

Effect of Pregnancy on Cytochrome P450 3a and P-Glycoprotein Expression and Activity in the Mouse: Mechanisms, Tissue Specificity, and Time Course

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ABSTRACT

The plasma concentrations of orally administered anti-human immunodeficiency virus protease inhibitors are significantly reduced during human and mouse pregnancy. We have shown that in the mouse, at gestational day 19, this reduction is due to increased hepatic cytochrome P450 3a (Cyp3a) protein expression and activity. In the current study, we investigated the mechanisms by which Cyp3a activity is increased by pregnancy and the time course of change in expression of Cyp3a and P-glycoprotein (P-gp) in various tissues. We found that hepatic transcripts of Cyp3a16, Cyp3a41, and Cyp3a44 were significantly increased during pregnancy, whereas those of Cyp3a11 and Cyp3a25 were significantly decreased. This resulted in a net increase in Cyp3a protein expression and activity in the liver during pregnancy. The increase in Cyp3a41 and

Cyp3a44 transcripts was positively correlated ($p < 0.05$) with hepatocyte nuclear factor 6 and estrogen receptor- α transcripts. The pregnancy-related factors that transcriptionally activated mouse Cyp3a isoforms also activated the human CYP3A4 promoter in pregnant CYP3A4-promoter-luciferase transgenic (CYP3A4-tg) mice. In contrast, intestinal Cyp3a protein expression was not significantly affected by pregnancy. No change in P-gp protein expression was observed in the liver or kidney during pregnancy, although a significant decrease was observed in the placenta. Because hepatic CYP3A activity also seems to be induced during human pregnancy, the mouse (including CYP3A4-tg mouse) seems to be an excellent animal model to determine the molecular mechanisms for such an induction.

To treat the pregnant woman and to prevent maternal-fetal HIV-1 transmission, HIV-1-infected pregnant women are routinely prescribed anti-HIV protease inhibitors as part of their highly active antiretroviral therapy regimen (Mofenson, 2003; Thorne and Newell, 2005). Several studies have indicated that pregnancy considerably reduces the exposure of these women to protease inhibitors (PIs) such as nelfinavir and saquinavir, compared with that of men, nonpregnant women, or postpartum women administered an equivalent dose of the drug (Angel et al., 2001; Acosta et al., 2004; Nellen et al., 2004). This reduction in exposure, as measured by plasma area under the curve or mean/median concentration

ratio, is in the order of 70 to 90% compared with postpartum or nonpregnant controls. Likewise, a perinatal study (PACTG 358) we have conducted in HIV-1-infected pregnant women has shown that the plasma area under the curve of indinavir is 73% lower antepartum (31 weeks) than that observed in the same women 6 weeks postpartum (Unadkat et al., 2007).

The bioavailability and systemic clearance of the PIs are primarily determined by the drug-metabolizing enzymes cytochrome P450 3A4/5 (CYP3A4/5) and the drug efflux transporter P-glycoprotein (P-gp) in the small intestine and liver (Kim et al., 1998; van Heeswijk et al., 2001). Several studies have indicated that pregnancy increases the activity/expression of P-gp and/or CYP3A. Tracy et al. (2005) have reported consistently increased CYP3A activity during all stages of pregnancy as measured by urinary metabolic ratio of dextromethorphan. Likewise, we have found that in pregnant

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ABBREVIATIONS: HIV, human immunodeficiency virus; PI, protease inhibitor; P-gp, P-glycoprotein; GH, growth hormone; TST, testosterone; PXR, pregnane xenobiotic receptor; CAR, constitutive androstane receptor; ER, estrogen receptor; mem PR, membrane progesterone receptor; GhR, growth hormone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; HNF, hepatocyte nuclear factor; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; WT, wild type; tg, transgenic; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGH, placental growth hormone.

women, hepatic and/or intestinal CYP3A activity is increased 2- to 3-fold in late pregnancy compared with postpartum, as demonstrated by reduced exposure to oral midazolam (Hebert et al., 2006). In the same cohort of women, net renal secretion of oral digoxin was significantly increased in pregnant women compared with postpartum, presumably indicating increased renal P-gp activity (Hebert et al., 2006).

