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Hepatocyte nuclear factor (HNF) 4 α transactivation of cytochrome P450 (Cyp) 2d40 promoter is enhanced during pregnancy in mice

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Abstract

We have recently reported that transactivation of cytochrome P450 (CYP) 2D6 promoter by hepatocyte nuclear factor (HNF) 4 α is enhanced during pregnancy, and this is triggered in part by altered expression of small heterodimer partner (SHP) and Krüppel-like factor 9 (KLF9). The objective of this study is to determine whether this is conserved for mouse endogenous Cyp2d gene(s). Among the eight Cyp2d homologs of mouse we examined, only Cyp2d40 expression was found induced (by 6-fold) at term pregnancy as compared to pre-pregnancy level. In mice where hepatic Hnf4 α was knocked-down, the pregnancy-mediated increase in Cyp2d40 expression was abrogated. Results from transient transfection, promoter reporter assays, and electrophoretic mobility shift assays indicated that HNF4 α transactivates Cyp2d40 promoter via direct binding to –117/–105 of the gene. Chromatin immunoprecipitation assay showed a 2.3-fold increase in HNF4 α recruitment to Cyp2d40 promoter during pregnancy. Results from mice treated with an SHP inducer (i.e., GW4064) and HepG2 cells co-transfected with KLF9 suggest that neither SHP nor KLF9 is involved in the increased HNF4 α transactivation of Cyp2d40 promoter during pregnancy. Together, our results indicate that while the underlying molecular mechanism is different from that for CYP2D6, Cyp2d40 is induced during pregnancy through enhanced transactivation by HNF4 α .

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Keywords

Cyp2d40; pregnancy; regulation; HNF4 α

1. Introduction

Cytochrome P450 (CYP) 2D6 is a major drug-metabolizing enzyme and expressed in the liver, intestine, kidney, and brain [1, 2]. CYP2D6 mediates hepatic metabolism of approximately 25% of marketed drugs including antidepressants and antipsychotics [3, 4]. Also, decreased CYP2D6 activity levels are associated with increased susceptibility to Parkinson's disease [5, 6] or higher blood perfusion levels in brain regions linked to alertness in humans [7]. The detailed roles of CYP2D6 in altered biological functions in extrahepatic tissues (such as brain) remains unclear, in part due to a lack of animal models to study CYP2D6 function *in vivo*. Transgenic mice such as CYP2D6-humanized (Tg-CYP2D6) mice that carry human CYP2D6 gene in the mouse genome exhibit robust CYP2D6 expression in liver and intestine [1, 8, 9]. However, CYP2D6 expression is absent in the brain of the transgenic mice [1, 9]. Identification and characterization of mouse Cyp2d homologs whose expression or activities are co-regulated as CYP2D6, if any, may potentially enable establishing study models to explore the biological functions of CYP2D6 in *in vivo* systems.

CYP2D6-mediated drug metabolism is increased during human pregnancy [10–12]. For example, metoprolol clearance increases 2–13 fold during pregnancy as compared to that after delivery [11]. We have recently shown that CYP2D6 expression is enhanced by 4-fold at term pregnancy (as compared to the pre-pregnancy or postpartum period) in Tg-CYP2D6 mice, and this was accompanied by increased transactivation of CYP2D6 promoter by hepatocyte nuclear factor 4 α (HNF4 α , NR2A1) [13]. HNF4 α is a nuclear receptor known to play a critical role in regulating expression of liver-specific genes, including drug-metabolizing enzymes [14–17]. Our studies also demonstrated that the increased HNF4 α transactivation of CYP2D6 promoter is in part attributed to two transcription factors, namely small heterodimer partner (SHP, NR0B2) and Krüppel-like Factor 9 (KLF9), whose hepatic expression is differentially regulated during pregnancy [13, 18]. SHP, a member of nuclear receptor superfamily, lacks the DNA-binding domain [19] and interacts with HNF4 α to function as a transcriptional repressor [20, 21]. During pregnancy, hepatic SHP expression decreases, and this leads to de-repression of CYP2D6 promoter [13]. On the other hand, KLF9 transactivates CYP2D6 promoter in synergy with HNF4 α [18]. During pregnancy, hepatic KLF9 expression increases, further potentiating HNF4 α transactivation of CYP2D6 promoter [18]. Importantly, these results provide a potential platform to identify and characterize mouse Cyp2d homologs that are regulated similarly as CYP2D6.

Humans express only one functional CYP2D (i.e., CYP2D6), but mouse genome harbors the following nine Cyp2d homologs: *Cyp2d9*, *2d10*, *2d11*, *2d12*, *2d13*, *2d22*, *2d26*, *2d34*, and *2d40*. How the expression of mouse Cyp2d genes is regulated remains largely unknown. Also unknown is whether the regulatory mechanisms leading to CYP2D6 induction during pregnancy is conserved for endogenous mouse Cyp2d genes.

In this study, we identified Cyp2d40 as an endogenous mouse Cyp2d gene whose expression is induced during pregnancy. We further evaluated the role of HNF4 α and its modulators (i.e., SHP and KLF9) in the regulation of Cyp2d40 expression during pregnancy, to explore conserved physiological/functional significance of CYP2D induction during pregnancy.

