Title page

Krüppel-like Factor 9 (KLF9) Promotes hepatic Cytochrome P450 (CYP) 2D6 Expression during Pregnancy in CYP2D6-humanized Mice

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Abbreviations: ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; HNF4α, hepatocyte nuclear factor 4α; KLF9, Krüppel-like factor 9; qRT-PCR, quantitative real time-PCR; SHP, small heterodimer partner;

Abstract

CYP2D6, a major drug-metabolizing enzyme, is responsible for metabolism of approximately 25% of marketed drugs. Clinical evidence indicates that metabolism of CYP2D6 substrates is increased during pregnancy, but the underlying mechanisms remain unclear. To identify transcription factors potentially responsible for CYP2D6 induction during pregnancy, a panel of genes differentially expressed in the livers of pregnant vs. nonpregnant CYP2D6-humanized (tg-*CYP2D6)* mice was compiled via microarray experiments followed by qRT-PCR verification. As a result, seven transcription factors (ATF5, EGR1, FOXA3, JUNB, KLF9, KLF10, and REV-ERBα) were found to be upregulated in liver during pregnancy. Results from transient transfection and promoter reporter gene assays indicate that KLF9 itself is a weak transactivator of CYP2D6 promoter but significantly enhances CYP2D6 promoter transactivation by HNF4α, a known transcriptional activator of CYP2D6 expression. The results from deletion and mutation analysis of CYP2D6 promoter activity identified a KLF9 putative binding motif at -22/-14 region to be critical in the potentiation of HNF4α-induced transactivation of CYP2D6. Electrophoretic mobility shift assays revealed a direct binding of KLF9 to the putative KLF binding motif. Results from chromatin immunoprecipitation assay showed increased recruitment of KLF9 to CYP2D6 promoter in the livers of tg-*CYP2D6* mice during pregnancy. Taken together, our data suggest that increased KLF9 expression is in part responsible for CYP2D6 induction during pregnancy via the potentiation of HNF4α transactivation of CYP2D6.

Introduction

Cytochrome P450 (CYP) 2D6 is a major drug-metabolizing enzyme expressed in the liver and extrahepatic organs (such as the brain, kidney, and intestine) and mediates the hepatic metabolism of approximately 25% of marketed drugs including antidepressants and antipsychotics (Yu et al., 2004; Zanger et al., 2004). In addition to metabolizing drugs, CYP2D6 is implicated in development of Parkinson's disease in Caucasian populations with decreased CYP2D6 activity (Lu et al., 2014). Also, decreased CYP2D6 activity levels in the brain are associated with higher perfusion levels in the regions linked to alertness or serotonergic function, suggesting a functional role of CYP2D6 in the brain (Kirchheiner et al., 2011). However, factors governing the regulation of CYP2D6 expression have been studied to only a limited extent. For example, pregnancy is known to induce hepatic elimination of CYP2D6 substrates (Hogstedt et al., 1985; Tracy et al., 2005; Wadelius et al., 1997). The underlying molecular mechanisms remain unclear, in part due to our lack of understanding of the transcriptional regulation of CYP2D6.

Hepatocyte nuclear factor (HNF) 4α is a transcription regulator of CYP2D6 expression (Cairns et al., 1996; Kharitonenkov et al., 2005; Lemberger et al., 1996). In HepG2 cells, ectopically expressed HNF4α enhances promoter activity of CYP2D6 by its binding to a promoter region (i.e., -53/-41 of CYP2D6) (Cairns et al., 1996). The knock-down of HNF4α expression significantly decreases the transcription of CYP2D6 (Corchero et al., 2001; Kharitonenkov et al., 2005). HNF4α harboring the G60D polymorphism is unable to transactivate CYP2D6 promoter activity in HEK293 cells, and it is associated with decreased metabolism of a CYP2D6 substrate in humans (Lee et al., 2008). These studies indicate key roles of HNF4α in CYP2D6 regulation. Of note, HNF4α controls constitutive expression of many genes involved in basic hepatic functions (e.g., nutrient metabolism and blood clotting), being a master regulator of hepatic genes (Gonzalez, 2008). This suggests that a mechanism that "fine-tune" HNF4α action on different HNF4α-target genes probably exists. The identities of such putative mechanisms remain unknown.

Krüppel-like factor (KLF) 9 is a member in the KLF transcription factor family, and it is capable of either activating or repressing target gene expression in a promoter-specific context. KLF9 is involved in various physiological functions. KLF9 modulates signaling pathways involving progesterone receptor (PR) (Zhang et al., 2003) such that Klf9-null female mice exhibit defects in the reproductive system (e.g., uterine hypoplasia and decreased litter size) (Simmen et al., 2004). In epidermis, KLF9 mediates the proliferation of keratinocytes by cortisol (Sporl et al., 2012). Biological actions of KLF9 are mediated either by its direct binding to the promoters of its target genes such as CYP1A1 (Imataka et al., 1992) or by coactivation of other transcription factors. In endometrial cells, KLF9 binds to PR and enhances transcriptional activation of PR target genes (Zhang et al., 2002; Zhang et al., 2003).

