Hormonal Regulation of CYP2B6 and CYP2D6 Expression

BY

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THESIS

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This thesis is dedicated to my parents for their endless support.

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This thesis shares the data I have gathered, and the work I have completed, in my graduate training, but it represents much more than that. Without the help, guidance, and selflessness of many others, I would not have been given the opportunities and encouragement to move further in my education and growth as a scientist.

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LIST OF ABBREVIATIONS

AF1	Activation Function 1
AF2	Activation Function 2
AP-1	Activator Protein 1
CAR	Constitutive Androstane Receptor
CYP	Cytochrome P450
DBD	DNA Binding Domain
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E_2	Estradiol
EMSA	Electrophoretic Mobility Shift Assay
ERα/β	Estrogen Receptor α/β
ERE	Estrogen Response Element
EtOH	Ethanol
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
HDAC	Histone Deacetylase
HNF4α	Hepatocyte Nuclear Factor 4α
mRNA	Messenger Ribonucleic Acid
NCoR	Nuclear Receptor Corepressor
OTC	Over The Counter
PCR	Polymerase Chain Reaction
PTM	Posttranslational Modification
PXR	Pregnane X Receptor
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element
RNA	Ribonucleic Acid

LIST OF ABBREVIATIONS (CONTINUED)

RXR	Retinoid X Receptor
SERM	Selective Estrogen Receptor Mediator
Sp1	Specificity Protein 1
TFF1	Trefoil Factor 1
UGT	UDP Glucuronosyltransferase

SUMMARY

Clinical observations have revealed an alteration in metabolic rate of many substrates during pregnancy, but to date, the responsible factors or the underlying mechanisms remain unknown. The goal of this thesis project was to identify the responsible plasma factors and elucidate the underlying molecular mechanisms for altered drug metabolism during pregnancy, with a focus on CYP2B6 and CYP2D6.

Previous research in the Jeong laboratory has shown that E_2, a major estrogen whose concentration increases over 100-fold during pregnancy, induces CYP2B6 expression in human hepatocytes. The molecular mechanism, however, remained unknown. Our results in HepG2 and HepG2-ER cells showed that E_2 induces CYP2B6 promoter activity in an ER-dependent manner. In HepG2 cells, E_2 enhanced CYP2B6 promoter activity only upon ER transfection. In HepG2-ER cells, E_2 increased CYP2B6 mRNA expression and co-treatment with ICI182,780 (an ER-degrading antiestrogen) abrogated the induction. To investigate whether the ER action on CYP2B6 is through classical [i.e., direct binding of ER to an estrogen response element in the upstream regulatory region of its target genes through ER's DNA-binding domain (DBD)] or nonclassical (i.e., ER exerting its genomic effects through third-party transcription factors such as AP-1) mechanisms, we performed luciferase reporter studies in HepG2 cells, transiently transfecting different ER mutants. Our results showed that the DBD is not required in the induction of CYP2B6 promoter activity by E_2, suggesting that a nonclassical mechanism of ER plays a role in CYP2B6's induction and a third-party protein is contacting the DNA. Results from deletion assays using 5'-deleted luciferase constructs of the CYP2B6 promoter revealed that the responsible cis-element(s) lie in the -1839 to -1461-bp region of CYP2B6. *In silico* analysis of the CYP2B6 promoter revealed two putative AP-1 binding sites within this region. Reporter assays revealed that when both binding sites are mutated, CYP2B6 expression is abolished after E_2 treatment. Taken together, our results show that CYP2B6 induction is driven by E_2, mediated by ER, and requires AP-1 proteins.

Pregnancy is known to induce CYP2D6-mediated drug elimination. The responsible factors and the underlying molecular mechanism are completely unknown, in part due to a lack of animal models recapitulating CYP2D6 induction during pregnancy. We have acquired CYP2D6-humanized mice carrying human CYP2D6 along with 2.5-kb of its upstream regulatory region, and have examined CYP2D6-humanized mice as a potential model to study CYP2D6 induction during pregnancy. Using liver tissues from these mice, collected from our collaborators at a different institution, we have previously shown that pregnancy induces CYP2D6 expression in these mice. In this study, the results were confirmed using mouse lines currently maintained at the University of Illinois at Chicago; we found that there was a ~2-fold induction in liver CYP2D6 mRNA at the 18th day of pregnancy as compared to the levels during virgin and postpartum periods. Together, we established CYP2D6-humanized mice as a potential animal model to study CYP2D6 induction during pregnancy.

HNF4a is a transcription factor known to play an important role in determining basal expression levels of CYP2D6. During human pregnancy, plasma levels of saturated fatty acids, ligands known to activate HNF4a, increase; however the roles of HNF4a in CYP2D6 induction during pregnancy have never been studied. In this study, we examined whether HNF4α DNA-binding activity was altered throughout pregnancy by using EMSA. Our preliminary results showed that nuclear extracts from liver tissue from the 18th day of pregnancy showed increased binding to a CYP2D6 probe harboring the known HNF4α binding sequence (from the CYP2D6 promoter) as compared to those from virgin or postpartum livers. This experiment suggests HNF4α has a role in CYP2D6 induction, but further studies are needed to verify this finding.

A recent report has shown a repressive effect of retinoic acid on CYP2D6 expression in HepG2 cells. Previously, the Jeong lab found, through a microarray, that multiple genes involved in retinoid regulation are differentially regulated during pregnancy. Pregnancy in humans and rodents has been shown to alter concentrations of retinol, a precursor to retinoic

acid, although it remains unknown whether retinoids are responsible for CYP2D6 induction during pregnancy. In a preliminary study, we aimed to verify the previously reported repressive effect of retinoic acid on CYP2D6 expression, to and examine the effects of various retinoids on CYP2D6 promoter activity. In HepG2 cells, retinoids showed a modest repression of both CYP2D6 promoter activity as well as mRNA expression after retinoid treatment. Further studies are needed to examine whether retinoids are responsible for CYP2D6 induction during pregnancy.

This thesis project took on two main areas of pregnancy's effect on drug metabolism. One project found a molecular mechanism, while the other set up a model and initial data to begin to deduce a mechanism that is almost entirely unknown. The molecular mechanism for CYP2B6 during pregnancy, elucidated in this study, will likely help us better understand drug metabolism changes in populations showing significant changes in hepatic estrogen concentrations, such as oral contraceptive users. On the other hand, the establishment of a mouse model of CYP2D6 induction creates a platform to further study CYP2D6 regulation during pregnancy. We also presented HNF4α and retinoids as a transcriptional regulator and a plasma factor, respectively, that are potentially responsible for CYP2D6 induction during pregnancy. Further elucidation of the underlying molecular mechanism in the future will likely help us better understand the well-known large interindividual variability in CYP2D6 activity.

I. INTRODUCTION

A. MEDICATION USE DURING PREGNANCY

1. Prevalence of medication use during pregnancy

Over 50% of pregnant women take at least one medication, excluding perinatal vitamins and iron [1]. Also, use of dietary supplements among pregnant women ranges from 13% to 36%, with users commonly taking more than one supplement [2, 3]. Another survey found that 96.9% of patients were taking at least one medication, with 76.5% of patients taking something other than prenatal vitamins and iron, and 62.8% of patients taking over-the-counter medications [4]. Taken together, it is clear that medication use during pregnancy is prevalent.

Drug selection, dosing, and monitoring are important considerations for any patient; however, these decisions in the pregnant mother become more difficult, and more critical, as we must consider the dynamic physiologic environment of the expecting mother and the developing fetus. The physiological changes during pregnancy can influence the choice of drug and its dosing, as these changes can have clinically-important effects on the mother and her child.

2. Altered pharmacokinetics of medications during pregnancy

Dosage regimens used during pregnancy are typically determined based on pharmacokinetic and pharmacodynamic studies obtained in men and/or nonpregnant women. Data from limited clinical studies, however, suggest that pregnancy alters pharmacokinetic profiles of drugs. This effect is potentially caused by many different physiological changes, including increased plasma volume, increased glomerular filtration, and in particular altered hepatic drug metabolism. For example, pregnancy is known to increase elimination of drugs metabolized by Cytochrome P450 (CYP)2A6, CYP3A4, CYP2C9, and CYP2D6, while decreasing elimination of CYP1A2 and CYP2C19 substrates [5].

CYP2D6 is a major drug-metabolizing enzyme that mediates hepatic metabolism of many drugs that are currently used during pregnancy including antidepressants,

antihypertensives, and antipsychotics. Of interest, clinical data indicate that pregnancy increases metabolic rates of CYP2D6 substrates in these drug classes [6-8]. For example, using dextromethorphan, a CYP2D6 probe drug that is metabolized to dextrorphan, researchers have shown an increase in the dextrorphan/dextromethorphan metabolic ratio during pregnancy, suggesting CYP2D6 activity is increased during pregnancy [9, 10]. Understanding the pharmacokinetic properties of CYP2D6-mediated metabolism during pregnancy is important in improving dosing for pregnant women, as well as predicting exposure of the patient and developing fetus. To better understand this process, knowing the causative factors involved is essential. Despite its clinical importance, the mechanisms underlying CYP2D6 induction during pregnancy are completely unknown.

3. Estrogen as a potential factor responsible for altered drug metabolism during pregnancy

One notable physiological change during pregnancy is a dramatic increase (up to 100-fold) in plasma concentration of estrogen [11]. Multiple studies have examined the effects of estrogen on hepatic drug metabolism in rodent models (reviewed in [12]); however, due to significant divergence in hepatic drug-metabolizing enzymes between humans and rodents [13, 14] as well as interspecies differences in pregnancy-related physiology (e.g., <10-fold increases in plasma concentrations of estrogen in rodents [15] vs. ~100-fold increase in pregnant women), the results are difficult to be extrapolated to humans. In human cells, estradiol (E_2), a major estrogen elevated during pregnancy [11], is shown to induce the transcription of CYP2A6 and UGT1A4 (a conjugating drug-metabolizing enzyme) by activating ERα transcriptional activity [16, 17]. To date, the effects of E_2 on other major drug-metabolizing enzymes and whether estrogen plays a role in altered drug metabolism during pregnancy remain to be determined.

4. Potential models to study altered drug metabolism during pregnancy

In vivo animal models can provide systems that are able to more accurately represent whole-body physiology. In the case of pregnancy, where many physiological changes occur simultaneously, *in vivo* models can provide a more robust model to elucidate molecular mechanisms. Previously, a transgenic mouse model of CYP3A4 provided initial insight into the regulatory mechanisms in place, as well as developmental- and tissue-dependent expression [18]. It also established a system responsive to known PXR and CAR activators, known transcription factors involved in CYP3A4 regulation. Furthermore, the transgenic mice were used to investigate changes in CYP3A-mediated drug metabolism during pregnancy, including mechanisms, tissue-specific expression, and gestational time course of CYP3A4 expression [19].

A less-developed model is the CYP2D6-transgenic mouse line. Due to interspecies differences in CYP2D genes, native rodent models do not provide a good system for CYP2D-mediated metabolism. This was overcome by the creation of mice expressing human CYP2D6 and 2.5-kb of its upstream regulatory element. It was found that these transgenic mice exhibit much faster debrisoquine (a CYP2D6 probe drug) clearance and a significantly reduced debrisoquine half-life, compared to wild-type animals [20]. Furthermore, decreased expression of HNF4α, a known activator of CYP2D6 expression, led to a significant decrease in CYP2D6 expression as well as CYP2D6-mediated debrisoquine metabolism. This data suggests that these mice provide a robust platform to examine the molecular mechanism of CYP2D6 regulation. To date, however, this mouse model has not been used to investigate the effects of pregnancy on CYP2D6 regulation. This model provides a system to investigate the molecular mechanism of pregnancy's induction of CYP2D6.

B. MECHANISMS OF GENE REGULATION

Gene regulation is a tightly controlled process. It typically involves the action of many transcriptional regulators, each enhancing or repressing the promoter activities of target genes. Nuclear receptors are a class of transcription factors which, upon binding their cognate ligand, translocate into the nucleus, bind to their respective recognition sequence on the DNA, and recruit coactivators, which have the ability to contact the basal level transcription machinery and induce transcription of target genes [21-23].