Materials and Methods

1.1. Animals

Tg-CYP2D6 and Hnf4 α /AlbCre transgenic mice were previously described [8, 22]. Adult female (8 weeks old) mice were mated with male mice of similar age. Mating between adult mice was confirmed by the presence of vaginal plugs (day 0). Male mice were separated from female mice immediately after a vaginal plug was found. Virgin mice were group-housed so that their estrous cycles were suppressed. All procedures were approved by the Institutional Animal Care and Use Committee in the University of Illinois at Chicago.

1.2. GW4064 treatment

Male C57BL/6J mice were injected with GW4064 (Sigma-Aldrich, St. Louis, MO) at 15 mg/kg/day or vehicle (olive oil) for 5 days. Livers were collected 6 hours after the last injection for examination of gene expression.

1.3. Plasmids

The upstream region of Cyp2d40 (GenBank accession no. NC_000081.6) was PCR-amplified using genomic DNA of C57BL/6J mouse and the primer set listed in Table 1. The PCR product was digested by *Kpn*I and *Sac*I restriction enzymes (New England Biolabs, Ipswich, MA) and cloned into promoterless pGL3-basic vector (Promega, Madison, WI) digested by the same enzymes, yielding pGL3-Cyp2d40 (-2000/+6). For cloning of 5'-deletion constructs, different regions of Cyp2d40 promoter were PCR-amplified using pGL3-Cyp2d40 (-2000/+6) as template and respective forward primers listed in Table 1. The PCR product was digested by *Kpn*I and *Sac*I restriction enzymes and cloned into pGL3-basic vector digested by the same enzymes. To construct mutant vectors of Cyp2d40 promoter at HNF4 α binding site, pGL3-CYP2d40 (-171/+6) was PCR-amplified by using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) and 5'-phosphorylated primers harboring mutation or deletion of the HNF4 α binding site listed in Table 1. The PCR products were then ligated by using T4 ligase (New England Biolabs, Ipswich, MA).

1.4. Cell culture and luciferase reporter assay

HEK293T cells were cultured in RPMI-1640 media supplied with 10 mM Hepes buffer, 100 μ M non-essential amino acids, 5,000 U penicillin/streptomycin (Life Technologies, Carlsbad, CA) and 10% fetal bovine serum (Gemini Bio-products, West Sacramento, CA). For assays, HEK-293T cells were seeded in 24-well plates at a density of 1×10^5 cells per well. On the next day, the cells were transfected with 0.3 μ g of luciferase construct, 0.1 μ g of expression plasmid (or empty vector as a control), and 0.002 μ g of *Renilla* expression vector (Promega, Madison, WI) per well, using Fugene HD transfection reagent (Promega, Madison, WI) according to the manufacturer's protocol. After 48 hours, the transfected cells

were harvested for determination of luciferase activities using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI).

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

Total RNAs were isolated from mouse liver tissues using Trizol (Life Technologies, Carlsbad, CA) and used as template for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA). Using the cDNA as template, qRT-PCR was performed using StepOnePlus™ Real-Time PCR System and the following TaqMan® Gene expression assays (Integrated DNA technologies, Coralville, IA) for Cyp2ds and mouse Actb. Shp and Gapdh mRNA levels were determined using SYBR® Green Real Time PCR Master Mix (Life technologies, Foster City, CA) and primer sets listed in Table 1. The relative expression in mRNA levels was determined after normalizing the gene expression levels by those of mouse Gapdh or Actb (2^{-Ct} method) [23].

1.6. Chromatin Immunoprecipitation (ChIP) assay

ChIP assays with mouse liver were performed as previously described [13]. Briefly, livers were finely minced and incubated in PBS containing 1% formaldehyde at room temperature for 15 min, and glycine was added to stop the crosslinking reaction. Cell pellets were resuspended in hypotonic buffer (15 mM HEPES, 60 mM KCl, 2 mM EDTA, 0.5% BSA, 0.15 mM spermine, 0.5 mM spermidine, 0.32 M sucrose, pH 7.9) and lysed by homogenization. Nuclei were pelleted and resuspended in nuclei lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 1% SDS, pH 8.0). The samples were sonicated to shear DNA at the length from 100 to 500 bp. After centrifuge, the chromatin samples were immunoprecipitated using magnetic beads coated with 2 µg antibody (HNF4α, sc-6556x, Santa Cruz Biotechnology, Dallas, TX) or immunoglobulin G (IgG, sc-2028, Santa Cruz, Dallas, TX) at 4°C overnight. The immune complex on the magnetic beads was collected and extensively washed, and the bound chromatin was eluted. Genomic DNA was purified by phenol chloroform extraction followed by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). qRT-PCR was performed using the genomic DNA and the primer set listed in Table 1.