Previously, we recapitulated CYP2D6 induction during pregnancy in an animal model (Koh et al., 2014). In tg-*CYP2D6* mice whose genome harbors the human CYP2D6 gene plus 2.5-kb of its upstream regulatory region, CYP2D6 expression was significantly enhanced at term pregnancy. This was accompanied by increased recruitment of HNF4α to the promoter of CYP2D6 (Koh et al., 2014). The enhanced HNF4α activity during pregnancy was attributed in part to the decreased expression of small heterodimer partner (SHP) (Koh et al., 2014), a corepressor that inhibits HNF4α activity via physical interaction (Zhou et al., 2010). Interestingly, our results showed that other target genes of HNF4α (such as Hes6 and ApoC2) did not exhibit similar increases in expression to CYP2D6 during pregnancy in tg-*CYP2D6* mice (Koh et al., 2014). These results suggest a potential role of promoter contexts in modulating HNF4α transactivation of its target genes and the presence of additional regulatory factors potentially involved in CYP2D6 regulation during pregnancy.

In this study, we show that KLF9 expression is upregulated during pregnancy in the livers of tg-*CYP2D6* mice. The roles of KLF9 in HNF4α transactivation of the CYP2D6 promoter as well as in CYP2D6 induction during pregnancy were investigated. Our results illustrate the interplay among hepatic transcription factors KLF9, HNF4α, and SHP contributing to the regulation of CYP2D6 expression during pregnancy.

Materials and Methods

Animals — tg-*CYP2D6* mice were previously described (Corchero et al., 2001). Adult female (8 weeks old) mice were mated with male mice of a similar age. Mating between adult mice was confirmed by the presence of vaginal plugs (day 0). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago.

Plasmids — Luciferase vectors harboring upstream regulatory regions of CYP2D6 were previously described (Koh et al., 2014). pGL3-CYP2D6 mutation constructs [i.e., mutations at K1 to K5 sites (see Fig. 3B for their locations), using pGL3-CYP2D6 (-205/+90) as a template] were made using a QuikChange XL site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. The primer sequences are listed in supplemental Table 1. Expression vectors for human REV-ERBα, KLF10, and FOXA3 were purchased from Thermo Scientific (Hanover Park, IL). An expression vector for KLF9 was purchased from Thermo Scientific and subcloned into pCMV-Sport6 vector. pcDNA3.1-ATF5 and pcDNA-JUNB were gifts from Drs. Nathalie Wong (the Chinese University of Hong Kong) and Jeremy J.W. Chen (National Chung-Hsing University), respectively. pcDNA3.1-his-EGR1 and pcDNA3-HNF4α (the longest isoform b) were received from Drs. Masahiko Negishi (NIEHS) and Frances M. Sladek (University of California Riverside), respectively. CYP2D6 promoter vector harboring mutated HNF4α response element was provided by Dr. H. Hara (Gifu Pharmaceutical University, Gifu, Japan).

Western blot — KLF9 protein expression levels were determined by using an antibody from Santa Cruz (sc-12996).

RNA isolation and quantitative real time-PCR (qRT-PCR) — Total RNA was isolated from mouse liver tissues using Trizol (Life Technologies, Carlsbad, CA) and converted to cDNA using High Capacity cDNA Archive Kit (Life Technologies). Resulting cDNA samples were subject to qRT-PCR analysis using StepOnePlus Real-Time PCR System and primers listed in supplemental Table 1. The fold increase in mRNA levels during pregnancy was determined after normalizing the gene expression levels to those of mouse β-actin ($2^{-\Delta\Delta Ct}$ method).

Microarray and promoter analysis — Total RNA was isolated from mouse liver tissues at pre-pregnancy (virgin), at term (21 days of pregnancy), and 7 days postpartum (PP7, n=2/time point) using Trizol. cDNA synthesis, modification, hybridization, and labeling on Affymetrix GeneChip MG430 2.0 arrays were performed using kits from Affymetrix (Santa Clara, CA) as described in the manufacturer's instructions. The raw microarray data were quantile normalized and summarized using Affymetrix Power Tools (GEO database: GSE50166). The LPE (local-pooled-error) method (Jain et al., 2003), implemented in the R Statistical Packages, was used to identify differentially expressed genes, considering its efficiency with a small number of replicated arrays. False discovery rate was controlled at 1% using rank-invariant resampling based estimation (Jain et al., 2005). The relative change in expression (fold change > 2.0 or 1.5) was used to obtain a list of differentially expressed genes at term pregnancy and postpartum, compared to the control group (virgin). The prediction of putative transcription factor binding sites in the CYP2D6 promoter was performed using the MatInspector software (Genomatix, Munich, Germany).