To determine the mechanistic basis for the reduction in exposure to anti-HIV PIs during pregnancy, we reproduced this phenomenon in the pregnant mouse (at gestational age day 19) using nelfinavir as our model PI (Mathias et al., 2006). In this animal model, the reduced exposure to nelfinavir during pregnancy was caused by reduced bioavailability as a result of enhanced expression and activity of hepatic Cyp3a enzymes. It is interesting that P-gp protein expression in the small intestine or liver was unaffected by pregnancy. To gain insight into the mechanisms by which Cyp3a (and perhaps P-gp) activity and expression may be increased during pregnancy, we 1) measured the expression of transcriptional factors that may be important in the regulation of these proteins; 2) studied the activity of the CYP3A4-promoter-luciferase activity in tissue of pregnant and nonpregnant transgenic (CYP3A4-tg) mice; and 3) measured the time course of change in activity and/or expression of Cyp3a and *mdr1* (genes that code for P-gp) isoforms in various tissues (liver, kidney, and placenta) of the pregnant mice.

Materials and Methods

Chemicals. Methyl-*tert*-butyl ether (HPLC grade), methanol (HPLC grade), testosterone, 11 α -OH-progesterone, phenyl methyl sulfonyl fluoride and NADPH were purchased from Sigma Chemical (St. Louis, MO). 6 β -OH testosterone was purchased from Steraloids Inc. (Newport, RI). D3-6 β -OH testosterone was purchased from Cerilliant Co. (Round Rock, TX). Potassium phosphate monobasic was purchased from J. T. Baker (Phillipsburg, NJ). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany). BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL). Primary antibodies C219, anti-rat CYP 3A2, and anti- β -actin were purchased from Alexis Biochemicals (San Diego, CA), Daiichi Pure Chemicals (Tokyo, Japan), and Sigma-Aldrich (St. Louis, MO), respectively. Secondary horseradish peroxidase-conjugated anti-mouse, anti-rabbit antibodies were purchased from Bio-Rad Laboratories (Hercules, CA). Hybond-ECL nitrocellulose membrane was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). All other reagents used in the experiments were purchased from Thermo Fisher Scientific (Waltham, MA).

Animals. Adult (8–10 weeks of age) wild-type (WT) FVB mice (Charles Rivers Laboratory, Wilmington, MA) and heterozygous CYP3A4-promoter-luciferase transgenic (CYP3A4-tg) FVB mice (a gift from Xenogen, Alameda, CA) (Zhang et al., 2003) were housed in the specific pathogen-free facility at University of Washington and were cared for in accordance with the Public Health Services policy for the *Guide for the Care and Use of Laboratory Animals*. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Washington. The animal experiment procedure was essentially the same as described previously (Mathias et al., 2006). The date that a vaginal plug was observed was assigned as gestational day 1. At gestational days 10, 15, and 19, WT pregnant mice were sacrificed under anesthesia (pentobarbital at 100 mg/kg i.p.), and then tissues (liver, small intestine, kidney, and placenta) were collected. The small intestine was cut open longitudinally on a cooled plate, cut into two (proximal and distal) pieces, and then the mucosae were removed by gently scraping the luminal surface using a fine tissue culture scraper. Tissues were flash-frozen in liquid N₂

and stored at -80°C until use. Age- and weight-matched nonpregnant female mice were used as controls (gestational day 0). Pregnant (day 17) and nonpregnant transgenic CYP3A4-tg mice were sacrificed, and tissues were collected as indicated above. As a positive control, tissues from CYP3A4-tg mice (2 male mice and 1 pregnant female mouse) were collected 6 h after the administration of a single dose of a Cyp3a inducer, dexamethasone (50 mg/kg i.p.).