1.7. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described [24]. Briefly, recombinant HNF4α protein (with C-terminal MYC/DDK tag; 0.2 µg; TP317863, OriGene Technologies, Rockville, MD) or nuclear protein extracts (5 µg) from HEK293T cells transfected with HNF4α expression vector (or empty vector) were preincubated with binding buffer (4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5) at room temperature in the presence or absence of unlabeled DNA probes or HNF4α antibody (2 µg, sc-6556x, Santa Cruz). After 10 min, the binding reaction was initiated by adding 0.035 pmol of 5'-end ³²P-labeled probes harboring Cyp2d40 putative HNF4α-binding sequence or CYP2D6 HNF4α-binding sequence. The reaction mixture was incubated at room temperature for 20 min. Protein-bound probes were separated from free probes on 4% (w/v) non-denaturing polyacrylamide gel. The gel was dried, and radioactivity visualized using PhosphorImager (GE Healthcare Bio-Sciences, Pittsburgh, PA).

1.8. Statistical analysis

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

2. Results

2.1. Cyp2d40 expression is enhanced during pregnancy

To identify the mouse Cyp2d genes that exhibit similar upregulation as CYP2D6 does during pregnancy, we examined the mRNA expression levels of the following Cyp2d homologs in livers of Tg-CYP2D6 or wild type mice at different gestational time points: *Cyp2d9*, *2d10*, *2d12*, *2d13*, *2d22*, *2d26*, *2d34*, and *2d40*. Cyp2d11 was not examined in this study because its expression is male-specific [25]. Cyp2d12 and Cyp2d34 expression was not detectable in the mouse liver tissues (data not shown). In the livers of nonpregnant mice, Cyp2d26 expression was most abundant, followed by Cyp2d10, Cyp2d22, Cyp2d13, Cyp2d9 and Cyp2d40 (Fig. 1A). Among the six Cyp2d isoforms, Cyp2d40 expression increased significantly at term as compared to pre-pregnancy level in both Tg-CYP2D6 and wild-type mice (by 6- and 4-fold, respectively) (Fig. 1B and 1C), suggesting that the presence of transgene (i.e., CYP2D6) does not impact Cyp2d40 regulation during pregnancy. On the other hand, mRNA expression of the remaining five Cyp2d isoforms slightly decreased during pregnancy (data not shown).

2.2. HNF4 α is critical for Cyp2d40 induction during pregnancy

We have previously shown that CYP2D6 induction during pregnancy is attributable to enhanced transactivation of CYP2D6 promoter by HNF4 α [13]. To define the role of HNF4 α in Cyp2d40 induction during pregnancy, we examined whether reduced hepatic HNF4 α expression alters the extent of Cyp2d40 up-regulation seen at term pregnancy. To this end, the pregnancy-mediated changes in Cyp2d40 expression were compared between mice carrying two vs. one copy of *Hnf4a* allele in the liver (noted as *Hnf4a(wt/wt)* and *Hnf4a(-/wt)* mice, respectively). Hepatic HNF4 α protein expression level in *Hnf4a(-/wt)* mice was previously shown to be about half of that in *Hnf4a(wt/wt)* mice [13]. In *Hnf4a(-/wt)* mice, the basal mRNA expression of Cyp2d40 decreased dramatically as compared to that in *Hnf4a(wt/wt)* mice (Fig. 2), indicating that HNF4 α is a major regulator of basal Cyp2d40 expression. More importantly, Cyp2d40 induction during pregnancy was abrogated in *Hnf4a(-/wt)* mice, suggesting a key role of HNF4 α in Cyp2d40 induction during pregnancy.

2.3. HNF4 α transactivates Cyp2d40 promoter

To further characterize the role of HNF4 α in the regulation of Cyp2d40 expression, transient transfection and luciferase reporter assays were conducted. Transfection of HNF4 α in HEK293T cells led to a dramatic increase in *Cyp2d40* promoter activity (Fig. 3A), indicating that HNF4 α transactivates *Cyp2d40* promoter. To map the *cis*-element(s) responsible for HNF4 α transactivation, a series of luciferase vectors harboring 5'-truncated upstream regulatory region of *Cyp2d40* were constructed, and luciferase assays were performed. Ectopic HNF4 α expression led to transactivation of *Cyp2d40* promoter as short

as -171 to +6 (Fig. 3B). However, the transactivation by HNF4 α was completely abrogated when the region between -171 and -86 was deleted (Fig. 3B), indicating the presence of key *cis*-element(s) for HNF4 α action within the region. *In silico* promoter analysis revealed a putative HNF4 α binding site in this region, which is conserved between CYP2D6 and Cyp2d40 (Fig. 3C). To further validate the putative HNF4 α binding site in Cyp2d40, mutation and deletion assays were performed. HNF4 α transactivation of Cyp2d40 promoter was completely abolished when the putative binding site of HNF4 α was deleted or mutated (Fig. 3D).

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

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

To examine the pregnancy-mediated changes in HNF4 α transactivation of Cyp2d40 promoter *in vivo*, ChIP assays were performed using mouse liver tissues collected at different gestational time points. The amount of HNF4 α -bound DNA was measured by qRT-PCR using a primer set that can detect the HNF4 α response element of Cyp2d40. The results showed that HNF4 α recruitment to Cyp2d40 was increased by 2.3-fold at term pregnancy as compared to pre-pregnancy (Fig. 5), suggesting that enhanced transactivation of Cyp2d40 promoter is in part responsible for Cyp2d40 induction during pregnancy.