Luciferase reporter assay — HepG2 or HEK293T cells were seeded in 12-well plates at a density of 4.5×10^5 cells/ml, and on the next day, transfected with 0.3 µg of luciferase construct, 0.1 µg of expression plasmid (or empty vector as a control), and 0.002 µg of *Renilla* expression vector (Promega) using Fugene HD transfection reagent (Promega) according to the manufacturer's protocol. The transfected cells were grown for 48 hr and harvested for determination of luciferase activities using a luciferase assay kit (Promega). At least two independent experiments were performed in triplicate.

Co-immunoprecipitation assay — HEK293T cells were co-transfected with expression vectors for 3xFLAG-tagged KLF9 and HNF4α, and lysed in lysis buffer (0.5% Nonidet P-40, 50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.25% Triton X 100, and protease inhibitor cocktail) and centrifuged at 13000 rpm for 30 min. The resulting supernatant was incubated overnight with magnetic beads (Dynabeads protein-A, Life Technologies) coated with HNF4α (Santa Cruz Biotechnology) or FLAG-M2 antibody (Sigma) at 4°C. The precipitated proteins were eluted in SDS-PAGE sample buffer, and subject to SDS-PAGE and western blot.

Chromatin immunoprecipitation (ChIP) assays — ChIP assays were performed as previously described with minor modifications (Fang et al., 2007). Briefly, livers were finely minced and incubated in PBS containing 1% formaldehyde at room temperature for 15 min, and glycine was added to stop the crosslinking reaction. Cell pellets were resuspended in hypotonic buffer (15 mM HEPES, pH 7.9, 60 mM KCl, 2 mM EDTA, 0.5% BSA, 0.15 mM spermine, 0.5 mM spermidine, 0.32 M sucrose) and lysed by homogenization. Nuclei were pelleted and resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% SDS). The samples were sonicated to shear DNA to the length ranging from 100 to 500 bp. After centrifugation, the chromatin sample was immunoprecipitated overnight with magnetic beads (Life Technologies) coated with KLF9 antibody (Santa Cruz Biotechnology, sc-12996x) or immunoglobulin G (IgG) at 4°C. The immune complexes were collected, and the magnetic beads were extensively washed, followed by elution of bound chromatin. Genomic DNA was purified by PCR Clean-up kit (Promega) and used as a template for qPCR. Primer sequences are listed in supplemental Table 1.

Electrophoretic mobility shift assay (EMSA) — EMSAs were performed following the manufacturer's protocol (Gel Shift Assay Systems, Promega). Briefly, nuclear extracts were prepared from HEK293T cells transiently transfected with KLF9 or empty vector (as a negative control) using the CelLytic Nuclear Extraction Kit (Sigma). A [^{32}P] dATP-labeled double stranded oligonucleotide (supplemental Table 1) was incubated with nuclear extract (20 μg) at room temperature in the presence or absence of nonradioactive DNA duplex probe (for competition) or KLF9 antibody (Santa Cruz Biotechnology). The reaction mixture was resolved on a native 4% (w/v) polyacrylamide gel. The signal was visualized by using PhosphorImager.

Statistical analysis — For comparison of two groups, statistical differences were determined by the Student's t-test. For statistical testing of more than two groups, one-way analysis of variance test followed by posthoc Dunnett's test was performed.

Results

KLF9 expression is enhanced during pregnancy — To identify potential transcription factors involved in CYP2D6 regulation during pregnancy, microarray gene expression profiling was performed using RNA samples extracted from the livers of tg-*CYP2D6* mice at various gestational time points (n=2/group): virgin, 21 days of pregnancy (G21), and 7 days postpartum (PP7). The results showed that 555 genes were upregulated at G21 (vs. virgin) and/or downregulated at PP7 (vs. G21), and 239 genes showed the opposite pattern (supplemental Tables 2 and 3; GSE50166). Subsequent qRT-PCR analyses (additional time points including virgin, G7/14/21, and PP7, n=4/group) led to the identification of seven upregulated genes (i.e., ATF5, EGR1, FOXA3, JUNB, KLF9, KLF10, and REV-ERBα) (Fig. 1). SHP was found to be down-regulated during pregnancy as we reported in a recent study (Koh et al., 2014).