1. Regulation of E_2 target genes

The biological effects of E_2 are typically mediated through two cognate nuclear receptors, ERα and ERβ, among which ERα is the major subtype expressed in the liver [24]. ER, being activated upon association with estrogen, may regulate expression of its target genes via binding to the promoter in different ways. ER may bind to its cognate *cis*-element, called the estrogen response element (ERE), which is considered classical mechanism of ER action, or alternatively, ER may associate with other transcriptional regulators and indirectly modulate expression of target genes (reviewed in [25]). The latter mechanism is considered a nonclassical mechanism of ER action. ER is composed of 3 key domains: the activating function 1 domain (AF1), DNA binding domain, (DBD), and activating function 2 domain (AF2) [26]. The DBD is responsible for direct interaction of ER with the recognition sequence (ERE). When ER acts through the nonclassical mechanism, its DBD is often dispensable, in part because nonclassical transactivation occurs through the tethering of ER to other transcriptional regulators, for which AF1 and/or AF2 domains of ER are necessary [27]. ER has been shown to pair with many transcription factor partners in nonclassical transactivation, and these proteins include members of the ATF family, the AP-1 family, the Sp1 family, NF-Y, and the USF family, with unknown partners still to be identified [27]. One particular family, the AP-1 family (activator protein 1), has been shown to be involved in many of ER's target genes and can play an

important physiological role [28]. The transcriptional regulators in the AP-1 family include c-Jun and c-Fos, which bind to an AP-1 motif in the upstream region of target genes and regulate gene expression.

2. Regulation by hepatocyte nuclear factor-4α (HNF4α)

HNF4α is a liver-enriched transcription factor that controls expression of many liver-specific genes. Recently, data has suggested that HNF4α binds an endogenous polyunsaturated fatty acid [29], and that HNF4α transcriptional activity is altered by the length and degree of saturation of the interacting ligand [30]. These data suggest that polyunsaturated fatty acids have a repressive effect on HNF4α, and saturated fatty acids have an inductive effect. It was further shown that these protein:ligand interactions occurred at physiologically relevant concentrations (~1 nM fatty acid), and that the ligand binding domain of HNF4α undergoes different conformational changes when bound to polyunsaturated fatty acids vs. saturated fatty acids [31]. This supports the finding that transcriptional activity is dependent on the degree of saturation of the fatty acid.

HNF4α has been shown to undergo multiple posttranslational modifications (PTM), some of which are able to alter its transcriptional activity. One report found, through proteomic analysis, 14 potential modification sites. *In vitro* testing showed that phosphorylation and acetylation of HNF4α repressed the transcriptional activity (in agreement with previously reported data [32, 33]. This analysis covered 81.2% of the HNF4α protein, however, suggesting other PTMs may exist. In fact, an acetylation site has been shown to be required for the nuclear translocation of HNF4α, as well as increases its DNA-binding affinity [34].

HNF4α has been shown to control a broad range of genes involved in lipid and carbohydrate homeostasis [35-37], including the regulation of multiple CYPs [38-40]. In particular, HNF4α, through a proximal *cis*-element in the CYP2D6 promoter, controls basal-level expression of CYP2D6 [41]. It has been reported that circulating lipid levels are altered in

composition and in concentration during pregnancy [42, 43]. Total fatty acids increased by 51% betweens weeks 10 and 40 of gestation, correlating with a 57% increase in saturated fatty acids, a 65% increase in monounsaturated fatty acids, and a 41% and 44% increase in two polyunsaturated acids. However, the relative amounts of these fatty acids changed during pregnancy. Saturated and monounsaturated fatty acids increased in percent of total fatty acid content, while polyunsaturated fatty acids decreased. Together, the effect of fatty acids on HNF4α-mediated transcription and the altered lipid composition during pregnancy suggest that HNF4α may be activated during pregnancy and this may lead to CYP2D6 induction. To date, HNF4α is the only known regulator of basal-level CYP2D6 expression.

3. Gene regulation by retinoids

Retinoids constitute a family of vitamin A derivatives that function in many diverse physiological processes, as well as serve in multiple important clinical roles (Figure 1). Retinoic acid and 9-cis retinoic acid, two pharmacologically active retinoids, elicit their effects through their cognate receptors, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), respectively. These are nuclear receptors that function to activate or repress gene transcription through retinoid binding. It has been shown that RAR and RXR repress many genes, including CYP4A11, c-jun and c-Fos, and apolipoprotein A-II (coincidentally RAR represses ApoAII by antagonizing RXR's transcriptional activation) [44-46].

In many cases of RAR and RXR repression, it occurs through the unbound receptor heterodimerizing and binding co-repressors such as NCoR or SMRT, recruiting HDACs, and effectively repressing transcription [47-49]. They are, however, able to induce transcription, as seen in the induction of CYP26A1 expression by RAR [50]. This happens through heterodimerization of RAR with RXR, which recognizes and binds a retinoic acid response element (RARE) in the CYP26A1 promoter and promote gene transcription [51, 52]. Other CYPs showing retinoid responsiveness include CYP2C7, CYP7A, and CYP24 [53-55].

However, potentially due a ligand-specific conformational change in the nuclear receptors, contradicting results have been found: in rat hepatocytes, the CYP1A2 showed no change in transcripts, and CYP3A family showed strong induction from retinoids [56], but *in vivo* models showed a decrease in CYP1A2 from both RAR- and RXR-selective ligands, and a decrease CYP3A transcripts from RAR-selective ligands [57]. Overall, retinoids show a range of transcriptional effects on CYP expression through multiple pathways, though the exact mechanism of regulation for each gene has yet to be revealed.

Of interest to our project, a previous study showed that retinoic acid decreases CYP2D6 expression in HepG2 cells [58]. Potentially, altered regulation of retinoid levels during pregnancy may be responsible for CYP2D6 induction. Limited data suggests that retinol, a retinoid capable of converting to retinoic acid, decreases throughout pregnancy and returns to basal levels during postpartum [59, 60]. Furthermore, results from microarray experiments performed in the Jeong lab showed that pregnancy increased hepatic mRNA expression of retinol binding protein and Cyp26b1, an all-trans retinoic acid metabolizing enzyme [61], and decreased expression of retinal dehydrogenases and lecithin-retinol acyltransferase as compared to virgin or postpartum period. All such changes can potentially lead to decreased level of bioavailable retinoic acid. These data, with the previous reports, suggest an alteration in retinoid regulation during pregnancy, but whether retinoic acids play a role in CYP2D6 induction during pregnancy remains unknown.

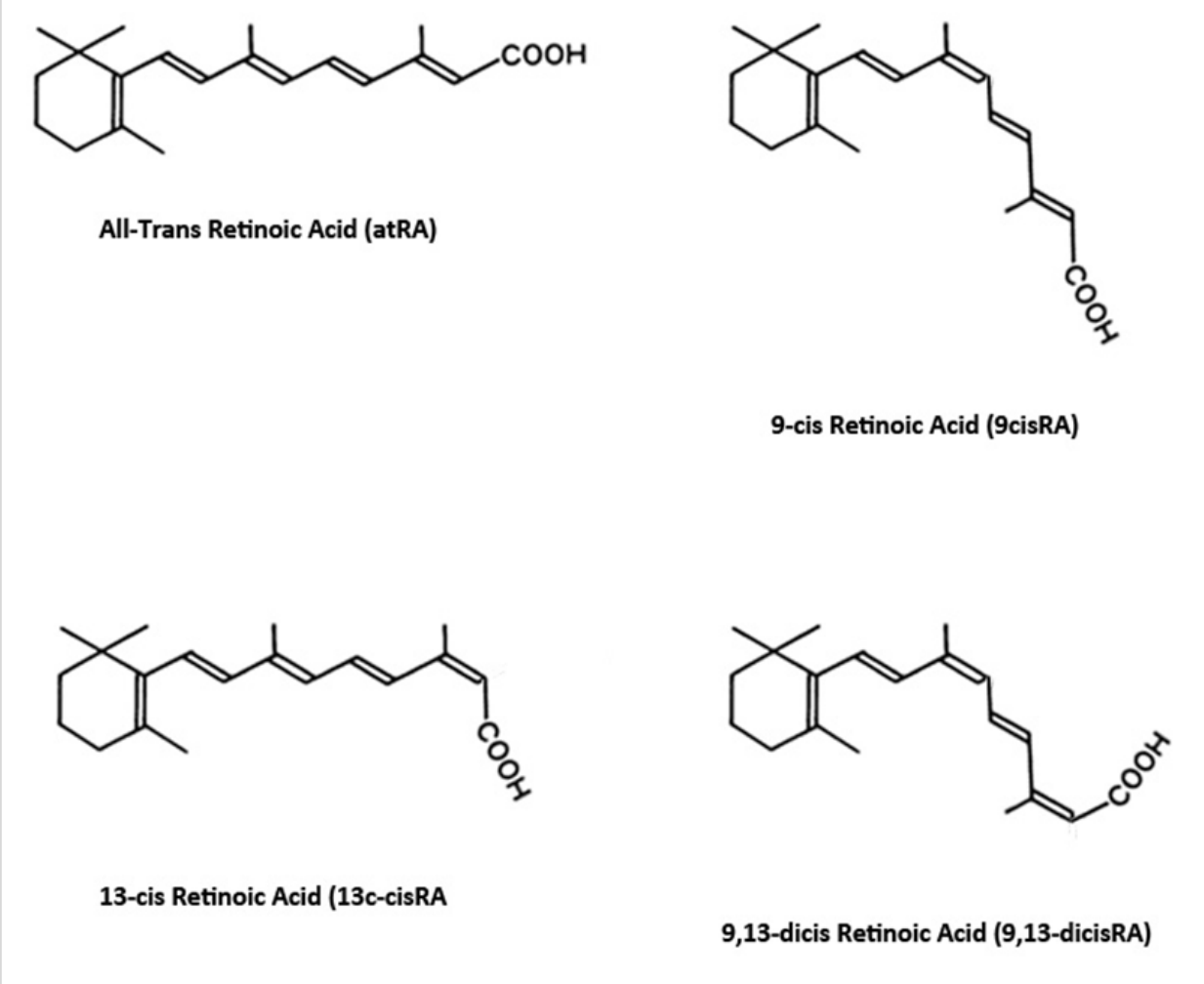

Figure 1. Structures of common endogenous retinoids; isomers of all-trans retinoic acid

C. SIGNIFICANCE OF THE PROJECT

Drugs and supplement use is prevalent in pregnant women [1-3]. While studies are performed to examine the safety of drugs for the mother and potential complications for the fetus, drugs deemed non-teratogenic may still have adverse affects during pregnancy, potentially due to altered pharmacokinetics of the drug. Altered pharmacokinetics can cause to an increased or decreased plasma concentration, and lead to adverse events to the mother as well as to the developing fetus.

Clinical results indicate pregnancy influences hepatic drug metabolism in a metabolic pathway specific manner [6-10]. Understanding the underlying mechanism and identification of the responsible factors will likely enable us to predict pharmacokinetic changes of drugs used in pregnant women, and may even predict the plasma concentrations. Furthermore, identifying such factors and elucidating the molecular mechanisms should allows us to identify not only any drugs that may interact with the induction/repression pathway, but also to individualize drug treatment based on each patient's expression, genotype, or concentration for a particular gene or circulating factor in the future. Furthermore, the information gained by knowing a molecular pathway used during induction/repression during pregnancy can then implicate the differential regulation to any physiological condition or disease state which might affect the involved factors or proteins.

D. HYPOTHESIS AND SPECIFIC AIMS

The overall goals of this thesis project were two-fold. First, we aim to understand the mechanism underlying upregulation of CYP2B6, an E2-responsive hepatic gene identified through our microarray experiment. We hypothesized that E_2 induces CYP2B6 expression through ER activation. Second, we aim to define the transcription factors or circulating plasma factors that potentially play a key role in CYP2D6 induction during pregnancy. We hypothesized

that HNF4α upregulated CYP2D6 expression and retinoic acid repressed CYP2D6 expression. We proposed the following specific aims.

Aim 1: To determine the molecular mechanism of E_2's induction of CYP2B6.

We have identified CYP2B6 as an E2-responsive gene through microarray experiment. Using various molecular tools, we established the important roles of ER in CYP2B6 induction by E2.

Aim 2: To determine mechanisms underlying CYP2D6 induction during pregnancy.

2.1. To establish CYP2D6-humanized mice as a working *in vivo* model to study CYP2D6 induction during pregnancy. Data from CYP2D6-humanized mice housed at the State University of New York showed a significant increase in CYP2D6 mRNA at term. We verified that the transgenic mice housed at this institution showed a similar induction of CYP2D6 at term.

2.2. To determine the role of HNF4α in CYP2D6 induction during pregnancy. We examined whether pregnancy affect HNF4a activity in mice by performing EMSA.

2.3. To determine the role of retinoids in CYP2D6 regulation. We examined the effects of retinoids on CYP2D6 expression in HepG2 cells.