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

Previously, we have shown that SHP represses and KLF9 potentiates HNF4 α transactivation of CYP2D6 promoter [13, 18]. To examine whether SHP and KLF9 modulate HNF4 α transactivation of Cyp2d40 promoter (similarly to CYP2D6 promoter), we performed promoter reporter assays in HEK293T cells. HEK293T cells were co-transfected with pGL3-Cyp2d40 along with HNF4 α and/or SHP expression vectors, and luciferase activity was measured. The results showed that SHP overexpression led to repressed HNF4 α

transactivation of Cyp2d40 promoter in HEK293T cells (Fig. 6A, lane 3 vs. 4). Considering that findings in HEK293T cells could result from super-physiological levels of SHP upon transient transfection, the role of SHP in Cyp2d40 regulation was further studied *in vivo*. To this end, mice were treated with GW4064, an inducer of SHP expression [21, 26], and mRNA expression levels of Cyp2d40 were measured. Cyp8b1 expression is known to be repressed when SHP is upregulated [27], and thus Cyp8b1 was included as a positive control. Unexpectedly, Cyp2d40 mRNA was not affected when SHP expression was induced in mice, whereas the expression of Cyp8b1 was significantly repressed in mice (Fig. 6B), suggesting that SHP does not modulate Cyp2d40 expression in *in vivo* systems. Results from promoter reporter assays for KLF9 showed that KLF9 did not affect HNF4 α transactivation of Cyp2d40 promoter (Fig. 6C, lane 3 vs. 4), unlike the previously-shown potentiation of HNF4 α transactivation of CYP2D6 promoter by KLF9 [18]. Together, these indicated that neither SHP nor KLF9 modulates Cyp2d40 expression, and the mechanism(s) underlying increased HNF4 α transactivation of Cyp2d40 promoter likely differ from that of CYP2D6.

3. Discussion

Our previous study in Tg-CYP2D6 mice showed that CYP2D6 induction during pregnancy is potentially triggered by enhanced HNF4 α transactivation of CYP2D6 promoter, attributable to decreased expression of transcriptional repressor (SHP) and increased expression of transcriptional activator (KLF9) during pregnancy [13, 18]. We hereby examined whether this regulatory mechanism is conserved for mouse endogenous Cyp2d genes.

Among the Cyp2d homologs, we found that Cyp2d40 expression is significantly up-regulated during pregnancy. This result is in part consistent with the previous report where expression of most Cyp2d genes examined in the study (i.e., Cyp2d11, 2d22, 2d26, and 2d40) was shown increased during pregnancy [28]. Interestingly, our results showed that expression of most Cyp2ds (except Cyp2d40) decreased during pregnancy. The discrepancy is potentially due to differences in mouse strains used in the studies (FVB vs. C57BL/6). While the directional changes in the expression of other mouse Cyp2ds remain to be verified, both our and the previous studies clearly demonstrated that mouse Cyp2d40 expression is increased during pregnancy similarly to human CYP2D6.

The finding of enhanced CYP2D6 and Cyp2d40 expression during pregnancy suggests that the physiological roles of these two enzymes may be conserved in mice and humans. CYP2D6 in the liver metabolizes and eliminates a variety of xenobiotics, and its enhanced activity during pregnancy may be an evolutionary consequence of providing protection against potentially harmful xenobiotics in developing fetus. CYP2D6 expressed in the brain, on the other hand, has been implicated in the synthesis of neurotransmitters [29, 30] although the roles of CYP2D6 in brain physiology remains to be verified. Whether pregnancy alters CYP2D6 expression in brain is currently unknown. CYP2D40 is highly homologous (i.e., 81%) to CYP2D6, and this suggests that CYP2D40 may play similar roles as CYP2D6 by participating in hepatic drug detoxification and modulating brain functions. Indeed, CYP2D proteins have been detected in mouse brain [1], and the contribution of

different mouse CYP2D isoforms (especially CYP2D40) to the CYP2D proteins expressed in the brain is yet to be determined.

Our previous study has shown that CYP2D6 induction during pregnancy is due to enhanced transactivation of CYP2D6 promoter by HNF4 α [13]. Results from this study showed that HNF4 α also plays a key role in enhanced expression of Cyp2d40 during pregnancy; Cyp2d40 induction during pregnancy was abrogated in mice with decreased hepatic HNF4 α expression, and HNF4 α recruitment to Cyp2d40 promoter increased at term pregnancy. We further showed that *Cyp2d40* is a direct target gene of HNF4 α , and mapped a HNF4 α response element to -117/-105 of *Cyp2d40*. Interestingly, the HNF4 α binding site was found conserved in the promoters of the other mouse Cyp2d genes (except Cyp2d22) (data not shown). This suggests that the presence of HNF4 α binding sequence in Cyp2d40 promoter alone does not explain the unique feature of Cyp2d40 regulation during pregnancy. HNF4 α transactivation of its target gene promoters is likely modulated in a Cyp2d gene-specific manner during pregnancy, similarly to other HNF4 α target genes whose expression is altered during pregnancy in a pattern different from that for CYP2D6 [13].