KLF9 potentiates HNF4α-mediated transactivation of CYP2D6 — To examine the functional impact of differentially regulated transcription factors on CYP2D6 transactivation, reporter gene assays were performed using a luciferase vector where *luc* expression is driven by 2.5-kb CYP2D6 promoter (i.e., pGL3-CYP2D6). HNF4α, a positive regulator of basal CYP2D6 expression (Cairns et al., 1996; Corchero et al., 2001; Lee et al., 2008), was included as a positive control and led to a marked increase in CYP2D6 promoter as expected (Fig. 2A). None of the transcription factors upregulated during pregnancy was found to directly modulate CYP2D6 promoter activity in a statistically significant manner. Although statistically insignificant, KLF9 appeared to enhance the CYP2D6 promoter activity (Fig. 2A).

Given the key role that HNF4α plays in CYP2D6 induction during pregnancy (Koh et al., 2014) and the evidence that HNF4α activity is functionally modulated by other transcription factors (Gonzalez, 2008), we examined whether the differentially expressed transcription factors affect HNF4α-mediated transactivation of CYP2D6 promoter. Our group has recently reported that SHP functions as a negative modulator of HNF4α action on CYP2D6 promoter (Koh et al., 2014). Indeed, SHP repressed the HNF4α-induced transactivation of CYP2D6 promoter as previously shown (Fig. 2B). Among the seven transcription factors tested, KLF9 exhibited the most prominent effect in enhancing HNF4α-induced transactivation of the CYP2D6 promoter (Fig. 2B); the co-expression of KLF9 and HNF4α led to a 304-

fold increase in CYP2D6 promoter activity while 7- and 69-fold increases in the promoter activity were shown in cells transfected with KLF9 or HNF4α alone, respectively. The rest of the transcription factors exhibited insignificant or minor effects on HNF4α activity.

Proximal regulatory region of CYP2D6 mediates the KLF9 action on CYP2D6 promoter — To map the *cis*-elements responsible for the potentiating effect of KLF9 on HNF4α-mediated CYP2D6 transactivation, promoter reporter gene assays were performed using 5′-deletion constructs of CYP2D6 promoter. The four deletion constructs of CYP2D6 promoter retained the potentiating effect of KLF9 on HNF4α-induced CYP2D6 promoter transactivation although the extent of potentiation slightly varied among the constructs (Fig. 3A). Given that 5′-deletion of the CYP2D6 regulatory region up to -94 did not abolish the potentiating effect of KLF9 on HNF4α-induced transactivation of CYP2D6 promoter, we further explored potential *cis*-element(s) responsible for KLF9 action within the proximal 94-bp region of the CYP2D6 promoter. *In silico* analysis of the proximal region (-100/+90) of CYP2D6 using MatInspector (Genomatix, Munich, Germany) revealed multiple putative binding sites (K1 to K5) for KLF9 (Fig. 3B) and the previously reported HNF4α response element (Cairns et al., 1996).

Prior to examining the functional importance of putative KLF9 binding sites, we confirmed the role of the HNF4α response element in mediating the KLF9 effect on CYP2D6 promoter activity. Reporter gene assays were performed using CYP2D6 promoter vectors harboring wild-type or mutated HNF4α response element sequences. The mutation of the HNF4α response element abrogated not only HNF4α-induced transactivation of CYP2D6 promoter activity, but also the potentiating effects of KLF9 (Fig. 3C, lane 2 vs. 8), indicating that KLF9 action on CYP2D6 promoter activity requires HNF4α binding to the DNA.

Next, reporter assays were performed using promoter constructs harboring mutated sequences at the putative KLF9 binding sites. Overall, mutations at putative KLF9 binding sites other than the K2 position did not have a major impact on the potentiating effect of KLF9 on the transactivation of CYP2D6 promoter activity (Fig. 3D, lane 3 vs. 7, 11, 15, 19, or 23). Mutations at the K2 position (mK2)

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substantially attenuated HNF4α transactivation of CYP2D6 promoter activity in KLF9-cotransfected cells (Fig. 3D, lane 4 vs. 12).

KLF9 binds to CYP2D6 promoter — The importance of the K2 site was further validated by examining its interaction with KLF9 via EMSA. Nuclear extracts were prepared from HEK293T cells transfected with a KLF9 expression vector (or empty vector as control) and incubated with radiolabeled K2 probe. Results from the KLF9 consensus sequence (a positive control) identified a band shift likely arising from KLF9 binding to the probe (Fig. 4A). The signal of this band was successfully competed away by the non-radiolabeled probe and blocked by KLF9 antibody (Fig. 4A, lanes 4 and 5). Similarly, the K2 probe exhibited a shifted band that can be competed away by the non-radiolabeled probe with intact K2 sequence (Fig. 4A, lanes 8 and 9). Signal of the band did not decrease when the non-radiolabeled probe contained the mutated K2 sequence (Fig. 4A, lane 10). Addition of KLF9 antibody to the mixture decreased the signal of shifted band (Fig. 4A, lane 11). Similar results were obtained when nuclear extracts were prepared from HEK293T cells transfected with 3xFLAG-tagged KLF9 (Fig. 4B). Together, these data support that KLF9 binds to the K2 site of CYP2D6 promoter.