II. MATERIALS AND METHODS

A. MATERIALS

Chemicals and Reagents

17β-Estradiol, all-trans retinoic acid, and 9-cis retinoic acid were obtained from Sigma (St. Louis, MO). ICI182,780 was purchased from Tocris Bioscience (Ellisville, MO).

B. CELL CULTURE

HepG2 cells from ATCC (Manassas, VA) were cultured as previously described [62]. HepG2 cells stably expressing ERα (HepG2-ER) were kindly provided by Dr. David Shapiro (University of Illinois at Urbana Champaign) and cultured in complete DMEM supplemented with 10% charcoal/dextran-stripped fetal bovine serum (Gemini, Woodland, CA), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% MEM nonessential amino acids.

C. METHODS

Plasmids

To construct the pGL3-CYP2B6 plasmid, the upstream region of CYP2B6 (-3290 to +8) was PCR-amplified using human genomic DNA as template and a pair of primers listed in Table 1. The PCR product was digested by *Kpn*I and *Hin*dIII restriction enzymes and cloned into promoterless pGL3-basic vector (Promega, Madison, WI) digested by the same enzymes, yielding pGL3-CYP2B6. CYP2B6 deletion constructs with different lengths of the 5′-flanking region of the human CYP2B6 gene (-1724 to +8, -1461 to +8, and -845 to +8) for deletion assay were prepared by PCR-amplification of relevant regions (using pGL3-CYP2B6 as template) followed by restriction digestion using *Kpn*I and *Hin*dIII and cloning into pGL3-basic plasmid. The PCR primers of the pGL3-CYP2B6 deletion constructs are listed in supplemental Table 1.

pGL3-ERE3 and expression vectors for ERα mutants [17] and wild-type ER expression vector [63] were previously described. pGL3-CYP2B6 [-1839/-12] was generously provided by Dr. Masahiko Negishi (NIEHS). Mutation constructs of pGL3-CYP2B6 (i.e., mutations at A1 and/or A2 sites or a mutation at ERE (see Fig. 4C for their locations)) were prepared using a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. All sequences were confirmed by sequencing. To construct the pGL3-CYP2D6 plasmid, the upstream region of CYP2D6 (-2455 to +90) was PCR-amplified using human genomic DNA as template and a pair of primers listed in Table 1. The PCR product was digested by *KpnI* and *NcoI* restriction enzymes and clones into the promoterless pGL3-Basic vector digest by the same enzymes, yielding pGL3-CYP2D6.

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

Total RNAs were isolated from cell lysates or fresh liver tissues using Trizol (Invitrogen, Carlsbad, CA). RNA was then used as a template for cDNA synthesis, using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed using StepOnePlus Real-Time PCR System and TaqMan® Gene expression assays (Applied Biosystems), and fold changes in mRNA levels of genes were determined after normalizing the gene expression levels by those of GAPDH or PPIA ($2^{-\Delta\Delta Ct}$ method) as previously done [64]. The PCR conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

Luciferase reporter assay

Luciferase reporter assays were performed in HepG2 or HepG2-ER cells as previously described [17, 62]. In short, HepG2 cells were plated in 12-well plates at a density of 4.5x10^^{5}/ml (day 0), transfected the following day with 0.3μg of reporter construct (pGL3-Basic, pGL3-ERE3, pGL3-CYP2B6 or related constructs, or pGL3-CYP2D6), 0.3μg of expression

plasmid (pcDNA3, pcDNA3-ERα or related construct), and 0.1μg of pcDNA3-β-galactosidase (day 1), and treated with vehicle or drug the following day (day 2). Plasmids were isolated using the Invitrogen Purelink© Quick Plasmid Miniprep Kit or the Promega PureYield Miniprep Kit, and cells were transiently transfected with Promega Fugene 6 or Fugene HD in a 3.5:1 Fugene:DNA ratio. Drug concentration is listed for each experiment, and lasted 24 hours in each case. In all cases, luciferase activity was normalized to β-galactosidase activity. At least two independent experiments were performed in triplicate.

Transgenic Mouse Study

All pregnant and virgin Tg-*CYP2D6* mice [20] were housed under the controlled temperature (20 ± 2°C), relative humidity (50-60%) and lighting (lights on 6:00 a.m. - 6:00 p.m.), with food and water provided *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Chicago. Adult female (at least 8 weeks old) mice were mated with male mice of the same age. The day after mating was assumed as gestational day 1 for those female mice demonstrating sperm plug. At gestational day 18 and 14 days after delivery, the Tg-*CYP2D6* female mice were sacrificed and tissues of liver were collected. The tissues were gently washed in 4°C PBS and then stored at -80°C before use. Tail snips were taken and digested using the previously described protocol [65]. Genotyping for CYP2D6 expression was performed by qRT-PCR using the custom taqman assay N000106.7.pt.CYP2D6 (Integrated DNA Technologies). Basal CYP2D6 expression levels were compared to an arbitrarily chosen sample given an expression of “1.00”, and expression levels at term and postpartum were corrected for with the relative basal-level expression value.

Electorphoretic Mobility Shift Assay (EMSA)

Nuclear proteins were prepared from transgenic mouse livers expressing human CYP2D6 using the CelLytic Nuclear Extraction Kit (Sigma-Aldrich) following the manufacturer's protocol. EMSA was performed using Gel Shift Assay Systems (Promega) following manufacturer's protocol. Briefly, nuclear proteins (10 μg) were preincubated with the provided reaction buffer at room temperature in the presence of radioactive DNA probe. DNA probe sequences are listed in supplemental Table 1. After 20 min, the binding reaction was initiated by adding 1.75 pmol of 5′-end ^{32}P-labeled HNF4α and CYP2D6 probes harboring consensus or putative HNF4α binding sequences. The reaction mixture was incubated at room temperature for 30 min. Protein-bound probes were separated from free probes on 4% (w/v) nondenaturing polyacrylamide gel. The gel was dried, and radioactivity visualized by using a PhosphorImager.

D. STATISTICAL ANALYSIS

In vitro experiments were performed at least twice in triplicate. Data for all figures is expressed as mean ± SD. Statistical comparisons between two groups was performed using a two-tailed independent Student's t-test, calculated using Microsoft Excel. Statistical comparisons between three groups were performed using a one-way ANOVA, followed by post-hoc comparison of different groups using t-test. One-way ANOVAs and EC_{50} values were calculated using Graphpad Prism 5.

TABLE I. PRIMERS USED IN DELETION CONSTRUCTS, PRIMERS USED IN MUTAGENESIS, AND PRIMERS USED AS EMSA PROBES

Construct name	**Sequences**
pGL3-CYP2B6 (-3290/+8)	(forward) 5'-ATGCGGTACCAGGCATAATTAGAAAGGCA-3' (reverse) 5'-ATCGAAGCTTGGTCCTGGTCTGACTGCC-3'
pGL3-CYP2B6 (-1724/+8)	(forward) 5'-ATCGGGTACCTCAGGGTCAGGAAAGTACAG-3' (reverse) 5'-ATCGAAGCTTGGTCCTGGTCTGACTGCC-3'
pGL3-CYP2B6 (-1461/+8)	(forward) 5'-ATCGGGTACCATGGGAGTCCAGTAGACATC-3' (reverse) 5'-ATCGAAGCTTGGTCCTGGTCTGACTGCC-3'
pGL3-CYP2B6 (-845/+8)	(forward) 5'-ATCGGGTACCGTAATCTTGGCTCACTGCAG-3' (reverse) 5'-ATCGAAGCTTGGTCCTGGTCTGACTGCC-3'
pGL3-CYP2B6 mA1	(forward) 5'-GCACCCAATCTTAGTGTCAGATTATATAGCACAGCAAGACCGAGGC-3' (reverse) 5-GCCTCGGTCTTGCTGTGCTATATAATCTGACACTAAGATTGGGTGC-3'
pGL3-CYP2B6 mA2	(forward) 5'-ACTGTTTGTCTGCTCCTCCTAGATTAAAGTAACTTCGGGTTCAGG-3' (reverse) 5'-CCTGAACCCGAAGTTACTTTAATCTAGGAGGAGCAGACAAACAGT-3'
pGL3-CYP2B6 dERE	(forward) 5'-CCTCCTGGGTCAAAGTCGGGTTCAGGTC-3' (reverse) 5'-GACCTGAACCCGACTTTGACCCAGGAGG-3'
pGL3-CYP2D6 (-2455/+90)	(forward) 5'-ATCGGGTACCCTTTCCGACATACACGCAAT (reverse) 5'-ATCGCCATGG ACCTGCCTCACTACCAAATG
Probe name	
HNF4α consensus	(forward) 5'-AGCAGAGGTCAAAGGTCATCATC (reverse) 5'-GATGATGACCTTTGACCTCTGCT
CYP2D6-derived HNF4α	(forward) 5'-AGCAGAGGGCAAAGGCCATCATC (reverse) 5'-GATGATGGCCTTTGCCCTCTGCT

III. Results

A. MECHANISM OF INDUCTION OF CYP2B6

1. E_2-Regulated, ER-Mediated Mechanism

A preliminary study in the Jeong laboratory to examine the effects of E2 on CYP expression in human hepatocytes has revealed that E2 upregulates CYP2B6 expression (data not shown). The underlying molecular mechanism remains unknown.

1.1. E_2's Induction of CYP2B6 Promoter Activity

To determine if ER is involved in the E_2-mediated induction of CYP2B6, luciferase assays were performed. To this end, a luciferase reporter plasmid was constructed where luciferase gene expression is driven by 3.3kb of the upstream regulatory region of the CYP2B6 gene (-3290 to +8), called pGL3-CYP2B6. HepG2 cells were transiently transfected with an ERα expression vector (or empty vector), pGL3-CYP2B6 reporter plasmid, and β-galactosidase expression vector (for normalization of transfection efficiency). The transfected cells were then treated with vehicle or 1 μM E_2 for 24 hours and luciferase activity was determined. As a positive control group, pGL3-ERE3, which is a luciferase reporter plasmid driven by 3 copies of the vitellogenin ERE, was transfected, and as a negative control group, pGL3-Basic, which is a promoterless reporter plasmid, was transfected. The results showed that E_2 induced CYP2B6 promoter activity by 12.5-fold as compared to vehicle treated cells, only in ER-transfected cells, indicating E_2 induces CYP2B6 promoter activity through ER. A similar finding was observed for cells transfected with the pGL3-ERE3, and a minor induction was seen in cells transfected with pGL3-Basic.

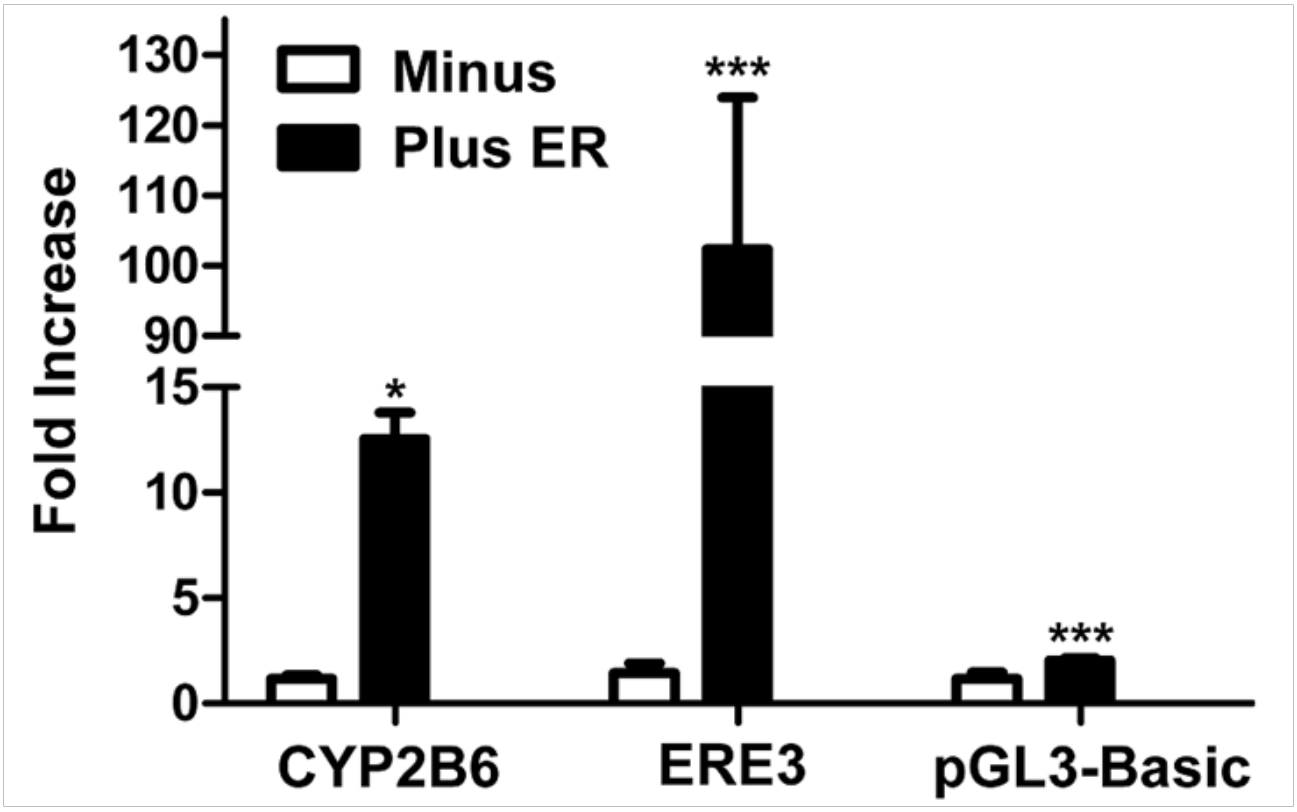

Figure 2. Luciferase activity of HepG2 cells co-transfected with a CYP2B6 reporter plasmid and an ERα expression vector after treatment with E_2. * = $p < 0.05$, ***= $p < 0.0005$ for "Plus ER" induction over "Minus ER".