Enhanced HNF4 α transactivation of CYP2D6 promoter is triggered in part by decreased SHP and increased KLF9 expression during pregnancy [13, 18]. However, results from this study suggest that neither SHP nor KLF9 plays a key role in modulating HNF4 α transactivation of Cyp2d40 promoter. This is potentially due to differences between CYP2D6 and Cyp2d40 in the promoter sequences, resulting in altered interplay among SHP, KLF9, and HNF4 α . Firstly, our EMSA results indicate that HNF4 α binding to Cyp2d40 promoter may be of lower affinity than that to CYP2D6 promoter. In an *in vivo* system, this may lead to dampened SHP action on HNF4 α transactivation of Cyp2d40 promoter. In accordance with this notion, although SHP significantly repressed Cyp2d40 promoter activity in HNF4 α -overexpressing HEK293T cells, such finding was not recapitulated in mice. Secondly, whereas *CYP2D6* harbors multiple binding sites for KLF9 that enhances HNF4 α transactivation of the promoter [18], *Cyp2d40* lacks such KLF9 binding sites in its promoter (based on *in silico* promoter analysis; data not shown). Our results from promoter reporter assays also correspond to the lack of KLF9 binding to *Cyp2d40*. The detailed molecular mechanisms underlying enhanced HNF4 α transactivation of Cyp2d40 promoter during pregnancy remain unclear. The roles of other known regulators of HNF4 α activity [31–35], such as PGC1 α , in the regulation of HNF4 α activity on Cyp2d40 promoter warrant further investigation.

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

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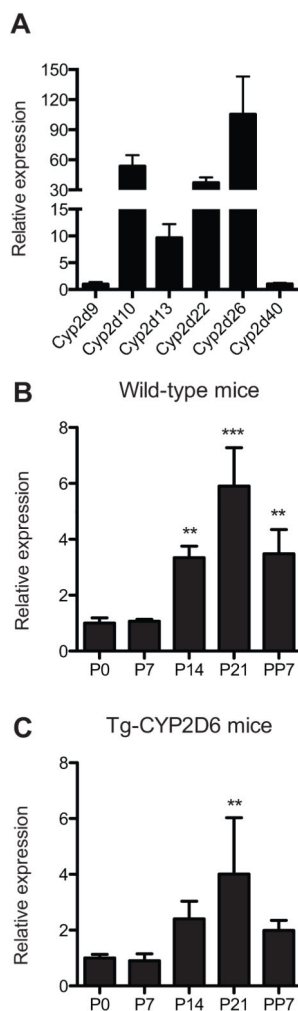


Figure 1. Hepatic Cyp2d40 is induced in both wild-type and Tg-CYP2D6 mice during pregnancy (A) Liver tissues of wild-type nonpregnant female mice were collected (n=4). mRNA expression levels of Cyp2ds were determined by qRT-PCR and normalized by Cyp2d40 expression. (B) and (C) Liver tissues of wild-type (B) and Tg-CYP2D6 (C) mice were collected at different gestational time points: pre-pregnancy (P0), 7, 14, or 21 days of pregnancy (P7, P14, P21, respectively), 7 days postpartum (PP7). mRNA levels of Cyp2d40 were determined by qRT-PCR and normalized by that in the pre-pregnancy group (n=4, mean \pm S.D.; **, $p < 0.01$, ***, $p < 0.001$ versus P0 group).