KLF9 does not physically interact with HNF4α — To examine whether KLF9 and HNF4α physically interact with each other for the synergistic activation of transcription from the CYP2D6 promoter, co-immunoprecipitation assays were performed using 3xFLAG-tagged KLF9. First, we verified that the FLAG-tagging of KLF9 did not interfere with the ability of KLF9 to potentiate the HNF4α transactivation of the CYP2D6 promoter activity using promoter reporter gene assays (Fig. 5A). To examine physical interaction between HNF4α and KLF9, HNF4α and 3xFLAG-KLF9 were ectopically expressed in HEK283T cells and then immunoprecipitation was performed using antibodies against HNF4α and FLAG, respectively. The pulled-down proteins were detected using western blot. The results showed a lack of KLF9 in proteins pulled down by HNF4α (or vice versa) (Fig. 5B), indicating that KLF9 and HNF4α do not directly bind to each other.

KLF9 functionally interacts with SHP — We have previously shown that SHP represses CYP2D6 promoter activity by inhibiting HNF4α-mediated transactivation and that SHP expression decreases during pregnancy (Koh et al., 2014). The current study, on the other hand, shows that KLF9 expression increases in mouse livers during pregnancy. Given that both KLF9 and SHP exert their actions on the CYP2D6 promoter via modulating HNF4α activity, we examined whether KLF9 and SHP interact functionally using co-transfection and promoter reporter gene assays. Additional expression of KLF9 enhanced CYP2D6 promoter activity as compared to the cells transfected with SHP and HNF4α (Fig. 6, lane 5 vs. 6). The potentiating effect of KLF9 was greater when cells were not transfected with SHP (Fig. 6, lane 6 vs. 7). This suggests the possibility that KLF9 may efficiently potentiate the HNF4α transactivation of CYP2D6 promoter activity during pregnancy due to the concomitant decline in SHP expression. The functional interplay among SHP, KLF9, and HNF4α was abrogated by the mutation of the HNF4α-binding response element in the CYP2D6 promoter (data not shown), indicating the key role of HNF4α in KLF9- and SHP-mediated regulation of CYP2D6 expression.

KLF9 recruitment to CYP2D6 promoter increases during pregnancy — Previously, we have shown that HNF4α recruitment to CYP2D6 promoter increases in liver at term pregnancy as compared to the pre-pregnancy level (Koh et al., 2014). To determine whether increased KLF9 expression contributes to the enhanced CYP2D6 expression during pregnancy, KLF9 recruitment to the CYP2D6 promoter was examined in tg-*CYP2D6* mouse liver tissues collected at different gestational time points. The increased KLF9 expression was first verified by using western blot (Fig. 7A). The ChIP results showed that KLF9 recruitment to the CYP2D6 promoter encompassing the K2 site increased during pregnancy and then returned to the pre-pregnancy levels after delivery, while KLF9 was not recruited to distal CYP2D6 genomic region without KLF9 binding site (Fig. 7B). We further examined whether the increased KLF9 expression during pregnancy can also influence other target genes. KLF9 is known to transrepress Cyp1a1 promoter via direct KLF9 binding to the promoter (Imataka et al., 1992). As expected, our result showed that KLF9 recruitment to Cyp1a1 promoter was significantly increased at term pregnancy (Fig.

7C), and this led to decreased Cyp1a1 expression (Fig. 7D). We also examined the recruitment of KLF9 recruitment to the promoters of previously known HNF4α target genes, Hes6 and ApoC2. While KLF9 recruitment to ApoC2 promoter was minimal (data not shown), KLF9 was unexpectedly found to be enriched on the promoter of Hes6 (Fig. 7E; KLF9/IgG signal ratio > 1), indicating the presence of sequences for direct or indirect KLF9 binding in Hes6 promoter. Interestingly, however, the extent of KLF9 recruitment to the promoter did not differ among different time points (Fig. 7E).

Discussion

CYP2D6-mediated drug metabolism is increased during pregnancy, but the underlying mechanisms remain to be fully elucidated. In our previous study, using tg-*CYP2D6* mice as an *in vivo* model, we recapitulated CYP2D6 induction during pregnancy and established the key role of HNF4α in CYP2D6 induction during pregnancy (Koh et al., 2014). In this study, we report that KLF9 is a novel regulator of CYP2D6 expression, enhancing HNF4α-mediated transcription from the CYP2D6 promoter and potentially contributing to CYP2D6 induction during pregnancy.