1.2. E_2's Induction of CYP2B6 mRNA Through ER

To investigate if CYP2B6 mRNA is induced through E_2-activated ER, HepG2-ER cells that stably express ERα were treated with vehicle, 1 μM E_2, 10 μM ICI 182,780 (ICI, an ER-degrading antagonist), or a combination of both 1 μM E_2 and 10 μM ICI, and qRT-PCR was performed for CYP2B6 and GAPDH. Relative CYP2B6 expression was increased 4-fold after E_2 treatment, but this induction was abrogated following combination E_2 and ICI treatment (Figure 3A). This trend was also seen in the ER-responsive gene TFF1, but not seen for either gene in the parental HepG2 cell line (ERα-negative) (Figure 3B). These results indicate that the ERα antagonist ICI represses the E_2-stimulated induction of CYP2B6 through ER, and suggest that CYP2B6 induction via E_2 requires ERα.

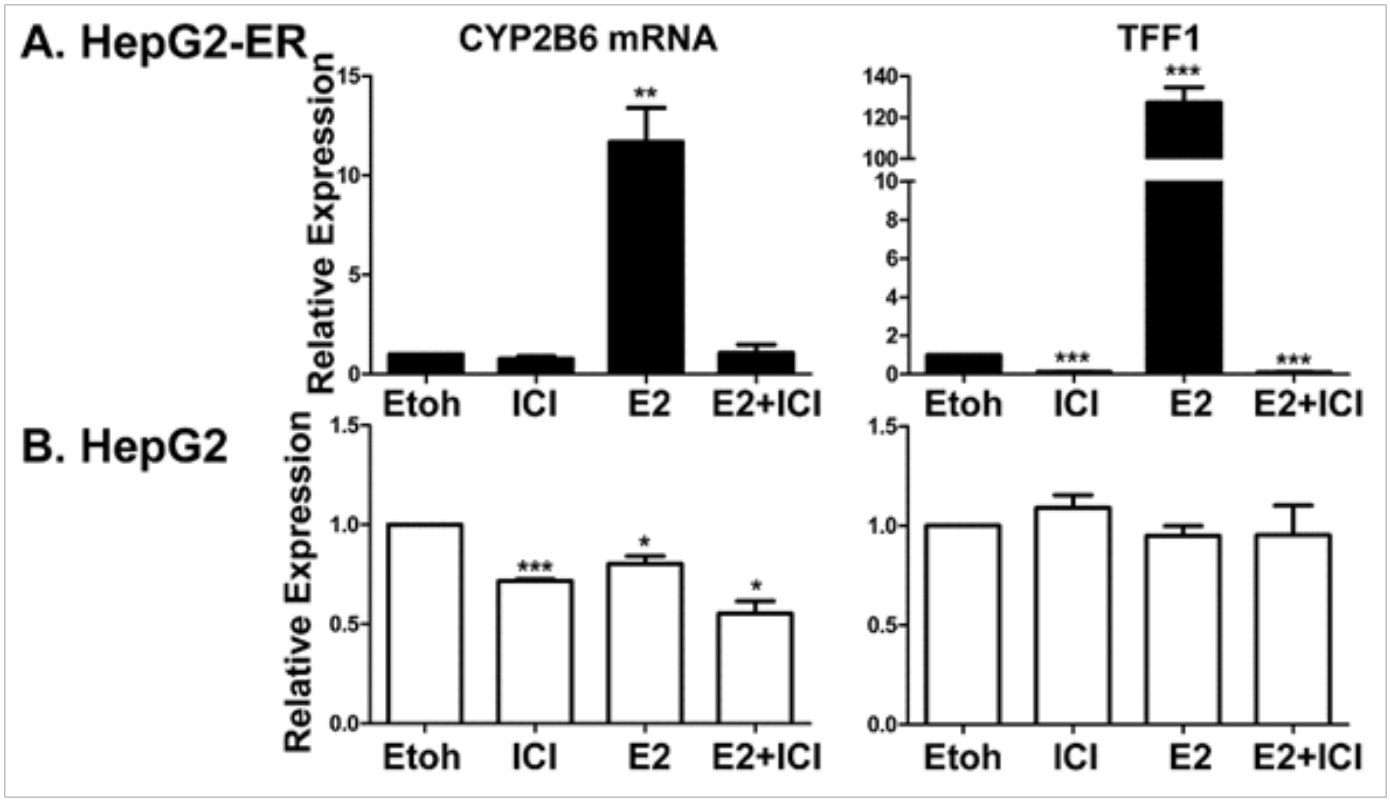

Figure 3. A. CYP2B6 and TFF1 mRNA expression in HepG2-ER cells after drug treatment. B. CYP2B6 and TFF1 mRNA expression in HepG2 cells after drug treatment. *p < 0.05, **p < 0.005, ***p < 0.0005 for drug treated cells over EtOH treated cells.

1.3. Dose-Dependent Relationship

To assess the relationship between E_2 concentration and CYP2B6 promoter activity, a luciferase assay was performed using pGL3-CYP2B6U3.3kb and concentrations of E_2 ranging from 100 pM to 1 µM. Luciferase data shows a concentration-dependent increase in CYP2B6 promoter activity, with an estimated EC_{50} value of 44 nM; a value similar to the EC_{50} of the positive control, ERE3 (35 nM) (Figure 4).

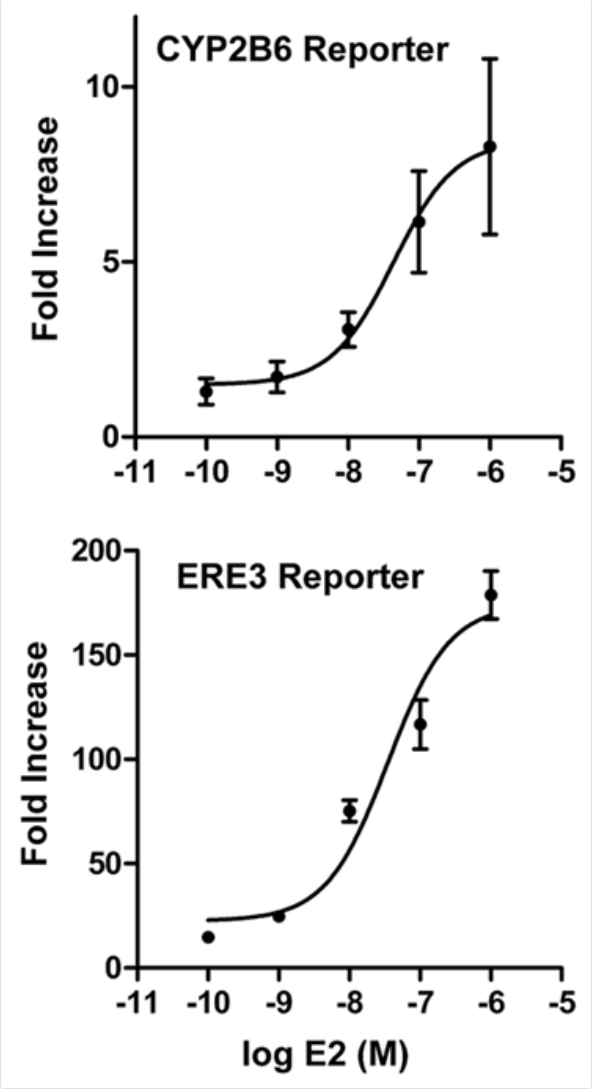

Figure 4. Dose-dependent response of CYP2B6-driven luciferase activity from E_2 treatment, ranging from 100 pM to 10 µM.

2. Nonclassical Mechanism of ER Transactivation

ER is able to exert its genomic effects through multiple molecular pathways. In classical ER transactivation, ER binds directly to its target DNA recognition sequence. In an alternative pathway, ER can tether to adaptor proteins and function through their respective DNA-binding activities, without itself directly binding to the DNA [27]. In the following, we looked to determine how and where ER functions to transactivate the CYP2B6 promoter.

2.1 Required ER Domain

To elucidate the mechanism of ER action, HepG2 cells were treated with 1μM E_2 and transfected with either wild type or mutated ER. The ER mutants used each contain a mutation that causes a loss of function in a specific domain of ER: mAF1-ER is missing AF1, mDBD-ER produces ER that is unable to bind DNA through its DNA-binding domain, and mAF2-ER decreases transcriptional activity without affecting DNA- or ligand-binding [66]. The results show a loss of E_2-mediated induction when the AF1 domain was mutated, but retained induction when the DBD was mutated (Figure 5). This is in contrast, however, to the results seen when mDBD-ER is co-transfected with pGL3-ERE3, confirming that mDBD-ER is unable to bind to EREs [17]. This result suggests that the DBD of ER is disposable in the induction of CYP2B6, and ER is acting through a nonclassical mechanism and not directly binding DNA.

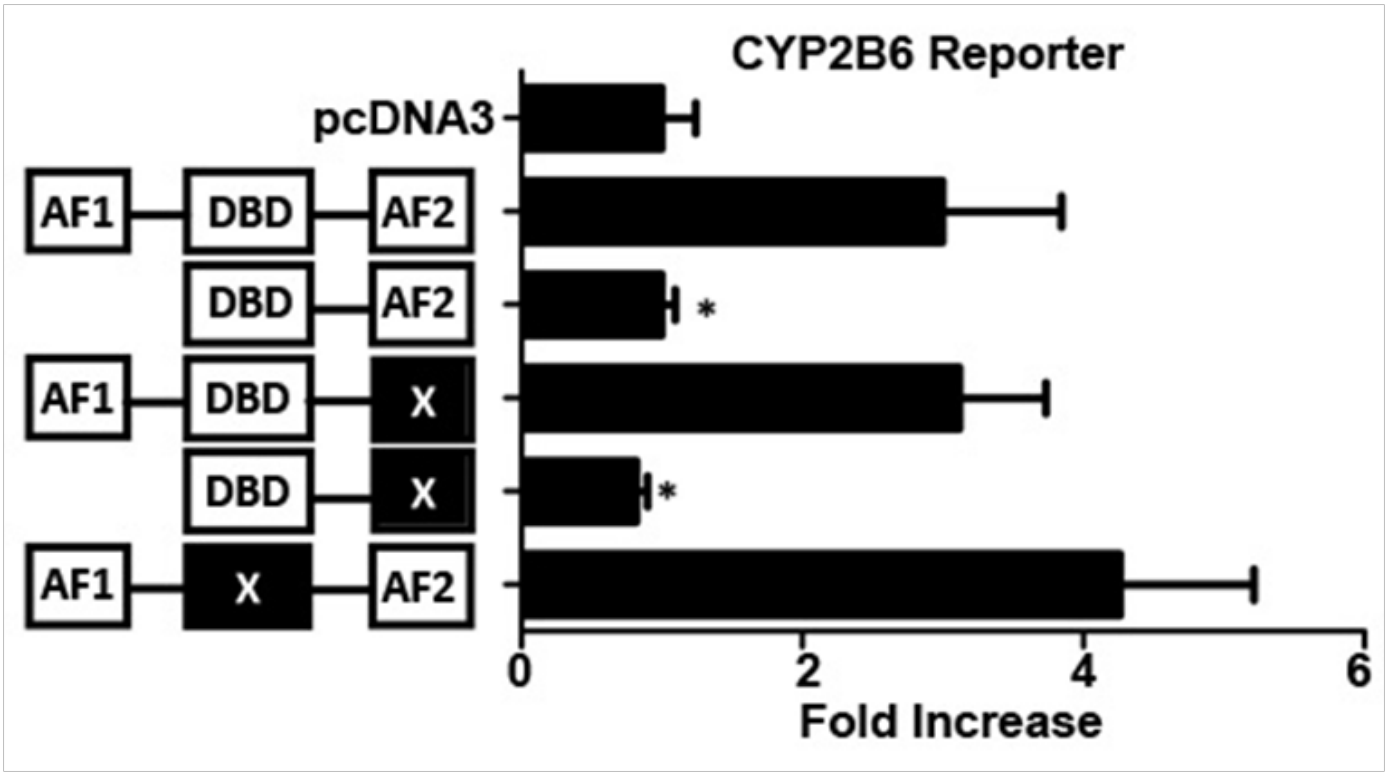

Figure 5. Luciferase activity of HepG2 cells co-transfected with a CYP2B6 reporter plasmid and a native or mutant ERα expression vector after treatment with 1 μM E_2. Data shown are fold increases in CYP2B6 promoter activity by E_2 treatment. *$p < 0.05$ vs. the wild type receptor.

