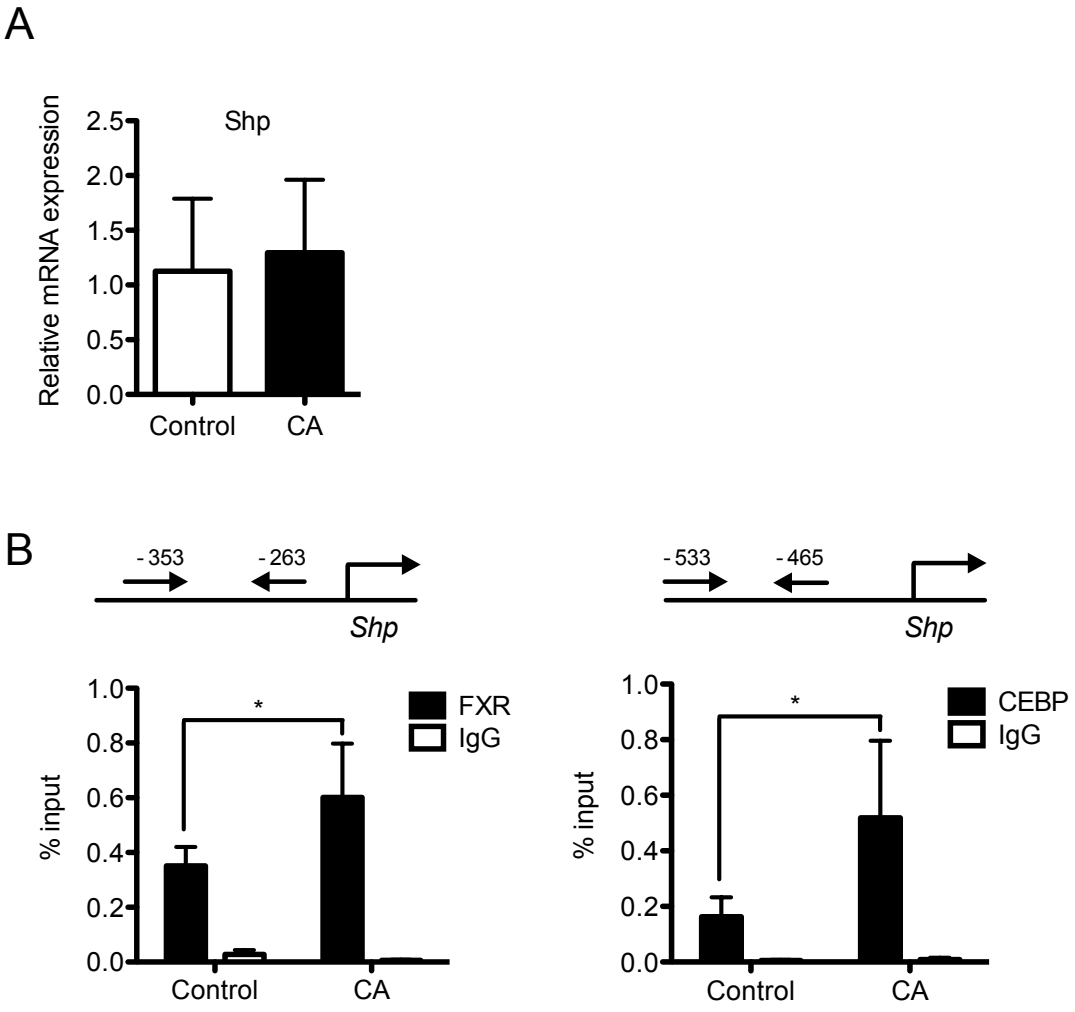

Figure 43. CA feeding does not alter hepatic mRNA expression level of SHP in Tg-*CYP2D6* mice.

Tg-*CYP2D6* mice were fed with normal chow (Control) or 1% (w/w) cholic acid-supplemented diet (CA) for 14 days (*n*=4). A, Hepatic Shp mRNA expression was determined by qRT-PCR. B, Recruitments of FXR and C/EBPα onto Shp promoter were analyzed by ChIP assays using mouse liver tissues, respectively. Values are presented as mean ± S.D. *$p < 0.05$; ** $p < 0.01$, versus control group.

To verify the lack of effects of CA feeding on Shp mRNA expression, SHP protein levels in CA-fed (or control diet-fed) mice were examined by western blot. Interestingly, the results showed that SHP protein was markedly decreased in CA-fed mice as compared to the control mice (Fig. 44A). Numerous studies have shown that microRNAs (miRNAs) influence mRNA stability and/or translation, creating a disconnect between mRNA and protein levels (188). To determine potential involvement of miRNA(s) in the regulation of SHP expression in CA-fed mice, *in silico* analysis was performed for 3′-untranslated region (3'-UTR) of *Shp* in search for putative miRNA binding sites. This revealed a putative binding site for one of the miRNAs previously known to be upregualted in cholestatic human liver tissues, i.e., miR-142-3p (189) (Fig. 44B). To determine whether miR-142-3p indeed shows increased expression in cholestatic mouse liver, its expression levels in CA-fed (or control diet-fed) mice were examined by qRT-PCR. The results showed that miR-142-3p expression was increased ~5-fold in the livers of CA-fed mice as compared to the control mice (Fig. 44C). Together these results suggest potential roles of miR-142-3p in regulating Shp expression in CA-fed mice.