Results from our previous study suggest that pregnancy enhances HNF4α transactivation in a gene-specific manner (Koh et al., 2014). For example, while mRNA level of CYP2D6 and HNF4α recruitment to CYP2D6 promoter were increased during pregnancy, these did not occur to other HNF4α target genes (e.g., Hes6). In our current study, we searched for transcription factors that may be involved in CYP2D6 regulation by "fine-tuning" HNF4α activity. In tg-*CYP2D6* mice, we found that expression of seven hepatic transcription factors (including KLF9) increases during pregnancy as compared to pre-pregnancy or after delivery. The identified transcription factors modulated CYP2D6 promoter activity to a minor extent when tested individually. However, when their effects on HNF4α-mediated transactivation of CYP2D6 promoter were examined, KLF9 was found to significantly enhance the HNF4α action on CYP2D6 promoter activity, indicating functional synergy between KLF9 and HNF4α. Results from deletion and mutation assays indicate that direct binding of KLF9 and HNF4α to the proximal region of

the CYP2D6 promoter likely contributes to the functional interactions between two transcription factors. Importantly, the HNF4α response element located at -53/-41 and KLF9-binding site at -22/-14 (i.e., K2) were found to play a key role. However, direct physical interaction between HNF4α and KLF9 appears unnecessary for this functional synergy based on the results from co-immunoprecipitation assays. Intermediary coregulators may be involved in the functional interaction between KLF9 and HNF4α. For example, a recent study showed that GATA4 forms a complex with KLF9 and HNF4α, and this interaction is important for activating genes involved in thyroid hormone homeostasis (Ohguchi et al., 2008). Taken together, our study revealed a novel role for KLF9 that it regulates CYP2D6 expression by enhancing HNF4α transactivation of CYP2D6 promoter activity.

What causes the upregulation of KLF9 during pregnancy remains unclear. Previous studies have shown that cortisol induces KLF9 mRNA expression in keratinocytes and the hippocampus (Bagamasbad et al., 2012; Sporl et al., 2012). Additionally, in developing rat brain, thyroid hormone upregulates KLF9, increasing the number and length of neurites (Denver et al., 1999). During pregnancy, it is shown that the plasma concentrations of free cortisol and thyroid hormones increase 1.5- to 2-fold by the third trimester as compared to the pre-pregnancy levels (Jung et al., 2011; Soldin et al., 2004). Our initial investigations using primary human hepatocytes showed that cortisol or thyroid hormone did not alter CYP2D6 expression (data not shown). In line with these findings, there is no clinical report on drug-drug interactions between corticosteroids (or thyroid hormones) and CYP2D6 substrates to date. Currently, we cannot rule out a possibility that these results are affected by as-yet-unknown, inherent limitations of primary hepatocytes as a model to study CYP2D6 regulation. Further investigations would be necessary to determine the role of rising concentrations of cortisol and thyroid hormone in CYP2D6 induction during pregnancy.

Previous studies have shown that growth factors functionally modulate KLFs by triggering post-translational modification or cytoplasm-to-nucleus shuttling of KLFs (Daftary et al., 2012; Lomberk and Urrutia, 2005). For example, the phosphorylation of KLF16 (one of the KLF family members) modulates

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its transcriptional activity assessed by reporter gene expression, and certain serum factors are shown to significantly decrease the nuclear translocation of KLF16 and its transcriptional activity (Daftary et al., 2012). Also, mutations of serines to alanines in the KLF9 protein were shown to significantly increase KLF9 transactivation of a target gene promoter (Pei et al., 2011), suggesting that phosphorylation of KLF9 may govern functionality of KLF9. During pregnancy, multiple physiological changes occur along with alterations in plasma concentrations of various hormones and growth factors. For example, placenta growth factor is a vascular endothelial growth factor uniquely produced by placenta, and its levels increase gradually over the course of pregnancy (Tjoa et al., 2001). Whether any of these pregnancy-specific growth factors could modulate KLF9 activity and subsequently CYP2D6 expression during pregnancy remains to be determined.

We have previously shown that SHP is a repressor of HNF4α transactivation of CYP2D6 promoter activity, and pregnancy is accompanied by decreased expression of SHP (Koh et al., 2014). Considering that both SHP and KLF9 modulate HNF4α activity, we examined whether any crosstalk exists between SHP and KLF9 for HNF4α-induced transactivation of CYP2D6. Results from our study indicate that the changes in expression of SHP and KLF9 (i.e., decreased and increased expression, respectively) lead to synergistic HNF4α-induced transactivation of CYP2D6 promoter (Fig. 8). This interplay may potentially account for the differential regulation of HNF4α target genes during pregnancy. Possibly, CYP2D6 expression is activated by synergistic functional interaction among SHP, KLF9 and HNF4α. This functional interaction appears to occur in a gene-specific manner during pregnancy. For example, we found Hes6 to be another gene where both KLF9 and HNF4α are enriched at its promoter; however, despite the increased expression of KLF9 at term pregnancy, KLF9 recruitment at the Hes6 promoter did not change during pregnancy. The underlying mechanisms for gene-specific interplay among KLF9, SHP, and HNF4α warrant further investigation.