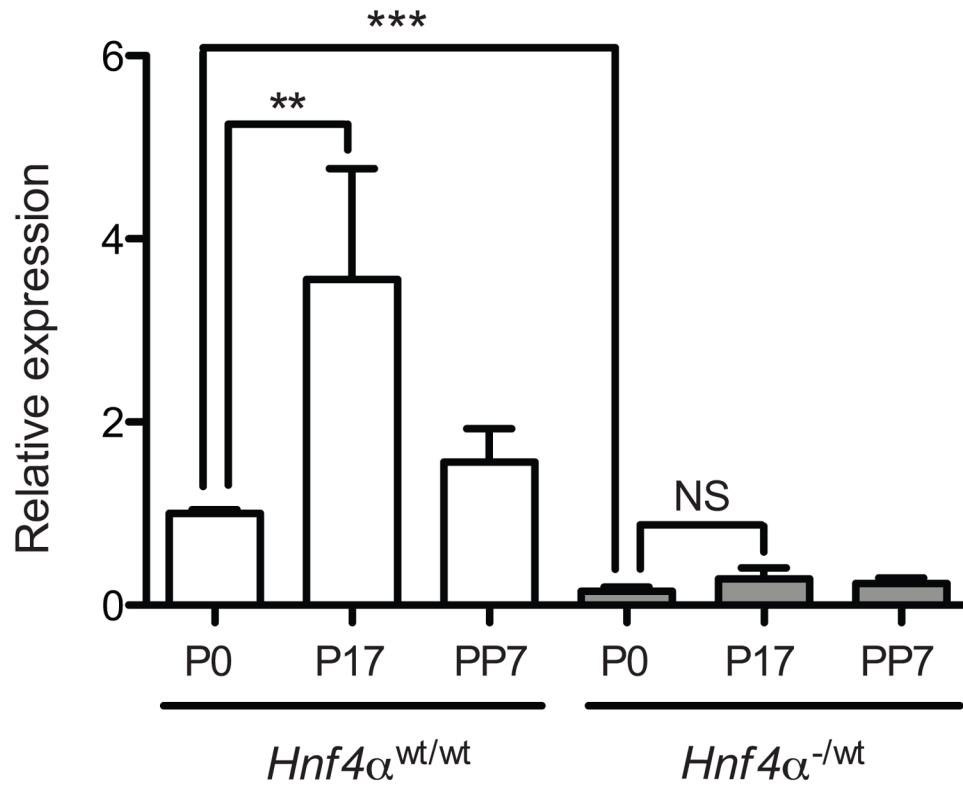
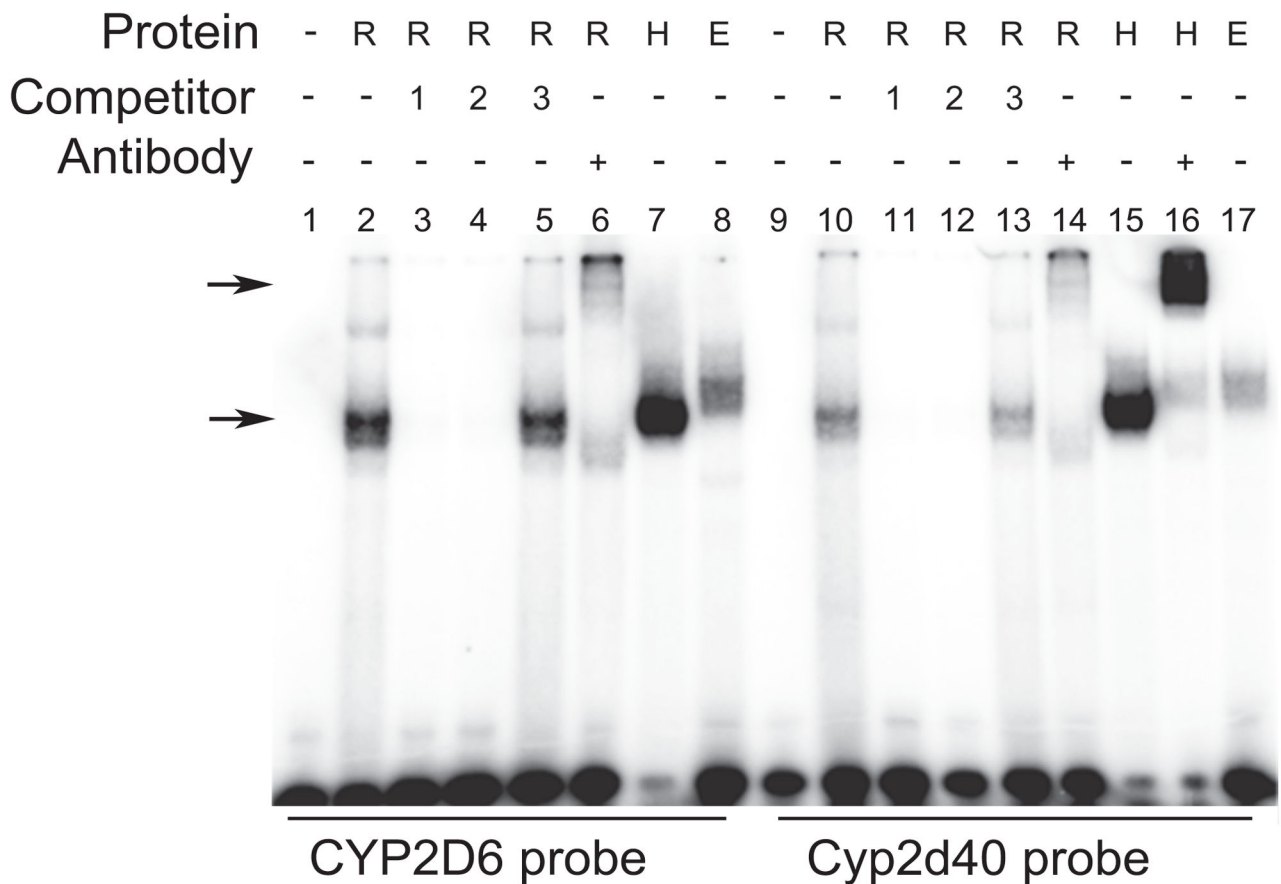


Figure 2. HNF4 α is critical for *Cyp2d40* basal expression and induction during pregnancy
Liver tissues were collected from *Hnf4 α (wt/wt)* and *Hnf4 α (-/-)* mice at pre-pregnancy (P0), 17 days of pregnancy (P17), and 7 days post-partum (PP7). mRNA levels of *Cyp2d40* were determined by qRT-PCR and normalized by that of *Hnf4 α (wt/wt)* mice at P0 (n=4, mean \pm S.D.; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant).