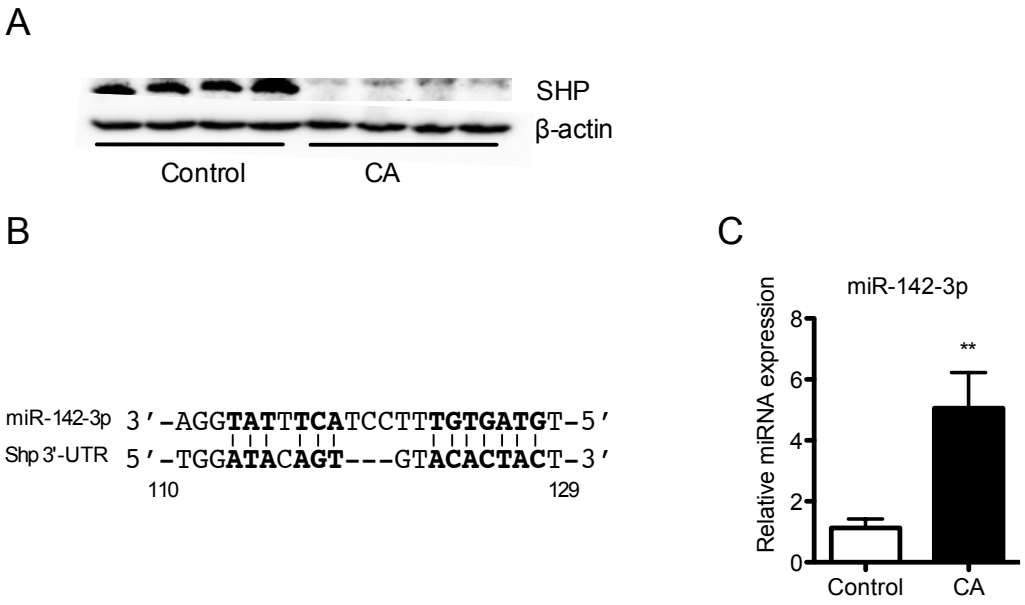

Figure 44. CA feeding leads to decreased SHP protein level in Tg-*CYP2D6* mice. Tg-*CYP2D6* mice were fed with normal chow (Control) or 1% (w/w) cholic acid-supplemented diet (CA) for 14 days (*n*=4). A, SHP protein expression level was determined by western blot. B, Schematic representation of miR-142-3p putative binding sites in *Shp* 3'-UTR. C, Hepatic miR-142-3p expression was determined by qRT-PCR. Values are presented as mean ± S.D. ** $p < 0.01$, versus control group.

5.3.2. Cholic acid feeding leads to increased CYP2D6-mediated drug metabolism in mice.

To determine how CA feeding alters CYP2D6 expression (potentially through modulating SHP expression), the expression levels of CYP2D6 were examined in CA-fed (or control diet-fed) mice by qRT-PCR and western blot. CYP2D6 mRNA and protein expression levels were significantly higher in CA-fed mice as compared to the control mice (Fig. 45A and 45B).

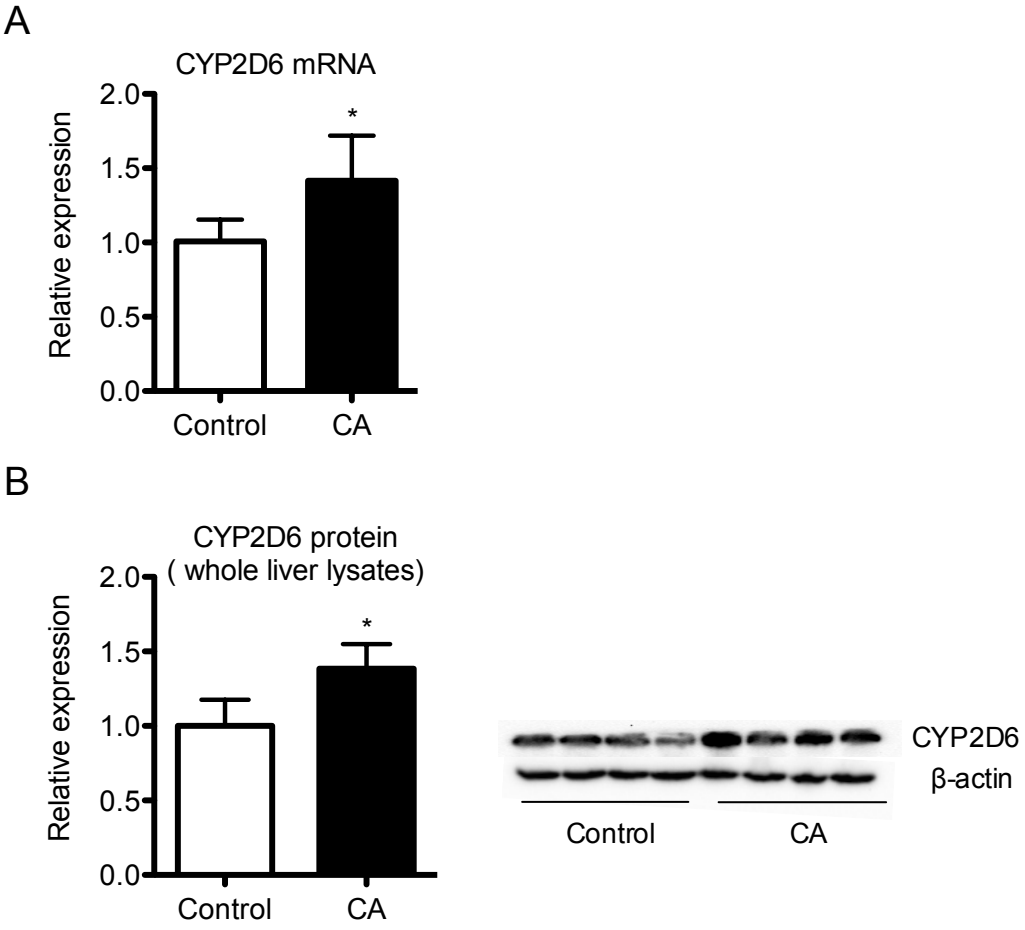

Figure 45. CA feeding increases CYP2D6 expression.
Tg-*CYP2D6* mice were fed with normal chow (Control) or 1% (w/w) cholic acid-supplemented diet (CA) for 14 days (*n*=4). A, Hepatic CYP2D6 mRNA expression was determined by qRT-PCR. B, Protein level of CYP2D6 in whole liver extract was determined by western blot. The image of western blot (right) is shown, and the band intensities (CYP2D6/β-actin, left) were quantified after normalized by CYP2D6 expression in control group. Values are presented as mean ± S.D. *$p < 0.05$, versus control group.