In conclusion, using CYP2D6-humanized mice, we identified KLF9 as a novel regulator of CYP2D6 expression during pregnancy, providing mechanistic insight into CYP2D6 induction during pregnancy.

These results may provide a basis to improve drug therapy during pregnancy. Also, considering the roles of CYP2D6 in the pathophysiology of neurological diseases (in addition to drug metabolism), better understanding of the regulation of CYP2D6 expression may allow for the identification of new drug targets. In this regard, whether the regulatory pathways involving KLF9, SHP, and HNF4α play a role in governing CYP2D6 expression in non-pregnant subjects is currently being investigated.

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Authorship Contributions

Participated in research design: Koh, Pan, McLachlan, and Jeong

Conducted experiments: Koh and Pan

Contributed new reagents or analytic tools: Urrutia

Performed data analysis: Koh, Pan, Zhang, McLachlan, and Jeong

Wrote or contributed to the writing of the manuscript: Koh, Pan, Zhang, McLachlan, Urrutia, and Jeong

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FOOTNOTES

KHK and XP contributed equally to the work.

Figure Legends

Fig. 1. Transcription factors are differentially expressed during pregnancy in the livers of tg-*CYP2D6* mice. Liver tissues were collected from tg-*CYP2D6* mice at pre-pregnancy (virgin), 7/14/21 days of pregnancy (G7, G14, and G21, respectively), or 7 days postpartum (PP7), and total RNA was isolated from the tissues. The mRNA expression levels of the respective transcription factors were determined by qRT-PCR (n=4, mean ± SD; *$p < 0.05$, **$p < 0.01$ vs. virgin).

Fig. 2. KLF9 enhances HNF4α-mediated transactivation of CYP2D6 promoter. (***A***) HEK293T cells were transfected with the pGL3-CYP2D6 vector (-2455/+90), an expression vector for each of the transcription factors (ATF5, EGR1, FOXA3, JUNB, KLF9, KLF10, REV-ERBα, or empty vector), and the pCMV-*Renilla* vector. After 48 hr, dual luciferase assays were performed (n=3, mean ± SD; **$p < 0.01$ vs. control). (***B***) HEK293T cells were transfected with the pGL3-CYP2D6 vector (-2455/+90), HNF4α expression vector plus an expression vector for each of the transcription factors (ATF5, EGR1, FOXA3, JUNB, KLF9, KLF10, REV-ERBα, or empty vector), and the pCMV-*Renilla* vector. After 48 hr, dual luciferase assays were performed (n=3, mean ± SD; *$p < 0.05$, **$p < 0.01$ vs. HNF4α alone).

Fig. 3. KLF9 action on CYP2D6 promoter requires *cis*-element(s) located in -94/+90 of CYP2D6. (***A***) HEK293T cells were transfected with the expression vectors (HNF4α and/or KLF9) and pCMV-*Renilla* vector, along with a 5′-deletion promoter construct harboring -2455/+90, -1068/+90, -205/+90, or -94/+90 of the CYP2D6 gene. After 48 hr, dual luciferase assays were performed (triplicate experiment, mean ± SD). Data shown are relative promoter activities in cells transfected with HNF4α and/or KLF9 vs. cells transfected with empty vector. (***B***) Putative KLF9- (K1 to K5) and HNF4α-binding site are underlined. (***C***) HEK293T cells were transfected with expression vectors (HNF4α and/or KLF9) and pCMV-*Renilla* vector, along with the pGL3-CYP2D6 vector carrying the wild-type (WT; lane 1-4) or mutated HNF4α

response element (lane 5-8). After 48 hr, dual luciferase assays were performed (triplicate experiment, mean ± SD). (*D*) HEK293T cells were transfected with expression vectors (HNF4α and/or KLF9) and pCMV-*Renilla* vector, along with the pGL3-CYP2D6 vector carrying the WT or mutated KLF9 binding sequences (i.e., mK1 to mK5). After 48 hr, dual luciferase assays were performed (triplicate experiment, mean ± SD).