- | | |
|----------------|-------------------------|
| 1. CYP2D6 | AGCAGAGGGCAAAGGCCATCATC |
| 2. Cyp2d40 | GGCAGAGGACAAAGGGCAACCTG |
| 3. Cyp2d40 mut | GGCAGATTGACAATTCACACCTG |

Figure 4. HNF4 α physically interacts with the putative HNF4 binding site in Cyp2d40 promoter Recombinant HNF4 α protein (R, 0.2 μ g) or nuclear proteins (5 μ g) prepared from HEK293T cells transfected with HNF4 α expression vector (H) or empty control vector (E) were incubated with 32 P-labeled DNA probes harboring putative HNF4 α *cis*-element of Cyp2d40 or the known HNF4 α binding sequence of CYP2D6, in the presence or absence of HNF4 α antibody or indicated unlabeled DNA probes as competitors (in 50-fold excess). The mixture was resolved on non-denaturing gel. The lower arrow indicates the location of shifted bands by apparent HNF4 α binding to DNA. The upper arrow indicates the super-shift complex. The probe sequences are shown below the gel image, with the mutated sequence underlined.

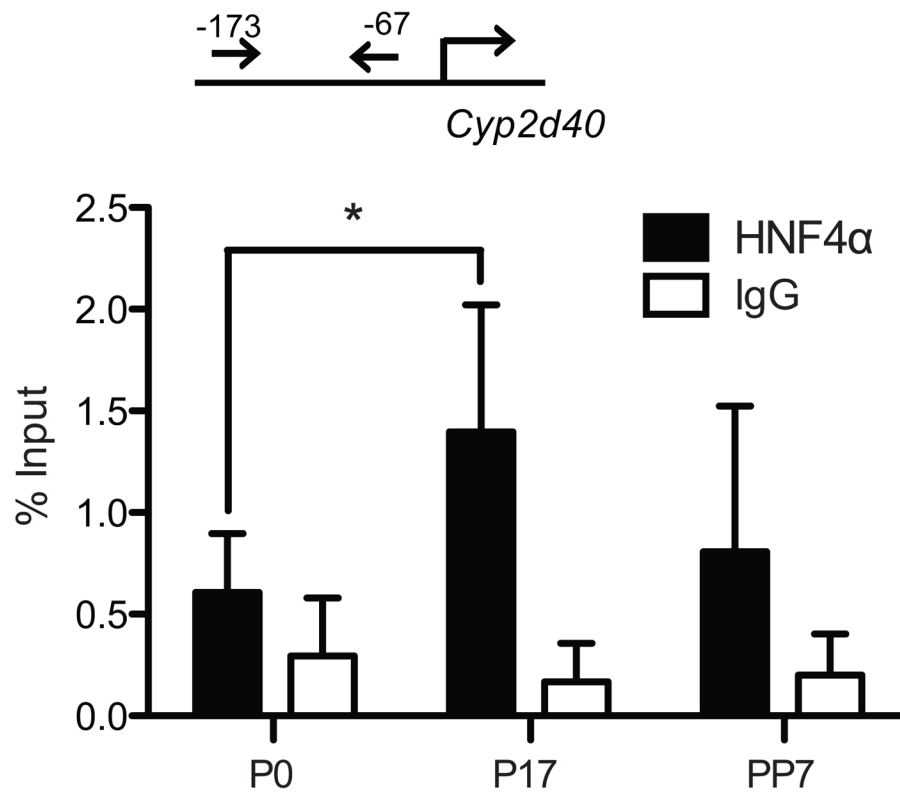


Figure 5. HNF4 α recruitment to *Cyp2d40* promoter increases at term pregnancy
Liver tissues were collected from Tg-CYP2D6 mice at pre-pregnancy (P0), 17 days of pregnancy (P17), and 7 days post-partum (PP7). ChIP assays were performed using HNF4 α antibody (or IgG as a control), and the pulled-down DNA was quantified by qRT-PCR using a set of primers that bind -171/-67 of *Cyp2d40* (n=7, mean \pm S.D.; *, $p < 0.05$).