To determine whether increased CYP2D6 expression leads to the change of CYP2D6 activity, S9 fractions were isolated from the livers of CA-fed mice and control mice. CYP2D6 activity in liver S9 fractions was measured using a CYP2D6 probe drug debrisoquine. The concentrations of 4-hydroxylated debrisoquine (a metabolite produced by CYP2D6) were measured by using LC-MS/MS. Interestingly, the data showed that CYP2D6 activity was increased in CA-fed mice by 4-fold (Fig. 46A), which is to a greater extent as compared to the increase in CYP2D6 expression levels (i.e., ~1.5-fold). Of note, CYP2C6 activity in CA-fed mice exhibited larger variability than in control mice (Fig. 46A). The large extent of increase in CYP2D6 activity was also reflected in CYP2D6 protein levels in hepatic S9 fractions (Fig. 46B).

To test the possibility that catalytic activity of CYP2D6 is potentiated by increased electron transferring in the reaction (190), the activity of cytochrome P450 reductase (CPR) and the concentration of cytochrome b_5 (Cyb5) were examined. CPR activity was determined by measuring CPR-mediated reduction of cytochrome c by β-NADPH, while Cyb5 content was determined by measuring β-NAPDH-oxidized and -reduced Cyb5. The results showed that CPR or Cyb5 levels did not differ between the CA-fed vs. control diet-fed mice (Fig. 46C and 46D). Of note, CPR activity in CA-fed mice was found more variable (than in control mice), suggesting that CPR may be responsible for the large variability in CYP2D6 activity in hepatic S9 fractions from CA-fed mice.

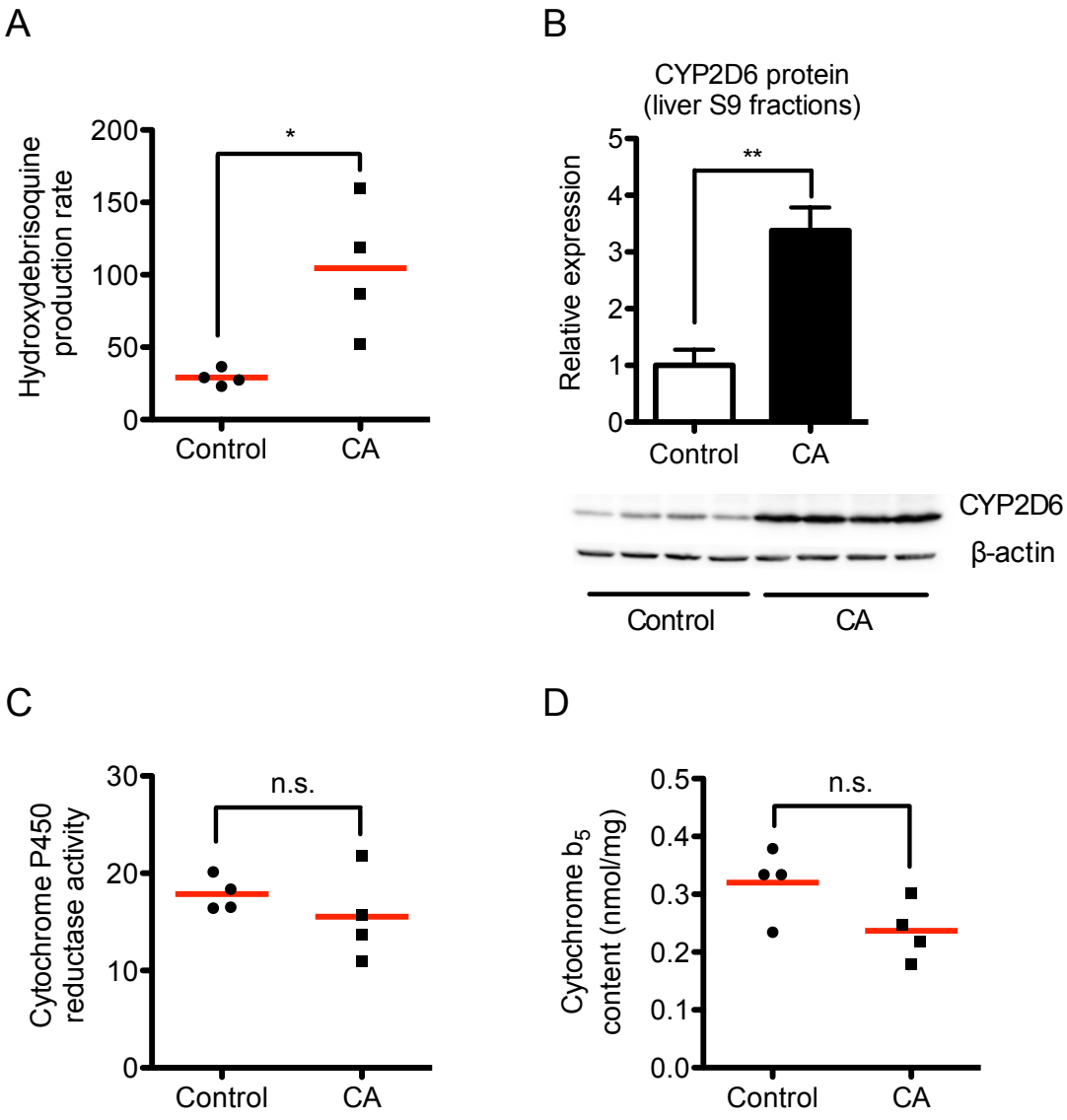

Figure 46. CYP2D6-mediated drug metabolism is induced in CA-fed Tg-*CYP2D6* mice.

Tg-*CYP2D6* mice were fed with normal chow (Control) or 1% (w/w) cholic acid-supplemented diet (CA) for 14 days (*n*=4). A, S9 fractions were prepared from the liver tissues, and CYP2D6 activity in S9 fractions was measured using debrisoquine (200 μM) as a probe drug. Data shown are metabolite production rates in pmol/min/mg protein. B, Protein level of CYP2D6 in hepatic S9 fractions was determined by western blot. The image of western blot (bottom) is shown, and the band intensities (CYP2D6/β-actin, top) were quantified after normalized by CYP2D6 expression in control group. C, Cytochrome P450 reductase activity was performed in hepatic S9 fractions. Data shown are cytochrome c production rate in nmol/min/mg protein. D, Cytochrome b_5 levels were measured in hepatic S9 fractions. Values are presented as mean ± S.D. *$p < 0.05$; ** $p < 0.01$.