Fig. 4. KLF9 binds to CYP2D6 promoter sequences. Cell lysates from HEK293T cells transiently transfected with KLF9 (*A*) or 3xFLAG-tagged KLF9 vector (*B*) (or empty vector as control) were incubated with ^{32}P-labeled probes harboring a consensus KLF9-binding sequence (left) or CYP2D6/K2 (right). The mixture was resolved on non-denaturing polyacrylamide gel. The lower and upper arrows indicate locations of shifted and super-shifted bands by apparent KLF9 binding to the DNA, respectively. The heavy band at the top likely represents nonspecific binding. The sequences of probes with the putative binding sequences underlined are shown in the bottom panel.

Fig. 5. KLF9 does not bind to HNF4α in vitro. (*A*) HEK293T cells were transfected with the pGL3-CYP2D6 vector (-2455/+90), the expression vectors (HNF4α and/or 3xFLAG-tagged KLF9), and pCMV-*Renilla* vector. After 48 hr, dual luciferase assays were performed (triplicate experiment, mean ± SD; **$p < 0.01$ vs. lane 1). (*B*) HEK293T cells were co-transfected with the 3xFLAG-tagged KLF9 and HNF4α expression vector. Cell lysates were prepared, and immunoprecipitation was performed using magnetic beads coated with antibodies against HNF4α (lane 1) or FLAG (lane 2). Immunoprecipitates were analyzed by western blot. One percent of the input (lane 3) was loaded onto the gel as a control.

Fig. 6. Functional interplay between SHP and KLF9 in regulating HNF4α-induced transactivation of CYP2D6 promoter. HEK293T cells were transfected with pGL3-CYP2D6 (-870/+65), expression

vectors (HNF4α, KLF9, or SHP) along with pCMV-*Renilla*. After 48 hr, luciferase assays were performed (triplicate experiment, mean ± SD; * $p < 0.05$, **$p < 0.01$ vs. lane 1).

Fig. 7. KLF9 recruitment to CYP2D6 promoter increases during pregnancy. Liver tissues were collected from tg-*CYP2D6* mice at pre-pregnancy (virgin), 17 days of pregnancy (G17), or 7 days postpartum (PP7). (*A*) Protein expression levels of the KLF9 and β-actin were determined by western blot ($n=4$, mean ± SD; *$p < 0.05$ vs. virgin). (*B, C and E*) ChIP assays were performed using antibodies against KLF9. KLF9 recruitment to CYP2D6 (*B*), Cyp1a1 (*C*) and Hes 6 (*E*) was examined ($n=4$/time point, mean ± SD; * $p < 0.05$, **$p < 0.01$ vs. virgin). The circle and rectangle in the gene promoters represent KLF9 and HNF4α binding sites, respectively. (*D*) mRNA expression level of Cyp1a1 was determined by qRT-PCR ($n=4$, mean ± SD; **$p < 0.01$ vs. virgin).

Fig. 8. Working model for CYP2D6 induction during pregnancy. The working model illustrates the interplay of SHP and KLF9 in regulating CYP2D6 expression.
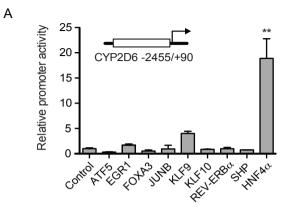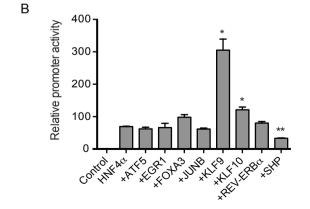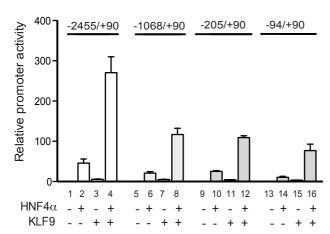
Figure 1

Figure 2

A

CYP2D6 -2455/+90

Figure 3

A

B

-100 5'- CTCTGCCACTGGCAGCACAGTCAACACAGCAGGTTCACTCACAGCAG<u>AGG</u>

-50 <u>GCAAAGGCCA</u><u>T</u><u>CATCAGCTCCCT</u>TTATA<u>AGGGAAGGG</u>TCACGCGCTCGGT
     HNF4α   **K1**   **K2**

+1   GTGCTGAGAGT<u>GTCCTGCCT</u>GGTCCTCTGTGC<u>CTGGTGGGG</u><u>TGGGGGTGC</u>
                K3   **K4**   **K5**

+51 CAGGTGTGTCCAGAGGAGCCCATTTGGTAGTGAGGCAGGT - 3'

C

D

Figure 4

A

B

CYP2D6/K2   CCTTTATAAGG GAAGGG TCACGCG
CYP2D6/mK2   CCTTTATAAGG GATTAT TCACGCG
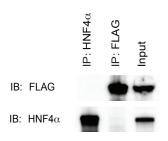Consensus KLF9-binding sequence   AGAGAAG GAGGCGTG GCCAACAGA

Figure 5

A

B

Figure 6

Figure 7

Figure 8