2.2. Localization of CYP2B6 promoter activity

To localize any functional *cis*-element(s) responsible for ER's transactivation, deletion constructs of the 3.3kb upstream CYP2B6 regulatory region were made with each construct containing less of the 5'-end than the previous (CYP2B6U3.3kb, -3290/+8; CYP2B6U1.8kb, -1839/-12; CYP2B6U1.7kb, -1724/+8; CYP2B6U1.5kb -1461/+8; CYP2B6U0.9kb and -845/+8). HepG2 cells were then co-transfected with an ERα expression vector and one of the CYP2B6 reporter constructs. Cells were then treated with 1 μM E_2 and cell lysates were collected. Luciferase data revealed promoter activity was strongly decreased between constructs CYP2B6U1.8kb and CYP2B6U1.7kb, and marginally decreased between CYP2B6 U1.7kb and CYP2B6U1.5kb (Figure 6), localizing the sites of transactivation to this ~400bp region**.**

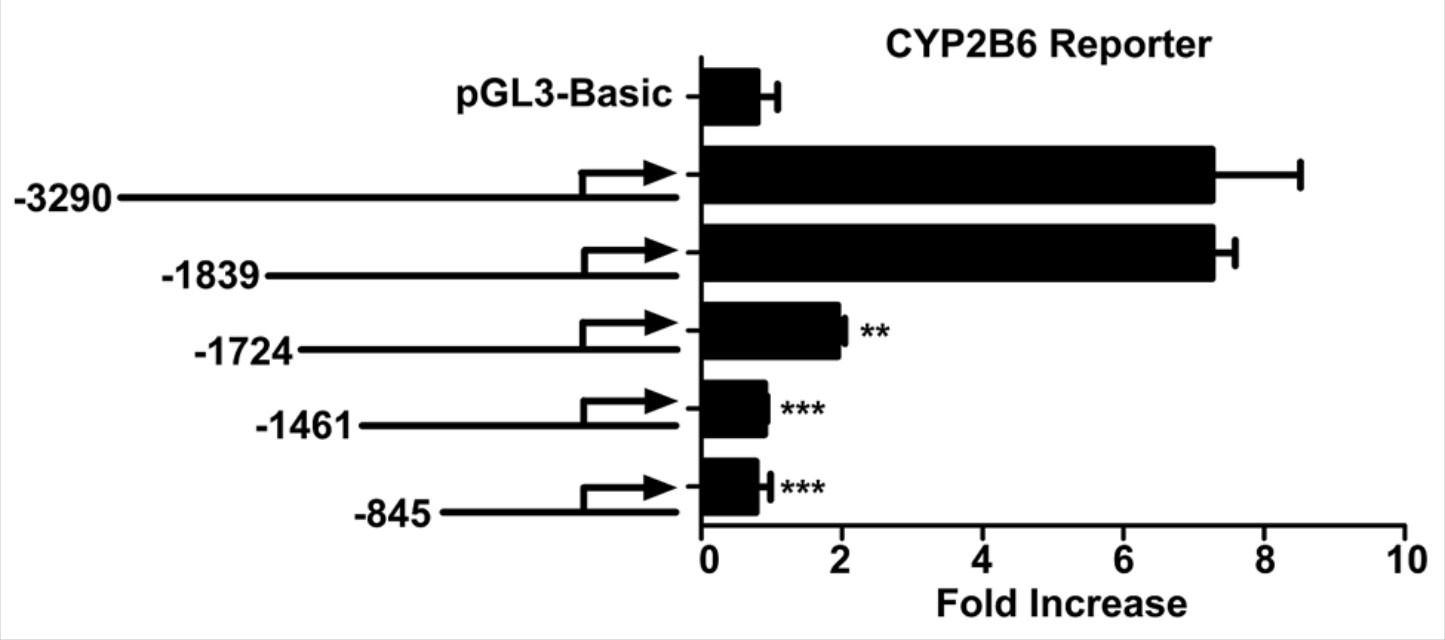

Figure 6. Luciferase activity of HepG2 cells co-transfected with a deletion construct of the CYP2B6 reporter plasmid and an ERα expression vector after treatment with E_2. Data shown are fold increases in CYP2B6 promoter activity by E_2 treatment. ** $p < 0.005$, ***$p < 0.0005$ vs. the -3290 promoter.

2.3. Putative Responsible *cis*-elements

To find putative transcription factor binding sites that could potentially be the functional cis-element(s) being utilized by ER, we performed *in silico* analysis of the CYP2B6 promoter using MatInspector of the Genomatix Suite. The results showed multiple AP-1 and Sp1 binding sites spread throughout the 3.3-kb region, and multiple AP-1 binding sites located specifically between -1560-bp and -1800-bp (Figure 7). The MatInspector results revealed that both previously identified DNA segments (-1724-bp to -1839-bp, and -1461-bp to -1724-bp) showing a decrease in E_2-induced luciferase activity correspond to an individual AP-1 binding motif (A1 and A2; consensus sequence of TGA(C/G)TCA), as well as a putative non-consensus ERE. One possible pathway of nonclassical transactivation is through ER tethering to an adaptor protein/protein complex to indirectly bind the CYP2B6 promoter.

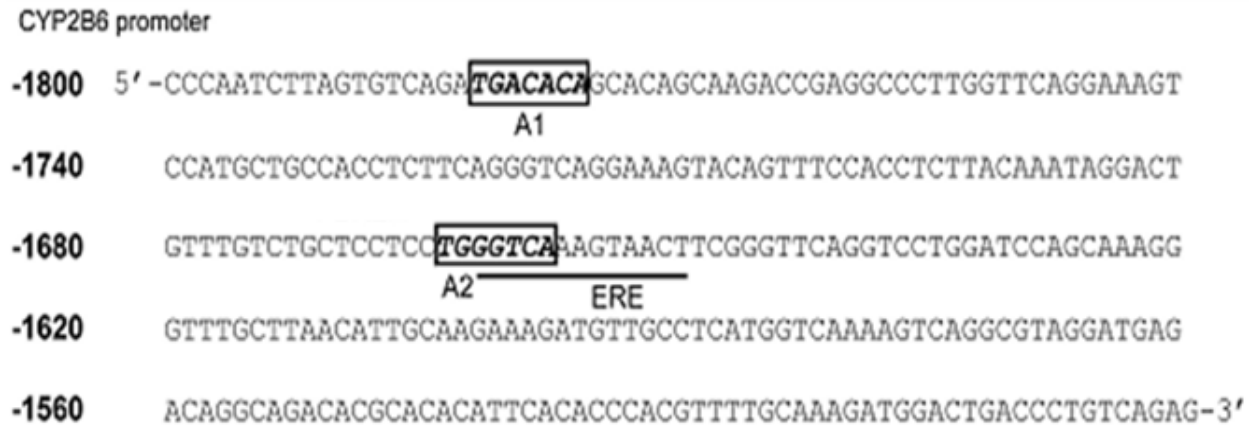

Figure 7. Key MatInspector results from the -1500 bp to -1800 bp upstream regulatory region. A1 and A2, putative AP-1 binding sites; ERE, estrogen response element.

2.4. Mutation of AP-1 Motifs in the CYP2B6 Promoter

To determine if either of the putative AP-1 binding sites was the responsible *cis*-element for ER's transactivation, mutation constructs of the CYP2B6 promoter were created to alter AP-1 protein binding to the CYP2B6 promoter. Each of the identified putative AP-1 *cis*-elements was altered by 3 bases individually, (CYP2B6mA1/CYP2B6mA2; primers in Table I), or together as a double-mutant, (CYP2B6mA1/mA2). HepG2 cells were co-transfected with an expression vector for ERα and either the native CYP2B6U3.3kb promoter or one of the four synthesized mutants. Cells were then treated with 1 μM E_2 for 24 hours and cell lysates were collected. The resulting promoter activity data revealed that the individual mutation constructs showed no significant change from the wild type promoter, however when both sites were mutated the induction was completely abolished (Figure 8). This result suggests that either individual AP-1 binding site is functional, and required, for the induction of CYP2B6 through E_2-activated ER, but that a cooperative activation complex involving both sites is not necessary for increased transcription.

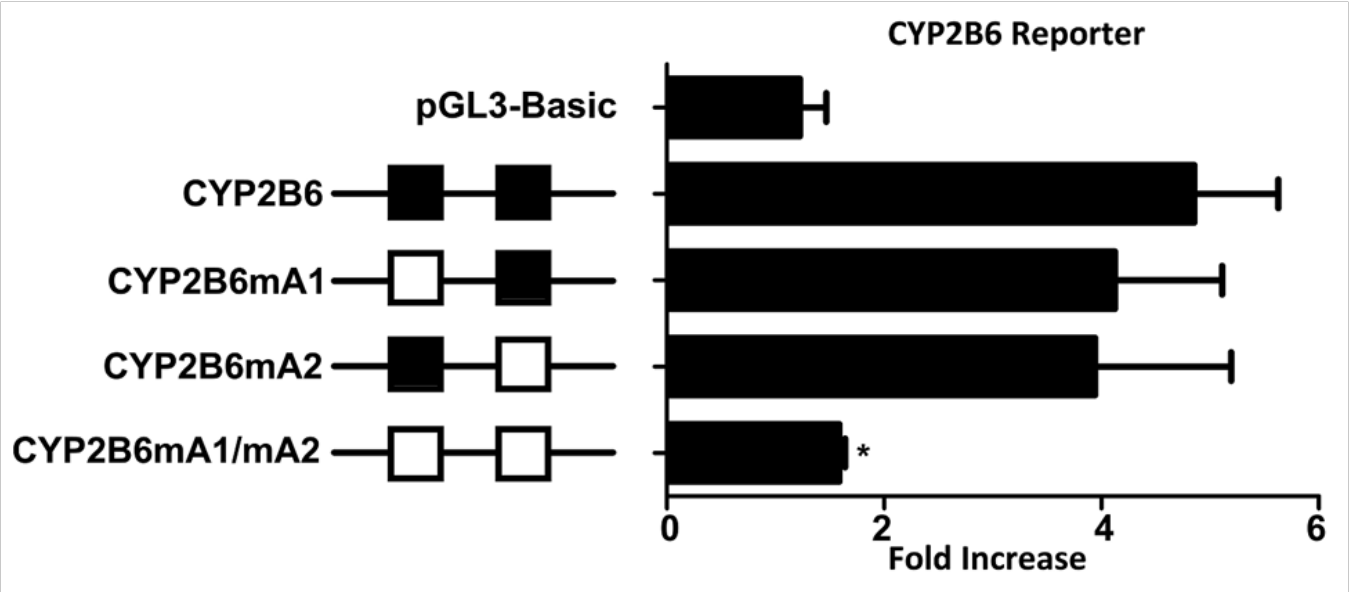

Figure 8. Luciferase activity of HepG2 cells co-transfected with a mutated CYP2B6 reporter plasmid and an ERα expression vector after treatment with E_2. Data shown are fold increases in CYP2B6 promoter activity by E_2 treatment. White squares represent mutated AP-1 binding sites. *$p < 0.05$ vs. the native promoter.