5.3.3. Cholic acid differentially regulates CYP2D6 and Cyp7a1 (or Cyp8b1) promoter

To determine whether decreased SHP protein levels upon CA feeding leads to altered HNF4α transactivation of CYP2D6 promoter, ChIP assays were performed using livers collected from CA-fed (or control diet-fed) mice. At the end, the recruitment of HNF4α, SHP, and RNA polymerase II (Pol II, a marker of transcription initiation) to CYP2D6 promoter was examined. As a control, the recruitment of the transcription factors on the promoters of representative SHP target genes, Cyp7a1 and Cyp8b1 (86,151), was also examined. The results showed that CA feeding increased the recruitments of HNF4α and Pol II to CYP2D6 promoter (Fig. 47), consistent with the increased expression levels of CYP2D6 mRNA (Fig. 45A). On the other hand, the recruitment of SHP onto CYP2D6 promoter was decreased in CA-fed diet although it did not reach a statistical significance.

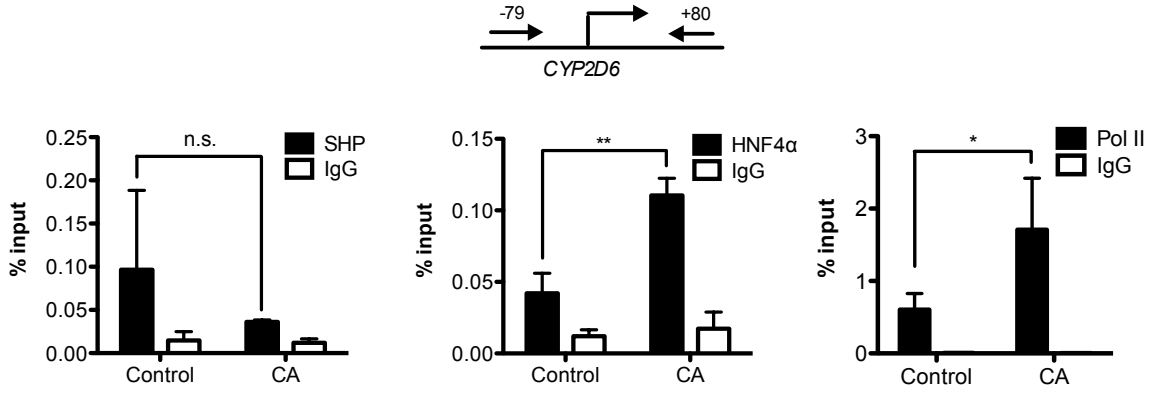

Figure 47. HNF4α transactivation of CYP2D6 promoter is increased in CA-induced cholestasis.
Tg-*CYP2D6* mice were fed with normal chow (Control) or 1% (w/w) cholic acid-supplemented diet (CA) for 14 days (*n*=4). Recruitment of SHP, HNF4α, and RNA polymerase II (Pol II) on to CYP2D6 promoter was analyzed by ChIP assay using mouse liver tissues. Values are presented as mean ± S.D. *$p < 0.05$; ** $p < 0.01$, n.s.= not statistically significant.

Interestingly, the recruitment of SHP, HNF4α, and Pol II to Cyp7a1 and Cyp8b1 promoter exhibited the opposite patterns as compared to those for CYP2D6 promoter; CA feeding led to increased SHP and decreased HNF4α and Pol II recruitment to Cyp7a1 and Cyp8b1 promoters (Fig. 48A and 48C). This was accompanied with significant decreases in hepatic mRNA levels of Cyp7a1 and Cyp8b1 in CA-fed mice (Fig. 48B and 48D), suggesting that despite overall decreases in SHP expression upon CA feeding, SHP action (e.g., its recruitment to target gene promoter) to Cyp7a1 and Cyp8b1 persists to repress the transcription of Cyp7a1 and Cyp8b1.