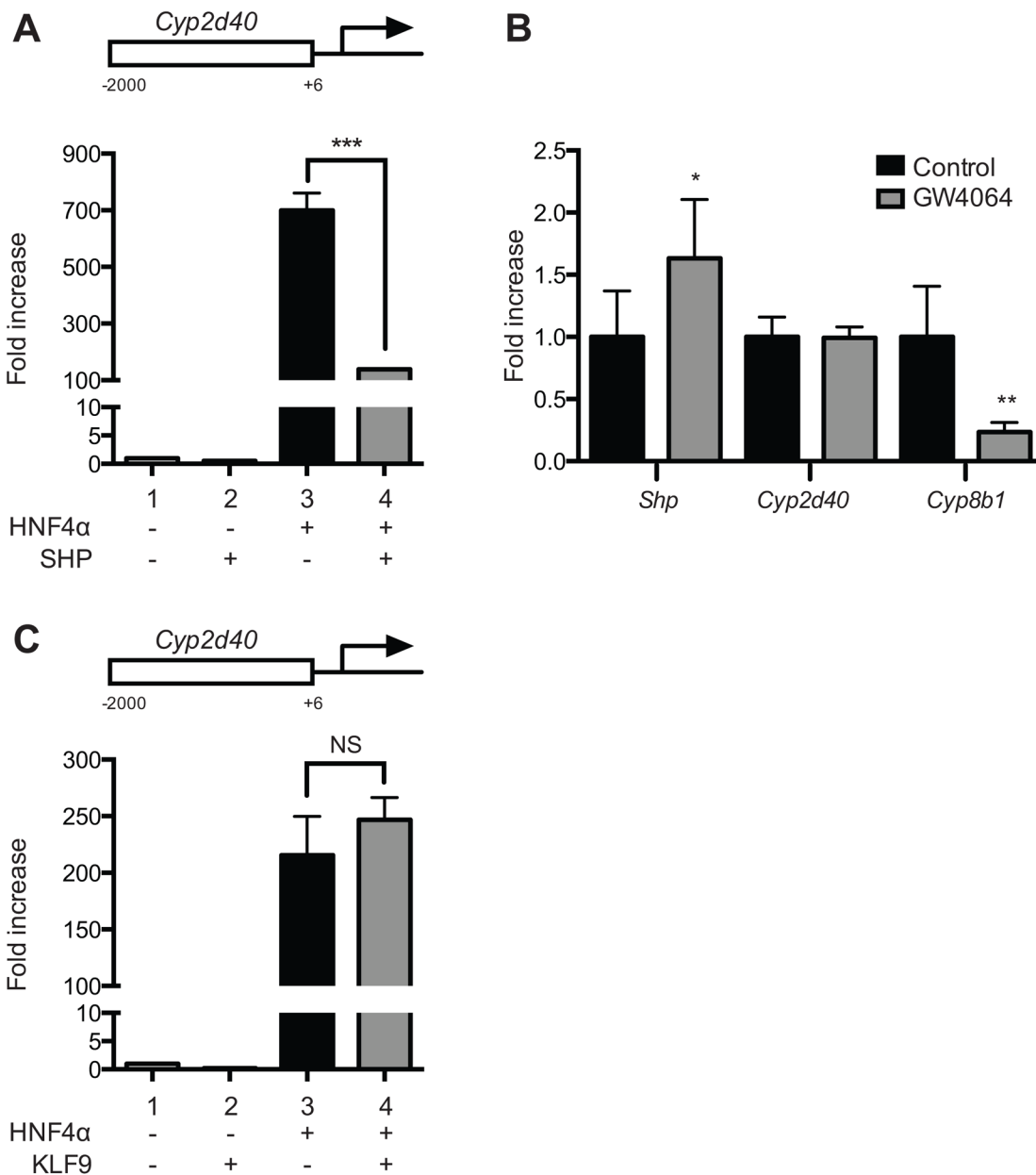


Figure 6. SHP and KLF9 do not modulate HNF4α transactivation of Cyp2d40
 (A) and (C), HEK293T cells were transfected with pGL3-Cyp2d40 with HNF4α and/or SHP expression vectors (A) or KLF9 expression plasmid (C), and dual luciferase assays were performed (n=3, mean ± S.D.). ***, $p < 0.001$; NS, not significant.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

Table 1

Primers used in the study

Cloning primers		
pGL3-Cyp2d40 (-2000/+6)	Forward	5'-ATCGGGTACCCACAGGACTGCAAAGCACTC -3'
	Reverse	5'-ATCGGAGCTCCCACTGCTTCCCAGGCTTCA -3'
pGL3-Cyp2d40 (-1451/+6)	Forward	5'-ATCGGGTACCAGCATCCTGAGACTGTCA -3'
pGL3-Cyp2d40 (-981/+6)	Forward	5'-ATCGGGTACCGGATCCAGCAAGGACATG -3'
pGL3-Cyp2d40 (-471/+6)	Forward	5'-ATCGGGTACCCTCTTCCAATGCCAAGAG -3'
pGL3-Cyp2d40 (-171/+6)	Forward	5'-ATCGGGTACCCTGGCCTGTCTCTACT -3'
pGL3-Cyp2d40 (-86/+6)	Forward	5'-ATCGGGTACCAAGGTGGTAGGATCCAAG -3'
pGL3-Cyp2d40 (-171/+6) HNF4 α site mutation	Forward	[Phos]5'-AATTCACACCTGGACACTCCTTTATAAGG -3'
	Reverse	[Phos]5'-GTCAATCTGCCCAACCCCAATCCT -3'
pGL3-Cyp2d40 (-171/+6) HNF4 α site deletion	Forward	[Phos]5'-ACCTGGACACTCCTTTATAAGG -3'
	Reverse	[Phos]5'-CTGCCCAACCCCAATCCT -3'
qRT-PCR primers		
Shp	Forward	5'-GGCCTCTACCCTCAAGAACAT -3'
	Reverse	5'-TGTC AACGTCTCCCATGATAGG -3'
Gapdh	Forward	5'-AGGTCGGTGTGAACGGATTTG -3'
	Reverse	5'-TGTAGACCATGTAGTTGAGGTCA -3'
ChIP PCR primers		
Cyp2d40	Forward	5'-TCTCTGGCCTGTCTCTACAC -3'
	Reverse	5'-GCTTGATCCTACCACCTTA -3'