2.5. Partial Deletion of a Putative ERE

To determine if the putative ERE played a role in CYP2B6 induction, a mutation construct was made (CYP2B6dERE) to partially delete the ERE to deter direct ER binding without affecting AP-1 binding. Cells were co-transfected with an ERα expression vector and either the wild type CYP2B6 promoter or CYP2B6dERE for 4 hours. Cells were then treated with 1 μM E_2 for 24 hours and cell lysates were collected. Luciferase activity showed no significant difference in promoter activity between reporter plasmids, further suggesting a nonclassical mechanism of ER transactivation (Figure 9).

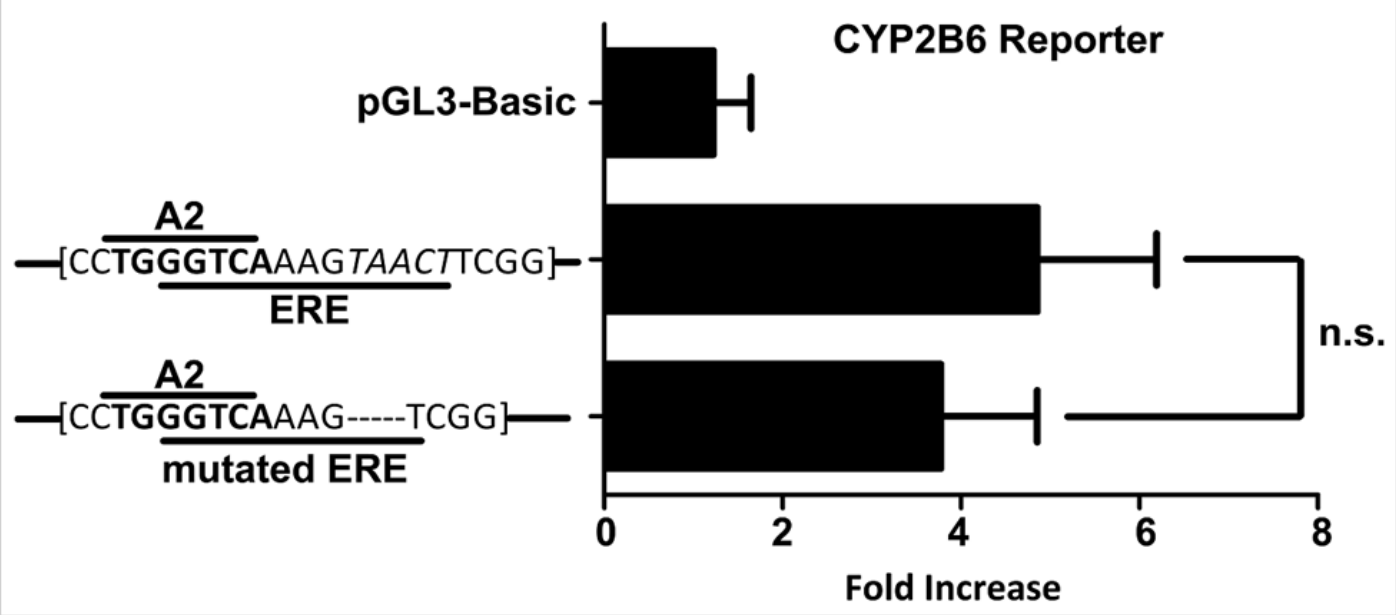

Figure 9. Luciferase activity of HepG2 cells co-transfected with a CYP2B6 reporter plasmid containing a mutated ERE and an ERα expression vector after treatment with E_2. Data shown are fold increases in CYP2B6 promoter activity by E_2 treatment. n.s., not significant

B. MODEL SYSTEM OF CYP2D6 INDUCTION DURING PREGNANCY

1. Transgenic Mouse Model

Clinical observations have revealed CYP2D6-mediated metabolism is increased during pregnancy [6-10]; however to date, the underlying mechanisms remain unknown in part due to a lack of an in vivo model which allows us to examine the system-wide effects of altered physiology during pregnancy on CYP2D6 expression. The objective here is to establish a mouse model, i.e., transgenic mice expressing human CYP2D6, as a system to probe the mechanism of increased CYP2D6-mediated metabolism during pregnancy.

A previous preliminary study in Jeong laboratory showed that CYP2D6 mRNA expression increased during pregnancy in CYP2D6-humanized mice (Figure 10; data provided by Dr. Kwi Hye Koh). The mice liver tissues collected at different gestational time points were provided by Dr. Aiming Yu (State University of New York at Buffalo). The results suggest that the transgenic mice may serve as a valid model system to elucidate the mechanism underlying CYP2D6 induction during pregnancy.

To verify that the transgenic mice housed at the University of Illinois at Chicago show a similar induction during pregnancy, we compared the CYP2D6 expression in the humanized mice at different gestational time points. Livers from the mice were collected at prepregnancy, at term, and during postpartum (n=2-3/group). RNA was isolated and CYP2D6 and β-actin expression was determined by using qRT-PCR. The CYP2D6 expression was normalized by the basal CYP2D6 level value (before pregnancy) (Figure 11).

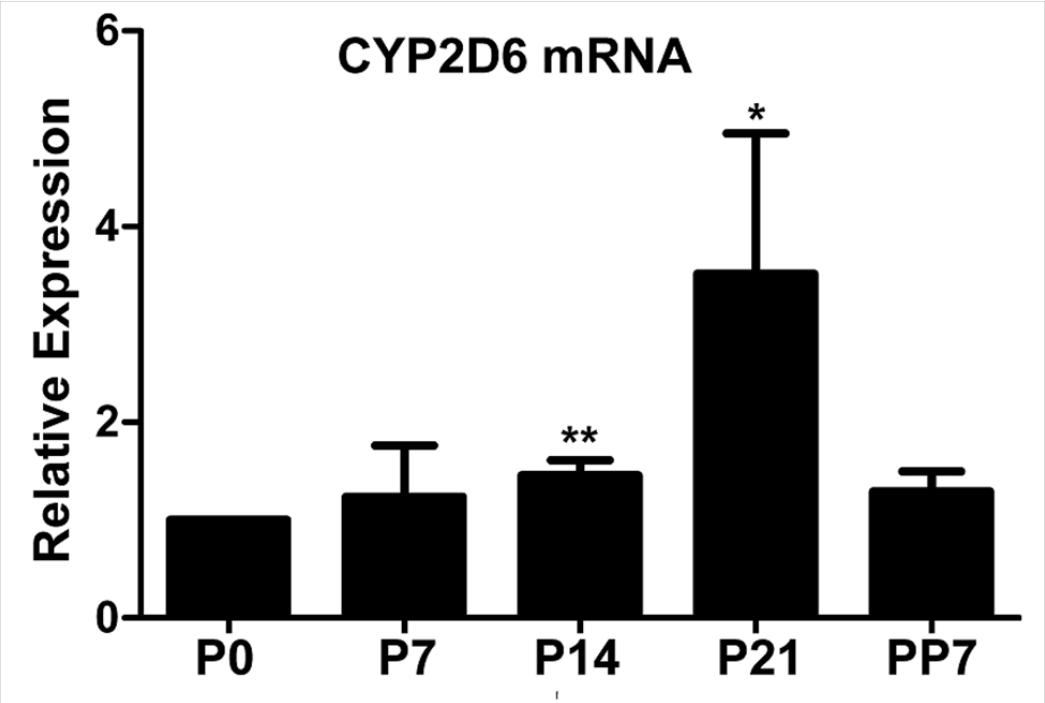

Figure 10. CYP2D6 mRNA expression from livers harvested from transgenic mice. P0 is virgin, P7, P14, and P21 indicate the gestational day, and PP7 is 7 days postpartum. *p < 0.05, **p < 0.005 vs. CYP2D6 expression at P0.

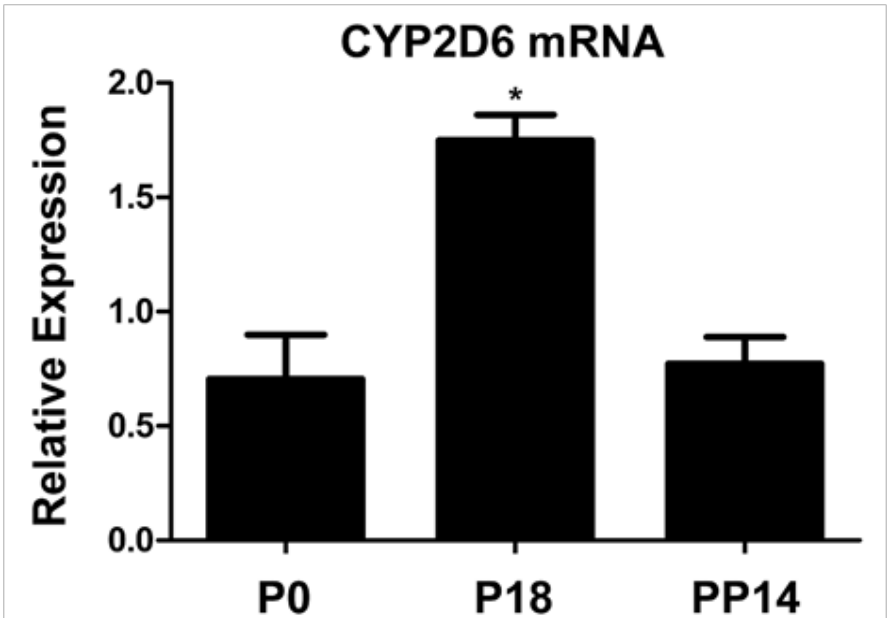

Figure 11. CYP2D6 mRNA expression from livers harvested from transgenic mice. P0 is virgin, P18 is term, and PP14 is 14 days postpartum. *p < 0.05 in P18 samples vs. P0 samples.

2. Involvement of HNF4α

HNF4α has been shown to regulate basal-level CYP2D6 transcription [41]. A preliminary study in the Jeong laboratory has revealed that pregnancy does not influence HNF4a expression at protein levels (data not shown). HNF4a is known to be activated by saturated lipids, the plasma concentration of which increase during pregnancy [42, 43]. This suggests that pregnancy may influence HNF4a activity even if it does not affect HNF4a expression. To determine whether pregnancy influences HNF4α activity, we performed EMSA using mouse liver extracts obtained at different gestational time points. To this end, a double stranded EMSA probe harboring an HNF4α recognition sequence in CYP2D6 promoter [41] (or HNF4α consensus sequence) was radio-labeled, incubated with nuclear protein extracts from virgin, term, and postpartum mouse livers, resolved on native polyacrylamide gels, and visualized using a phosphorimager. The results showed multiple shifted bands for both CYP2D6 and HNF4a consensus probes, and the band intensity increased in livers collected at term. These results suggest that HNF4α DNA-binding is increased at term (Figure 12).