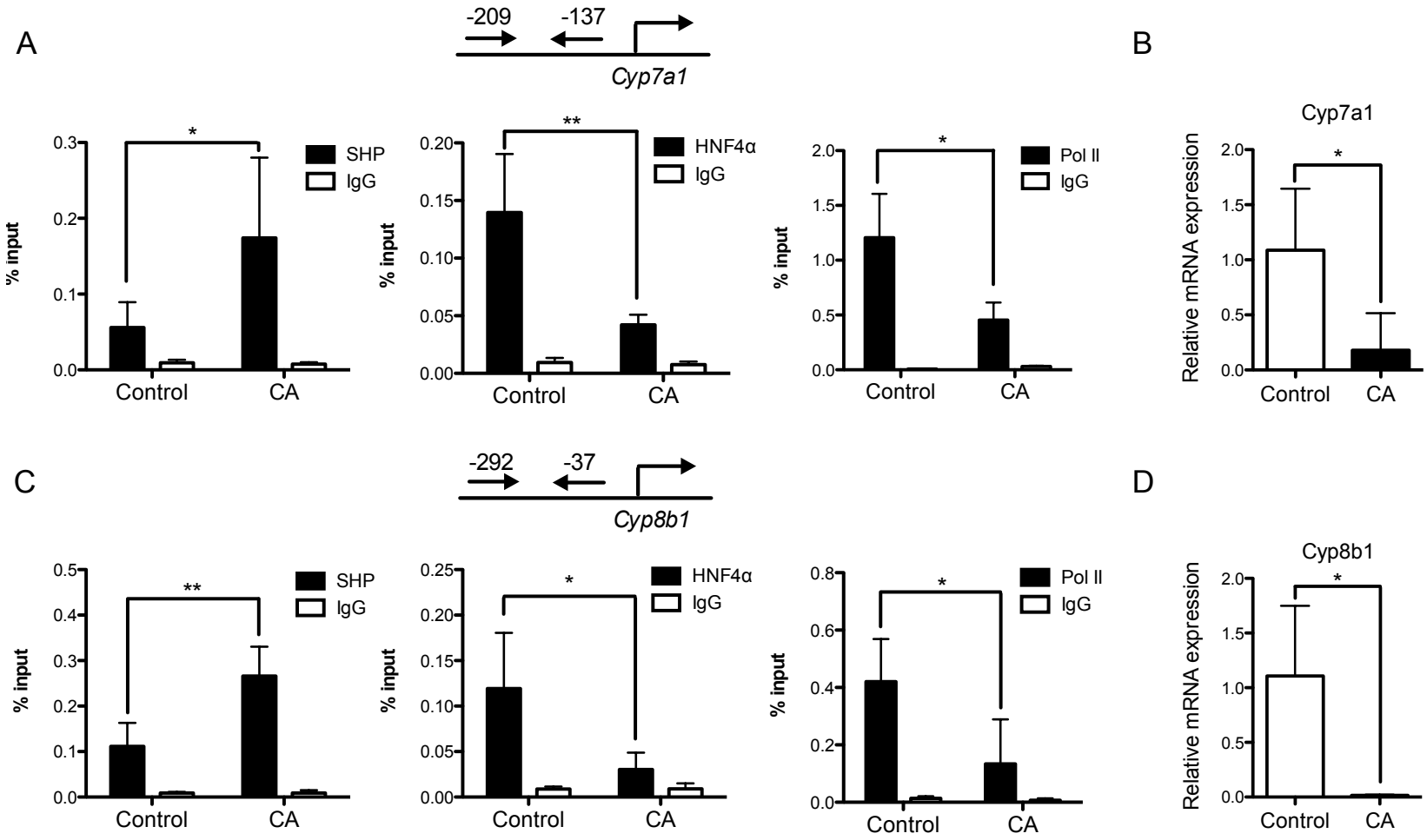

Figure 48. CA feeding down-regulates Cyp7a1 and Cyp8b1 expression likely by suppressing promoter activity.
Tg-*CYP2D6* mice were fed with normal chow (Control) or 1% (w/w) cholic acid-supplemented diet (CA) for 14 days (*n*=4). A and C, Recruitment of SHP, HNF4α, and RNA polymerase II (Pol II) on to (A) Cyp7a1 promoter and (C) Cyp8b1 promoter were analyzed by ChIP assays using mouse liver tissues. B and D, Cyp7a1 and Cyp8b1 mRNA expression levels were measured by qRT-PCR. Values are presented as mean ± S.D. *$p < 0.05$; ** $p < 0.01$.

Previous studies have demonstrated that bile acid induces the recruitment of histone deacetylase (HDAC) and histone methyltransferase G9a that methylates lysine 9 of histone 3 (H3K9), leading to “closed” chromatin and Cyp7a1 silencing (95,191). To explore potential mechanisms underlying differential regulation of CYP2D6 and Cyp7a1/Cyp8b1, chromatin modification was examined for these genes. The recruitments of HDAC and dimethylated H3K9 (diMet-H3K9) to the promoters of CYP2D6, Cyp7a1, and Cyp8b1 were measured by ChIP assays using liver tissues from CA-fed mice and control mice. The results showed that the recruitments of HDAC and diMet-H3K9 to Cyp7a1 promoter and Cyp8b1 promoter were increased in CA-fed mice (Fig. 49A and 49B), indicating the presence of closed chromatin structure at Cyp7a1/Cyp8b1 promoter region. On the other hand, the recruitment of HDAC to CYP2D6 promoter was decreased upon CA feeding (Fig. 49C). These results indicate that differential chromatin modification is associated with dissimilar regulation of CYP2D6 and Cyp7a1/Cyp8b1 expression upon CA-feeding.

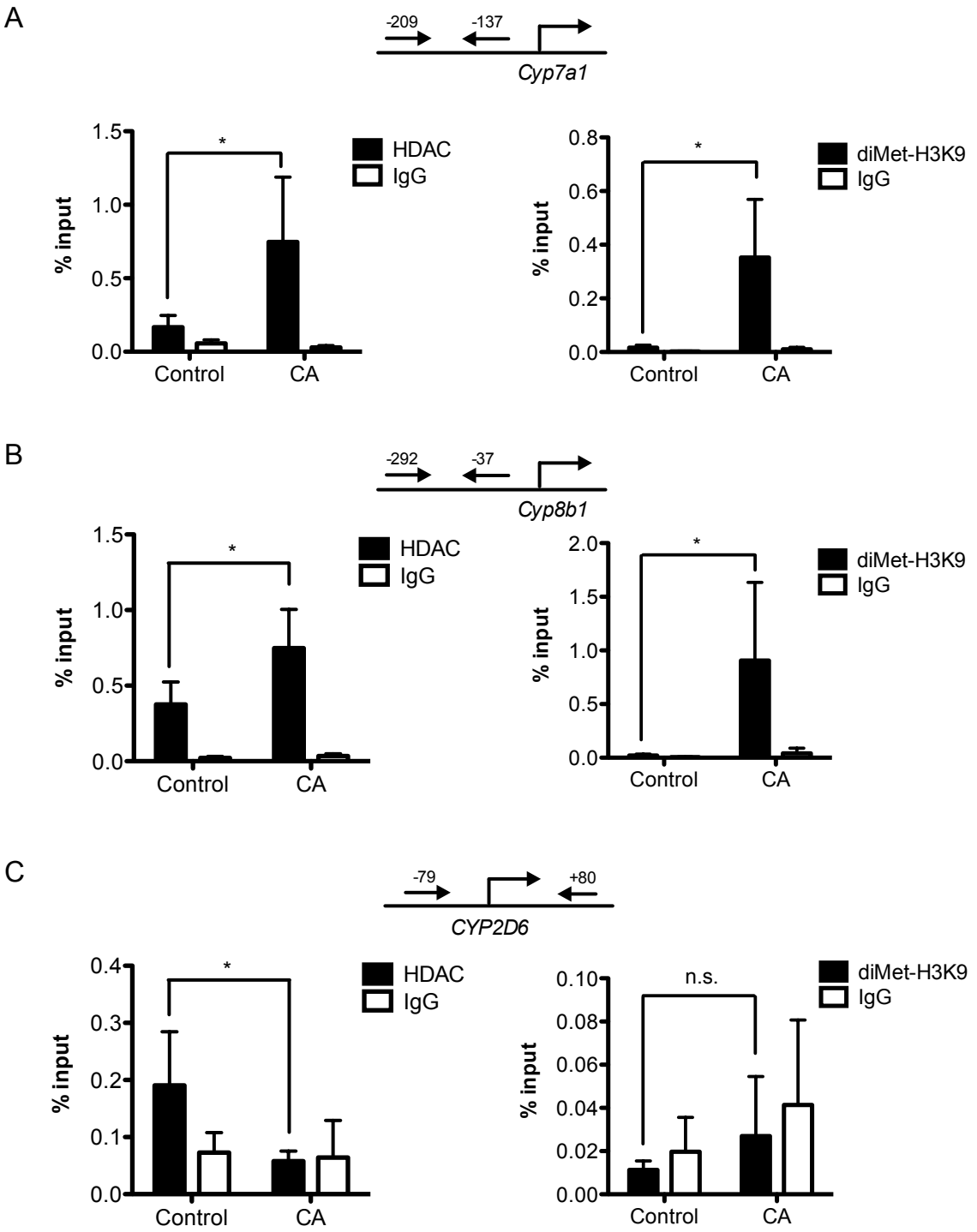

Figure 49. CA-induced cholestasis causes differential chromatin modification of CYP2D6 and Cyp7a1/Cyp8b1.

Tg-*CYP2D6* mice were fed with normal chow (Control) or 1% (w/w) cholic acid-supplemented diet (CA) for 14 days (*n*=4). Recruitments of HDAC and dimethylated H3K9 (diMet-H3K9) on to (A) Cyp7a1 promoter, (B) Cyp8b1 promoter, and (C) CYP2D6 promoter were analyzed by ChIP assays using mouse liver tissues. Values are presented as mean ± S.D. *$p < 0.05$, n.s.= not statistically significant.

5.4. Discussion

We have previously shown that SHP is a transcriptional repressor of CYP2D6 expression (144). Also, the activation of FXR (by using a synthetic agonist) and subsequently increased SHP led to decreased CYP2D6 expression and activity (176). Here we examined whether increased levels of endogenous FXR ligands (i.e., bile acids) in cholestasis alter CYP2D6 expression in mice. Considering that cholestasis typically lasts days to weeks in clinical setting, a feeding period of 2 weeks was employed. The important finding of this study is that CA feeding-induced cholestasis increased CYP2D6 expression and activity, accompanied by decreased SHP protein levels.

SHP is a representative target gene of FXR; SHP expression is minimal in Fxr-null mice (17), and Shp mRNA level was induced 2-fold within 3~24 hours of CA feeding (95,148). Unexpectedly, in this study, SHP mRNA levels were not increased in CA-fed mice. Different time points of sample collection after CA feeding may explain the discrepancy. Shp is an early response gene whose mRNA expression peaks within 2 hours upon promoter transactivation and then normalizes to the basal levels in 24 hours in mouse liver (141). In most previous studies, liver tissues were collected within hours after feeding of CA (95,148) when SHP induction by FXR activation is likely maximal. In contrast, the 2-week time point used in this study may allow time for normalizing SHP expression to basal level, as in a previous study where 12-week CA feeding had no effects on mRNA levels of Shp (192). While molecular mechanisms underlying this process remain to be elucidated, transcriptional repressor of SHP may play a role. Recent studies have revealed C/EBPα as a transcriptional repressor of SHP; phosphorylation of C/EBPα by phosphatidylinositol 3 kinase (PI3K) represses human SHP gene via directly binding to C/EBPα response element located at -476/-466 bp of *SHP* (187). Our *in silico* analysis showed this putative C/EBPα binding site is conserved

in mouse SHP promoter (-503/-493 bp). Our results showed that CA feeding increases C/EBPα recruitment to Shp promoter, in addition to the FXR recruitment to Shp promoter (likely through increased bile acid levels). Potentially, activated PI3K pathway triggered by CA feeding (193-195) enhances C/EBPα action on Shp promoter, and this offsets FXR transactivation of Shp promoter.

While mRNA levels of SHP did not differ between the CA-fed vs control diet-fed mice, protein levels of SHP were significantly decreased upon CA feeding. This suggests that post-transcriptional regulation may play a role in the gene regulation, which is often mediated by miRNAs. miR-142-3p was previously shown to exhibit increased expression in cholestatic human liver tissues (189). In this study, we also showed that hepatic expression of miR-142-3p is increased in CA-fed mice, and *in silico* analysis of 3'-UTR region of Shp revealed the presence its putative binding site. These findings strongly suggest that miR-142-3p is possibly involved in the regulation of SHP expression in CA-fed mice. Studies to determine whether miR-142-3p indeed regulates SHP expression by using transient transfection and luciferase reporter assays are currently ongoing.

CA feeding led to significant increases in CYP2D6 expression at mRNA and protein levels, consistent with the notion that SHP is a negative regulator of CYP2D6 expression (144) and decreased SHP protein levels in CA-fed mice. Interestingly, the extent of changes in CYP2D6 activity in S9 fraction of CA-fed mice was much greater than that in CYP2D6 expression. Our results indicate that this is likely due to a greater extent of increases in CYP2D6 protein levels in S9 fraction by CA feeding as compared to those in whole liver lysate. Changes in targeting of CYP2D6 protein to endoplasmic reticulum upon CA feeding may explain the findings. Recent studies have shown that in

addition to endoplasmic reticulum, CYP2D6 also localizes to mitochondria (196). Of note, S9 is composed of cytosol and endoplasmic reticulum but not mitochondria. Studies have shown that low protein kinase A (PKA) conditions facilitate CYP2D6 translocation on endoplasmic reticulum membrane, while CYP2D6 is guided to mitochondria in high PKA conditions (196-198). PKA activity is known to be highly dependent on cellular levels of cAMP whose synthesis can be repressed in cholestasis in hamsters (199). Whether CA feeding indeed represses cAMP, deactivates PKA, and facilitates CYP2D6 targeting to endoplasmic reticulum need to be further studied.