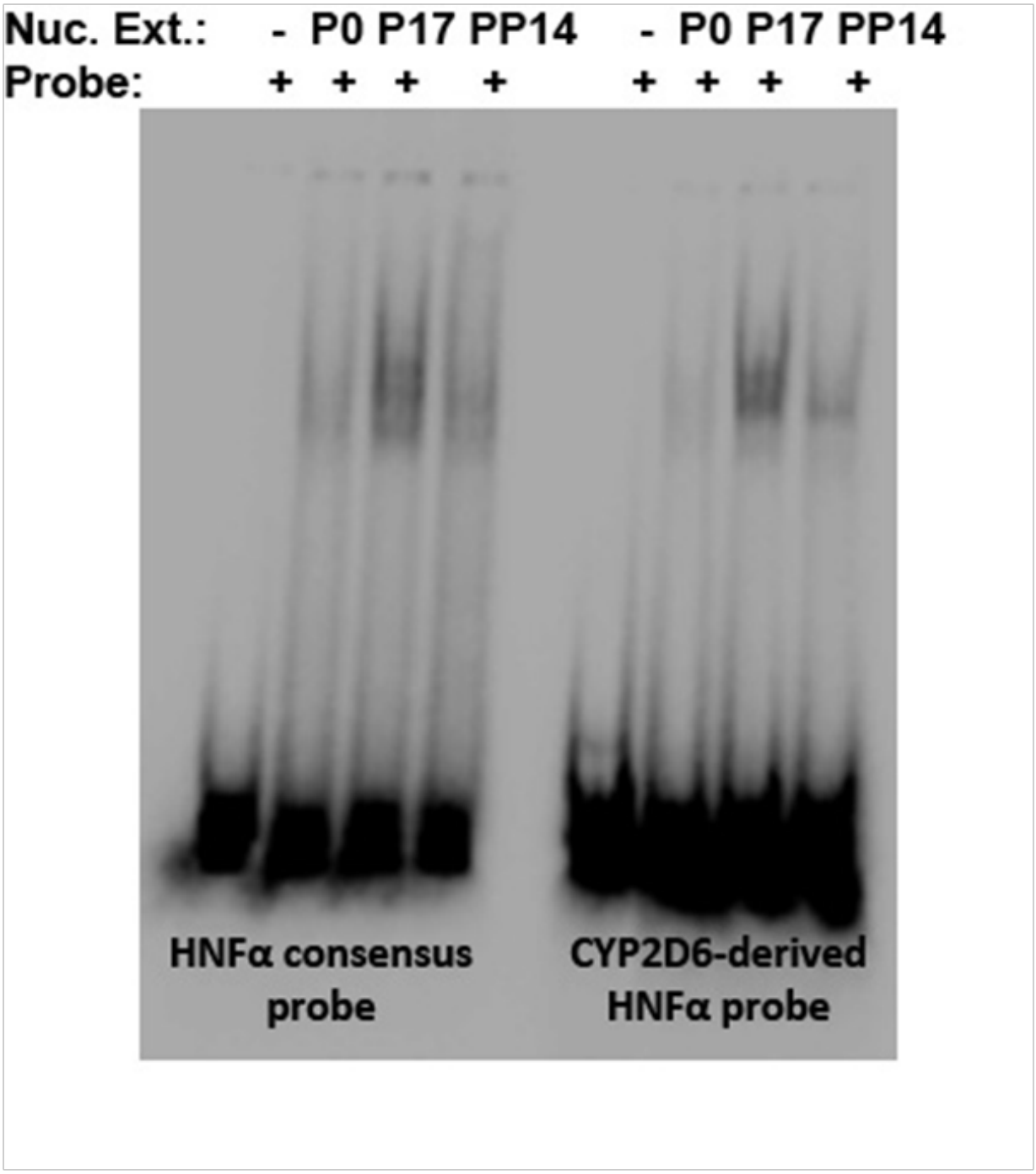

Figure 12. EMSA gel using a consensus HNF4α probe and a CYP2D6 probe and liver nuclear extracts from transgenic mice. The 1st lane in each group consisted of labeled probe only, the 2nd lane of labeled probe and virgin extract, the 3rd lane of labeled probe and term extract, and the 4th lane of labeled probe and postpartum extract.

C. INHIBITORY EFFECT OF RETINOIDS ON CYP2D6 EXPRESSION

To examine the effects of retinoic acid on CYP2D6 promoter activity, we performed a luciferase reporter assay. To this end, a luciferase vector harboring 2.5-kb of the upstream region of CYP2D6 was transiently transfected into HepG2 cells with an expression plasmid for β-galactosidase (for normalization of transfection efficiency). The transfected cells were then treated with all-trans retinoic acid (in concentrations ranging from 0.1 nM to 1000 nM) for 24 hours, after which luciferase activity was measured and normalized to β-galactosidase activity. The result showed that CYP2D6 promoter activity was decreased by ~40% after retinoid treatment in all concentrations tested (Figure 13). The results suggest that retinoids likely repress CYP2D6 promoter activity at basal physiological concentrations.

To examine whether retinoids also decrease CYP2D6 expression at the mRNA level, HepG2 cells were treated with DMSO, 1μM all-trans retinoic acid, 1μM 9-cis retinoic acid, or a combination of both retinoids for 72 hours. RNA was collected and expression levels of CYP2D6 and GAPDH were determined using qRT-PCR. The results showed that all-trans retinoic acid had an insignificant effect on CYP2D6 expression, a result inconsistent with that from the luciferase assay (Figure 13). On the other hand, a similar but less pronounced effect (~25% reduction) was observed when cells were treated with 9-cis retinoic acid, and 9-cis retinoic acid plus all-trans retinoic acid (Figure 14). CYP26A1, a known retinoid-responsive gene, was significantly induced by all three retinoid treatments (Figure 15), but apolipoprotein CIII, a positive control for 9-cis retinoic acid, was not significantly induced (data not shown).

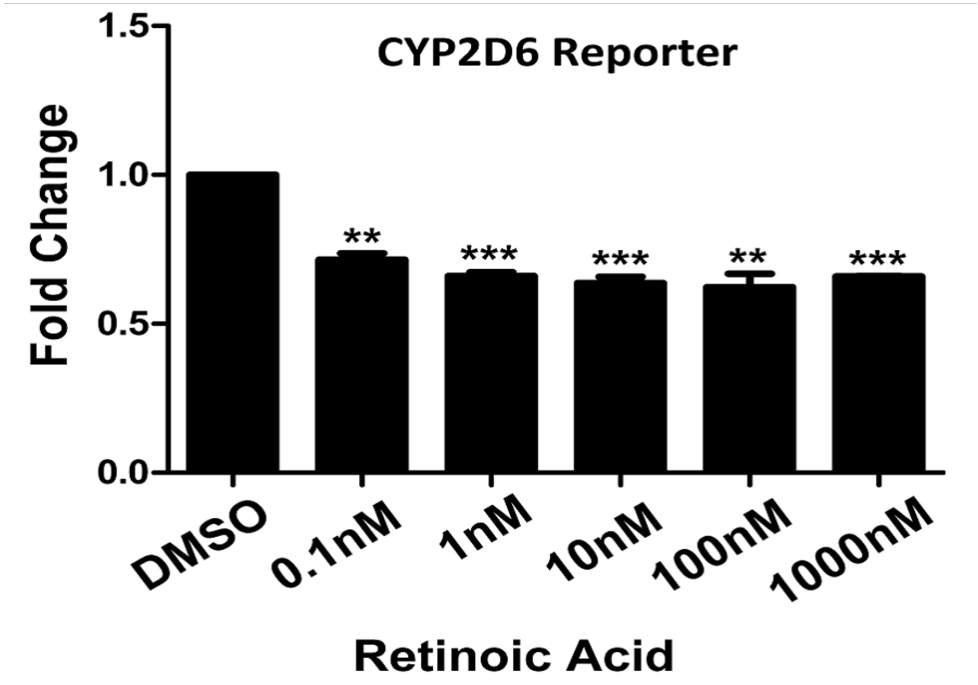

Figure 13. Luciferase activity of HepG2 cells transfected with a CYP2D6 reporter plasmid after treatment with concentrations of all-trans retinoic acid ranging from 100pM to 1µM. Data shown are fold decreases in CYP2D6 promoter activity by all-trans retinoic acid treatment. **p < 0.005, ***p < 0.0005 vs. DMSO

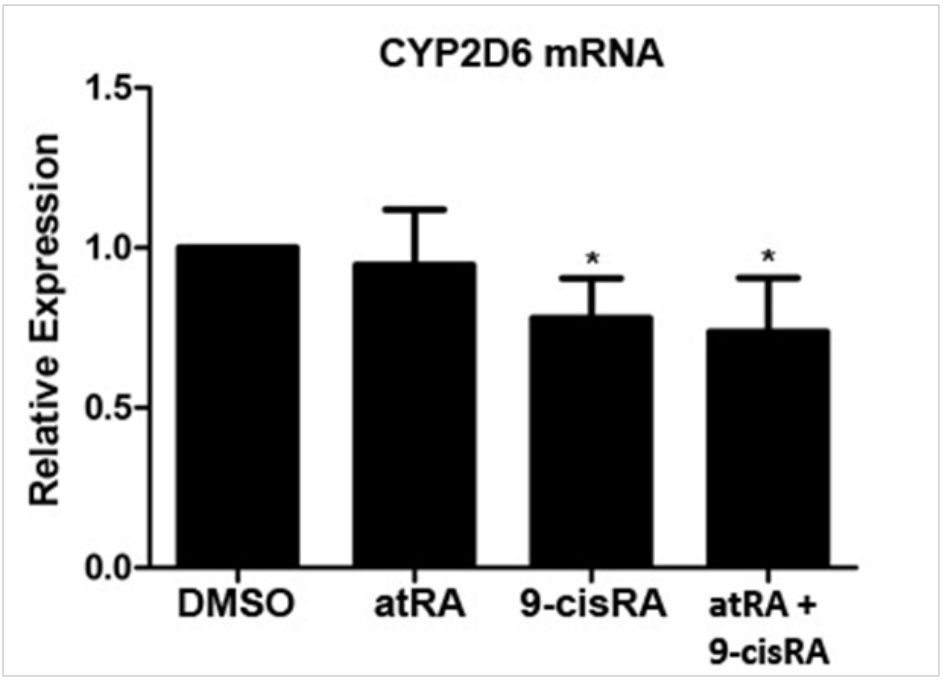

Figure 14. CYP2D6 mRNA expression in HepG2 cells after treatment with 1µM of varying retinoids. Data shown are fold decreases in CYP2D6 promoter activity by retinoid treatment. *p < 0.05 vs. DMSO

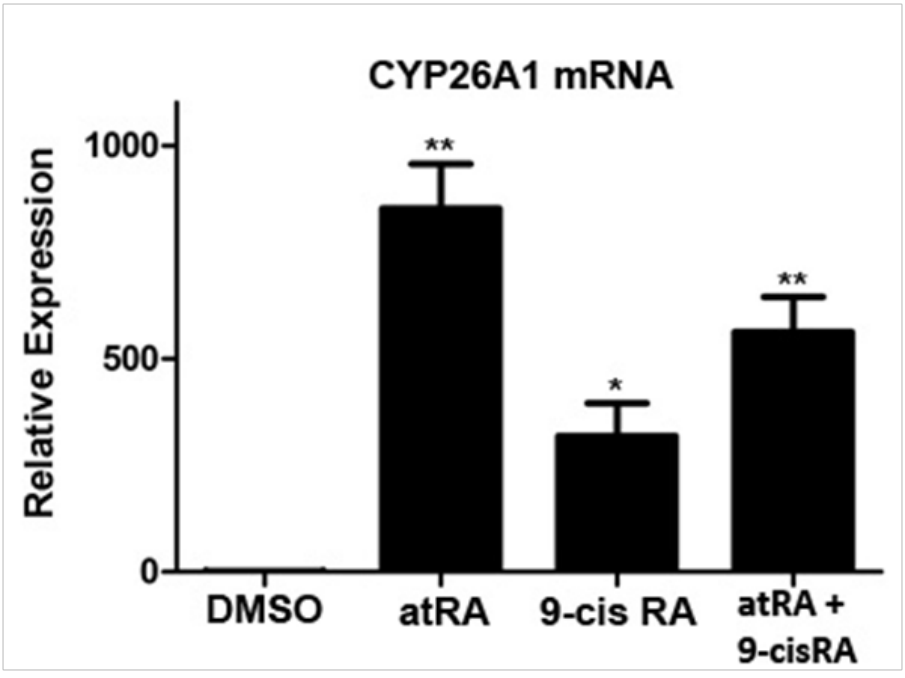

Figure 15. CYP26A1 mRNA expression in HepG2 cells after treatment with 1µM of varying retinoids. Data shown are fold increases in CYP2D6 promoter activity by retinoid treatment. *p < 0.05, **p < 0.005 vs. DMSO

IV. DISCUSSION

Altered pharmacokinetics of drugs can influence their efficacy, tolerability, and toxicity. Accurate pharmacokinetic information of a drug is essential for effective and efficient dosing. Many environmental or genetic factors can influence the pharmacokinetic properties of drugs, leading to interindividual variability in drug disposition. Such factors include coadministration of drugs, gender, and genetic variations. Pregnancy has been shown to alter pharmacokinetics of drugs and affect hepatic drug metabolism in a CYP pathway-dependent manner.

Clinically, the effect of pregnancy on differential regulation of these CYPs has shown altered metabolic ratios, with an outcome requiring dosage adjustments to achieve desired plasma concentration. To date, factors responsible for the altered drug metabolism of many CYP substrates have not been identified, nor have in-depth molecular mechanisms elucidated. In this work, we elucidated an ER-mediated regulatory mechanism for the CYP2B6 gene, providing a mechanistic basis for potential increases in CYP2B6-mediated drug metabolism during pregnancy. Additionally, we examined roles of potential plasma factors or transcriptional regulators responsible for CYP2D6 induction during pregnancy. Knowledge of these mechanisms provides information for multiple critical areas of research: a better ability to accurately predict plasma concentrations of drugs, provides the potential to alert us to other conditions that may be implicated in the mechanism, and allows for individualizing prescriptions based on personal genotype/phenotype and the determined mechanism of induction.