Results from this study demonstrate a disconnect between SHP protein levels and SHP activity as a transcriptional repressor, in a gene-specific manner. Despite the decreased SHP protein levels in CA-fed mice, SHP recruitment to the promoter regions of Cyp7a1 and Cyp8b1 was found increased but not for CYP2D6 promoter. Corresponding changes in SHP-induced chromatin modifications that lead to the "closed" chromatin structure (i.e., HDAC and dimethylation of H3K9) were found for Cyp7a1 and Cyp8b1, but not or CYP2D6. These results suggest that altering SHP expression alone is not sufficiently for Cyp7a1 and Cyp8b1 repression in cholestasis and that additional factors may be involved in Cyp7a1 and Cyp8b1 repression in cholestasis. Indeed, bile acid-induced repression of Cyp7a1 and Cyp8b1 expression was maintained in *Shp*-null mice (149). Subsequent studies demonstrated that SHP-independent mechanisms including cytokine signaling pathway (173,200) and intestinal-liver signaling pathway FGF15/19-FGFR4 (201-203) play critical roles in bile acid repression of Cyp7a1 and Cyp8b1. Whether the activation of cytokine signaling or intestinal FGF15/19 pathways is responsible for the chromatin modifications of Cyp7a1 and Cyp8b1 gene regulatory regions in CA-fed mice remains to be determined.

Previously, we demonstrated that CYP2D6 expression were repressed (accompanied by increased SHP expression) in estrogen-induced intrahepatic cholestasis (204). However, in present studies using CA-fed mice to mimic cholestatic conditions, we observed enhanced CYP2D6 expression and repressed SHP expression. The opposite impacts on CYP2D6 expression may be associated with differential gut environment between EE2-induced cholestasis and CA feeding induced cholestasis in mice. Bile (mainly bile acids) is secreted from gallbladder to the gut, and maintains the proper bacterial growth in intestine (205,206). Of note, EE2-induced cholestasis remarkably blocked bile secretion, and subsequently led to intestinal bacteria overgrowth (20), while CA feeding abolished intestinal bacterial overgrowth in cholestatic rodents by induced secretion of bile (25,207-209). Studies have shown abnormally intestinal bacterial growth influences signaling pathways in the liver. For example, AMP-activated protein kinase (AMPK) signaling pathway was activated in the livers of germ-free mice (210). Activation of AMPK signaling pathway repressed SHP expression through FXR *in vitro* and *in vivo* (211). Whether (and how) gut environment contributes to SHP/CYP2D6 regulation needs to be investigated.

In conclusion, we showed that CA-feeding increases CYP2D6 expression and activity in Tg-*CYP2D6* mice potentially through decreased SHP protein expression. While the detailed molecular mechanisms underlying the phenomenon remain to be elucidated, potential involvement of miRNA and C/EBPα has been postulated (Fig. 50). Studies are currently ongoing to define the role of miR-142-3p in post-transcriptional regulation of SHP in CA-fed mice.

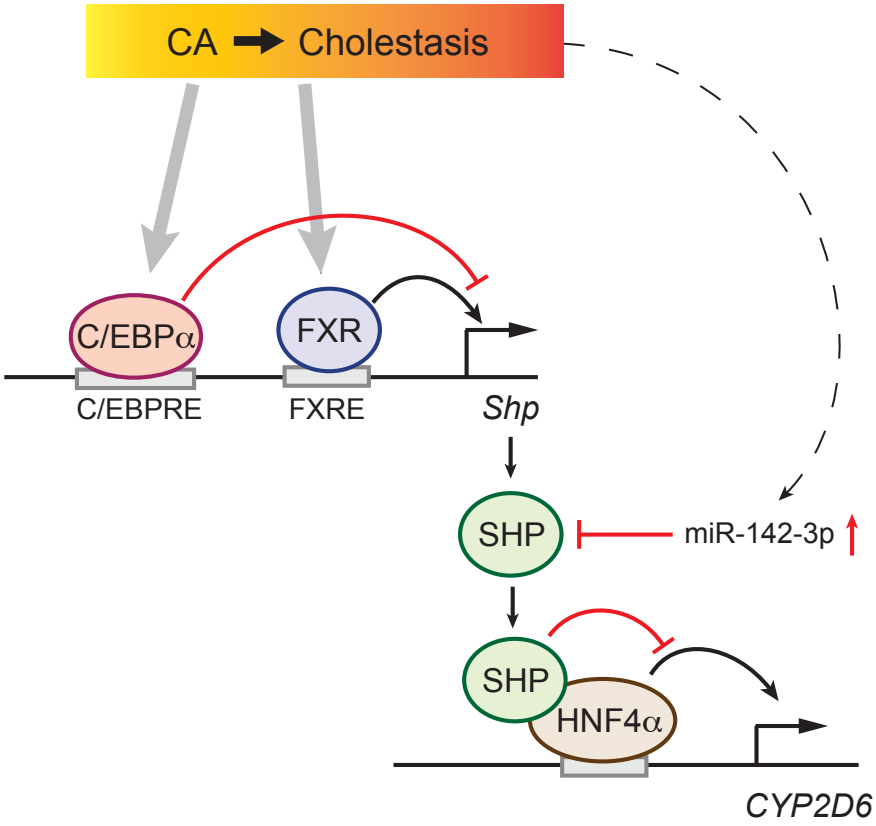

Figure 50. Working model for CYP2D6 regulation in CA feeding-induced cholestasis.
The working model illustrates the opposite actions of C/EBPα and FXR on Shp promoter. In CA feeding, C/EBPα and FXR recruitments to Shp promoter are increased, leading to offsetting Shp mRNA expression to basal level. Induced level of miRNA-142-3p in the liver may be responsible for decreased protein level of SHP. Decreased SHP level in turn de-represses HNF4α transactivation of CYP2D6 promoter, leading to CYP2D6 induction.