A. UPREGULATION OF CYP2B6 BY E_2

One of the major physiological changes during pregnancy is an alteration in circulating hormone levels, particularly an increase in E_2. Previously, we showed that E_2 induces CYP2B6 expression in hepatocytes. This work aims to elucidate the mechanism responsible for this increased expression.

Our results obtained in HepG2 and HepG2-ER cells indicate that CYP2B6 induction by E_2 is mediated by ER. Classical ER transactivation requires an intact DBD; however, we found that the DBD is dispensable for induction of CYP2B6 promoter activity by E_2, suggesting a nonclassical mechanism of ER activation in CYP2B6 regulation by E_2. Results from deletion assays revealed the -1839 to -1461-bp upstream region of CYP2B6 mediates E_2 action on CYP2B6. *In silico* analysis revealed this region harbors two putative AP-1 binding sites. Results from the mutation assay revealed that the putative AP-1 binding sites are critical in CYP2B6 induction by E_2. Together, these data suggest that a nonclassical mechanism of ER action, likely via AP-1, plays an important role in CYP2B6 induction by E2.

Recently, a report by Lo et. al. has shown that in Huh-7 cells, CYP2B6 is upregulated in an E_2-dependent, ER-mediated manner through an ERE located ~1600-bp upstream in the CYP2B6 promoter [67]. This, however, does not agree with our findings in HepG2 cells. Interestingly, in our HepG2 cells, deletion of part of this ERE, rendering a half ERE, caused no significant change in E_2's ability to induce CYP2B6 transcription (Figure 9). It has been shown that ER binds half EREs with 50- to 100-fold lower affinity as compared to the full ERE [68], suggesting that mutation of the ERE to an hERE would abolish any significant ER-binding. This discrepancy in the ER-mediated CYP2B6 transactivation can be attributed to the following factors. First, cell-line differences in activity of AP-1 proteins or expression of ER co-regulators are one possible explanation [69]. For example, it has been shown that there are cell-line differences in c-jun activity [70], a major component of AP-1 protein complexes, and a potential difference in AP-1 protein activity exists between Huh-7 cells and HepG2 cells. Alternatively, in HepG2 cells, both ER mechanisms (i.e., direct ERE binding and AP-1 mediated binding to CYP2B6 promoter) may be functional, each compensating the other when one mechanism is inhibited. The overlap between the ERE and an AP-1 site supports this notion of dual-mechanisms.

A more global clinical impact of knowing the induction is mediated through AP-1 activity is important because of the altered activity of E_2:ER due to the third-party protein complex. Tissue distribution of AP-1 proteins may alter the effect of ER-mediated transcription of CYP2B6 in extrahepatic tissues. CYP2B6 has been shown to be expressed in extrahepatic tissues such as lung [71] and brain [72] in humans, and AP-1 expression has been found in both of these tissues, among others, in mice transgenic for human AP-1 [73]. With this in mind, AP-1-mediated induction of CYP2B6 may not be restricted to the liver, but may occur in other tissues as well. For example, CYP2B6 has been shown to play a role in C-oxidation of nicotine to cotinine [74], suggesting that an induction of CYP2B6 expression in lung tissue may alter the pharmacokinetics of nicotine and lead to an increase in cigarette use to acquire the desired effect. Although smoking during pregnancy has been known to have negative effects on the fetus for a long time, estimates from 2001 say that 12-25% of expecting mothers in the US still smoke [75]. A potential induction of CYP2B6 in the lung suggests a larger investment in smoking cessation with pregnant mothers may be crucial to prevent birth defects.

The finding that CYP2B6 expression is regulated by E_2 has clinical implications in any physiological state where estrogen levels are disturbed from their baseline concentration. This implicates not only pregnancy as a state of altered CYP2B6 expression, but also during the menstrual cycle, women taking oral birth control pills, and post-menopausal women taking hormone replacement therapy.

B. REGULATION OF CYP2D6 EXPRESSION

HNF4α is a liver enriched transcription factor that is responsible for regulation of many genes, and also the only transcription factor to date that has been shown to control CYP2D6 expression. Results from our preliminary study showed that HNF4α expression was not induced during pregnancy. Considering the possibility that HNF4a transcriptional activity can be induced during pregnancy (in spite of no changes in HNF4a expression), we investigated

whether pregnancy influenced HNF4a activity by performing EMSA. The preliminary result suggests that HNF4α DNA-binding activity increases at term. Of note, a wide range of nuclear receptors expressed in the liver have been shown to bind the HNF4α consensus sequence [76-79], thus a supershift experiment is needed to verify that the shifted bands represent HNF4a binding.

Because HNF4α controls many hepatic genes, a global increase in HNF4α activity is an unlikely explanation for CYP2D6 induction during pregnancy. If this occurs, many important genes involved in lipid and carbohydrate homeostasis would be upregulated, and metabolism homeostasis would be seriously disturbed. In fact, our results from microarray experiments showed that pregnancy does not affect expression of a representative HNF4α target gene apolipoprotein CIII (data not shown). Transcriptional activity of transcription factors can be promoter-specific, and it's possible that HNF4α may play a role in CYP2D6 induction during pregnancy.

A recently published report claims that retinoic acid represses CYP2D6 mRNA expression in HepG2 cells. To verify the data, we examined in HepG2 cells whether retinoic acid affects CYP2D6 expression and promoter activity. CYP2D6 promoter activity after treatment with a wide range of retinoic acid concentrations shows a ~2-fold reduction in promoter activity, suggesting potential involvement of retinoic acid and/or its isomers in the differential regulation of CYP2D6 during pregnancy. On the contrary, treatment with all-trans retinoic acid did not alter CYP2D6 expression, while treatment with 9-cis retinoic acid, alone or in combination with all-trans retinoic acid, reduced CYP2D6 transcripts, contradicting the results from luciferase assays. The discrepancy may be in part due to physicochemical properties of retinoic acids. All-trans and 9-cis retinoic acids are isomers of each other, and are interchangeable when exposed to time, light, and relatively unknown intracellular mechanisms thought to be isomerases [80, 81]. Due to the different endpoints of each experiment (luciferase protein vs. CYP2D6 mRNA), our luciferase data were obtained after 24 hours of

exposure to 1 µM all-trans retinoic acid, but mRNA data were obtained after 72 hours of exposure to retinoids. Examination of the effect of 9-cis retinoic acid on CYP2D6 promoter activity may shed light on this issue.

Two caveats to using retinoids in cell culture is the isomerization that occurs amongst them, and their solubility in culture media. Our conflicting results fail to determine which retinoid is responsible for CYP2D6 repression. It has been observed that in fasted human volunteers, atRA concentrations ranged from 1.1-1.9 ng/mL, or ~3 to 6 nM [82]. Previously, it was shown that in hepatocytes, 1 µM atRA led to the isomerization of atRA to 9-cisRA, and an accumulation of 1.2% of 9-cisRA inside of cells, and 4.7% of 9-cisRA in the media (percentage of initial atRA) [83]. Our data shows that at 100 pM, all-trans retinoic acid repressed CYP2D6 expression nearly ~40%(Figure 13). Our mRNA data, on the other hand, suggests that CYP2D6 repression occurs via 9-cisRA, though atRA had a small but statistically insignificant effect (Figure 14). This suggests that isomerization of 1 µM atRA, potentially producing ~1 nM intracellular and ~5 nM extracellular 9-cisRA, may produce an effect via 9-cisRA on CYP2D6 expression. If 9-cisRA is the responsible retinoid, RXR would be implicated in the regulation of CYP2D6, as RXR is the target nuclear receptor for 9-cisRA. On the other hand, upon administration of 9-cisRA to humans, the 9,13-dicisRA isomer was found, suggesting further metabolism of 9-cisRA is possible [84], and it has been observed that atRA can be converted to 11-cisRA *in vitro* [85]. Due to extensive isomerization, it is not clear which compound(s) is responsible for the effects seen on CYP2D6 expression, however retinoids appear to affect the basal expression level of CYP2D6.

A second potential caveat of retinoid use is their solubility in culture media. Retinoids are practically insoluble in water, so stock solutions had to be made in DMSO. Our promoter activity results show that over 5 orders of magnitude, CYP2D6 repression was relatively constant. This result suggests that once the varying drug concentrations were made in culture medium, a portion of the drug precipitated out, leaving each of the applied concentrations with

the same effective concentration in solution. On the other hand, there is a possibility that the repressive effect was already at maximum level at 100 pM. To overcome this issue, synthetic retinoids with higher solubility may be used, and/or overexpression studies of retinoid receptors can be performed.

V. CONCLUSIONS

In summary, E_2 was found to significantly induce CYP2B6 expression and promoter activity in HepG2 cells transfected with an ERα expression vector. While previous studies have shown that E_2 functions to increase CYP2B6 expression in hepatocytes, this work initially established HepG2 cells as a working model to elucidate the molecular mechanism involved. Using mRNA expression and luciferase reporter assays, we found that E_2's effects on CYP2B6 expression are mediated through ERα. Once ligand-bound, ER functionally transactivates the CYP2B6 promoter in a nonclassical manner, leaving the DNA-binding domain dispensable. *In silico* analysis revealed multiple putative AP-1 and Sp1 binding sites in the 3.3-kb upstream region. Deletion constructs of the CYP2B6 reporter plasmid showed the region of -1461 bp to -1839 bp to be crucial in ER's transactivation. Two AP-1 motifs were located within this region, as well as a putative ERE. After mutation of both AP-1 motifs, promoter activity was abolished, but deletion of a single motif had no significant effect on promoter activity, indicating an AP-1-mediated mechanism that does not require both AP-1 binding sites for induction. A partial deletion of the ERE, leaving an in-tact AP-1 motif, produced no significant difference in promoter activity between the native promoter and the mutated construct. Together the results indicate E2 induces CYP2B6 via an AP-1/ER-mediated mechanism.

To better understand CYP2D6 induction during pregnancy, we first aimed to verify previous results from a different set of transgenic mice that showed CYP2D6 induction during pregnancy. Our preliminary results from a small set of mice show a ~2-fold induction of CYP2D6 mRNA level at term as compared to the levels at pre-pregnancy or postpartum period. This suggests these mice are a valid model and can be used to further pursue the mechanism of CYP2D6 induction during pregnancy. To determine whether pregnancy influences activity of HNF4α, a known regulator of CYP2D6 expression, we performed EMSA assays using nuclear extracts from mouse liver tissues collected at different gestational time points. We found that nuclear protein binding to a known HNF4α-binding motif of the CYP2D6 promoter region

increased at term and returned to basal level in postpartum samples. Whether the shifted bands represent HNF4α binding remains to be verified by using supershift assays. Lastly, to determine the roles of retinoids in CYP2D6 induction during pregnancy, we verified a recently published result implicating retinoids in basal-level CYP2D6 mRNA expression. Our results show that all-trans retinoic acid represses CYP2D6 promoter activity almost 2-fold, and that 9-cis retinoic acid represses CYP2D6 mRNA roughly 25%.

VI. FUTURE DIRECTIONS

CYP2B6 is regulated via an E_2, ER-dependent, AP-1-mediated mechanism. With the knowledge that oral birth control users experience high intrahepatic hormone levels, it would follow that CYP2B6 may undergo differential regulation during their hormone/placebo cycle. An experiment to answer this question is to examine CYP2B6-mediated drug metabolism in oral contraceptive users, at different time points in their hormone/placebo cycle.

Another question that is brought up is the effect of antiestrogens on CYP2B6 activity. Antiestrogens used in preventative care and in chemotherapy known as selective estrogen receptor modulators, or SERMs, are known to block ER:ERE transcriptional activity, but to activate ER:AP-1-mediated transcriptional activity. To see if this has any significant effect, HepG2 cells can be treated with physiologic concentrations of different SERMs, and CYP2B6 expression can be determined.

CYP2D6 transgenic mice show an increase in CYP2D6 mRNA at term. The next step to take would be to look at the mechanism. By performing chromatin immunoprecipitation (ChIP), it is possible to determine if a particular transcription factor is binding more or less at term than during basal-level expression. One such factor to investigate is HNF4α, due to the preliminary result obtained by EMSA.

Retinoids slightly decrease CYP2D6 promoter activity and mRNA expression. Due to isomerization and solubility, however, these results need to be confirmed. Treating HepG2 cells with RAR- and RXR-selective agonists may provide a more concrete answer. Furthermore, RAR and RXR have been shown to interact with DNA at the same locations as HNF4α. Performing ChIP for these two transcription factors may also provide insight into the molecular mechanism of CYP2D6 induction.

VII. CITED LITERATURE

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