6. CONLUSION AND FUTURE DIRECTIONS

The large interinidividual variability in CYP2D6 activity is a major obstacle in achieving optimal drug therapy for CYP2D6 substrate drugs. Improved prediction of CYP2D6 activity through better understanding of factors determining CYP2D6 expression should enable personalized medicine for CYP2D6 substrate drugs. We have identified a novel transcriptional regulator of CYP2D6, namely SHP. We have further shown that inducers of SHP expression (atRA, GW4064, and estrogen-induced cholestasis) lead to decreases in CYP2D6 expression and activity (Fig. 51). These results suggest different levels of SHP modulators may contribute to large interindividual variability in CYP2D6 activity.

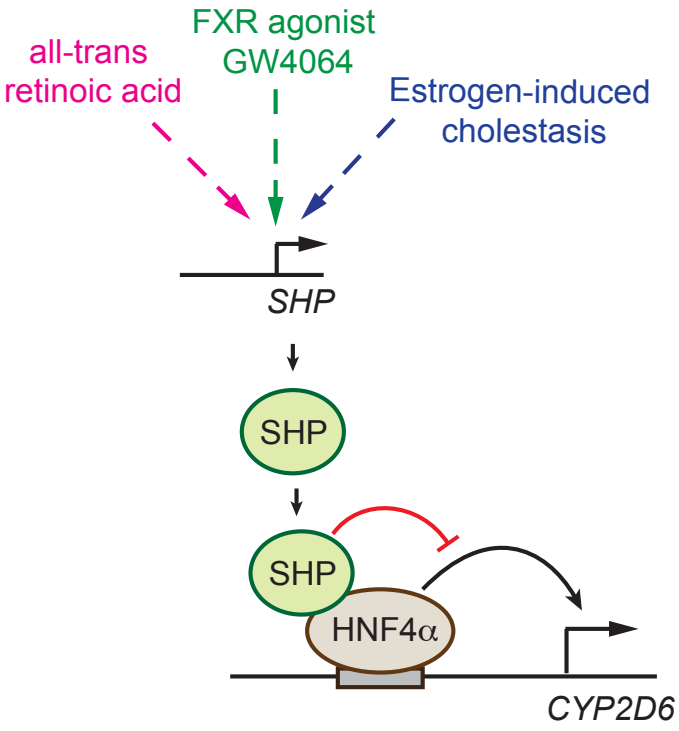

Figure 51. Potential novel factors involved in transcriptional regulation of CYP2D6. This illustrates that the inducers of SHP expression (all-trans retinoic acid, FXR agonist GW4064, and estrogen-induced cholestasis) decrease CYP2D6 expression through repressing HNF4α transactivation of CYP2D6 promoter.

Our future study will focus on determining the portion of CYP2D6 variability in humans that can be explained by altered levels of SHP expression/activity. Specifically, we will measure levels of atRA and bile acids in a large number of healthy human liver tissues, and examine their relative contribution to CYP2D6 variability in the tissues. In addition, considering that many genetic variants of SHP are associated with altered expression and functional changes of SHP (212-216), we will examine whether and how SHP genetic polymorphisms are linked to altered CYP2D6 expression and activity in human liver tissues. The results are expected to help us identify major factors determining CYP2D6 activity and develop a predictive model for CYP2D6 activity in an individual. Together, this effort should decrease adverse drug events from using CYP2D6 substrates.

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ABSTRACTS:

1. 6/11/2015-6/13/2015 Inducers of small heterodimer partner expression lead to decreased CYP2D6 expression and activity. 47th Annual Pharmaceutics Graduate Student Research Meeting (PGSRM), Lexington, KY
2. 3/28/2015-4/1/2015 Inducers of small heterodimer partner expression lead to decreased CYP2D6 expression and activity. Experimental Biology meeting, Boston, MA
3. 2/27/2015 Differential expression of small heterodimer partner potentially contributes to interindividual variability of CYP2D6. UIC College of Pharmacy Research Day, Chicago, IL
4. 10/19-10/23/2014 Differential expression of small heterodimer partner potentially contributes to interindividual variability of CYP2D6. 19th North America International Society for the Study of Xenobiotic (ISSX) Annual Meeting, San Francisco, CA
5. 6/27-6/29/2014 Altered expression of small heterodimer partner governs CYP2D6 induction during pregnancy in CYP2D6-humanized mice. 46th Annual Pharmaceutics Graduate Student Research Meeting (PGSRM), Chicago, IL
6. 6/6-6/11/2014 Altered hepatic retinoic acid content and small heterodimer partner expression underlie CYP2D6 induction during pregnancy. Gordon Research Conference (Drug Metabolism), Holderness, NH
7. 5/15-5/16/2014 Altered hepatic retinoic acid content and small heterodimer partner expression underlie CYP2D6 induction during pregnancy. Annual Meeting of the Great Lake Drug Metabolism and Disposition Group Meeting, Indianapolis, IN
8. 4/28/2014 Altered hepatic retinoic acid content and small heterodimer partner expression underlie CYP2D6 induction during pregnancy. UIC Women's Health Research Day, Chicago, IL
9. 3/7/2014 Altered expression of small heterodimer partner governs CYP2D6 induction during pregnancy in CYP2D6-humanized mice. UIC College of Pharmacy Research Day, Chicago, IL
10. 4/20-4/24/2013 Induction of CYP2D6 Expression during Pregnancy Is Associated with an Increased Activity of Hepatocyte Nuclear Factor 4α. Experimental Biology Meeting, Boston, MA
11. 2/22/2013 Induction of CYP2D6 Expression during Pregnancy Is Associated with an Increased Activity of Hepatocyte Nuclear Factor 4α. UIC College of Pharmacy Research Day, Chicago, IL
12. 5/2011 A PBPK model characterizing mechanism-based inhibition of CYP1A2 for predicting theophylline/antofloxacin interaction in both rats and humans. International Symposium on Microsomes and Drug Oxidation, Beijing, China

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