S-9 Fraction Isolation and Protein Quantification. Tissue homogenate was prepared as described previously (Mathias et al., 2006). In brief, flash-frozen tissues were thawed on ice and homogenized in a Wheaton homogenizer with 3 to 5 \times volume of homogenization buffer (10 mM KH₂PO₄ and 250 mM sucrose, pH 7.4) containing protease inhibitors. Liver, kidney and placenta homogenates were centrifuged at 10,000g for 30 min at 4 $^{\circ}\text{C}$. Intestinal homogenates were briefly spun (600g for 5 min), and the supernatant was then centrifuged at 10,000g for 30 min at 4 $^{\circ}\text{C}$. Protein concentration of the supernatant (S-9 fraction) was determined by BCA protein assay. The S-9 fractions were stored at -80°C in aliquot until further use.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot. A 2- to 20- μg (for Cyp3a detection) or 40- to 80- μg (for P-gp detection) sample of S-9 fraction was separated by 10% (for Cyp3a) or 4 to 15% (for P-gp) precast Criterion Tris-HCl gel (Bio-Rad Laboratories). Proteins were transferred to Hybond-ECL nitrocellulose membrane (Amersham). Western detection followed the ECL procedure according to the manufacturer's instructions (Amersham). In brief, blots were soaked in PBS buffer for 10 min and then placed in blocking buffer (5% nonfat dry milk, 0.05% Tween 20 in PBS buffer) for 1 h at room temperature. C219 (1/800), anti-rat 3A2 (1/2000), or anti- β -actin antibody (1/5000–10,000) was added to the blocking solution, and incubation was continued for another hour. The blots were then rinsed in wash buffer (0.05% Tween 20 in PBS buffer) and washed twice for 10 min each. The blots were then incubated in blocking buffer with secondary antibodies (1/10,000–20,000 horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG; Bio-Rad Laboratories) for 1 h. The blots were washed in PBS buffer twice for 20 min each. ECL plus reagent (Amersham Biosciences) was then applied to the membrane according to the manufacturer's instructions. Chemiluminescent signal was recorded on an X-Omat Blue XB-1 film (PerkinElmer Life and Analytical Sciences, Rochester, NY). The relative intensity of each protein band was determined using Bio-Rad Chemi-Doc and Quantity One Program by following the manufacturer's instructions.

Cyp3a Activity Assay. Cyp3a activity was measured by minor modification of testosterone (TST) 6 β -hydroxylation assay as described previously (Mathias et al., 2006). S-9 fraction (1–4 mg/ml) was preincubated at 37 $^{\circ}\text{C}$ for 5 min in 100 μl of phosphate buffer (100 mM potassium phosphate, pH 7.4, and 5 mM MgCl₂) containing 150 μM substrate TST. Total organic solvent (methanol) content was <3%. Reaction was initiated by the addition of freshly prepared NADPH (final concentration, 1 mM) and terminated at 30 min by the addition of 100 μl of ice-cold acetonitrile containing 10 μl of internal standard 11 α -OH progesterone (54 $\mu\text{g}/\text{ml}$, HPLC/UV method) or D3-6 β -OH TST (10 $\mu\text{g}/\text{ml}$, LC/MS method). Samples were incubated on ice for 30 min and then were vortex-mixed and centrifuged at 15,000g for 3 min. A 20- μl portion of the supernatant was directly injected onto HPLC/UV or LC/MS.

6 β -OH TST Detection by HPLC/UV. HPLC/UV was modified from a method described previously (Mathias et al., 2006). In brief, separation of the substrate TST, product 6 β -OH TST, and internal standard 11 α -OH progesterone was carried out on an HPLC system consisting of a Shimadzu LC-600 Liquid Chromatograph, interfaced with a Shimadzu SPD-6A UV detector and a Waters 717 auto sampler. The chromatography was performed on an Econosil C-18 reverse phase column (250 \times 4.6 mm, 5 μm ; Alltech Associates, Deerfield, IL) with a guard column (7.5 \times 3.2 mm, 5 μm ; Alltech) at room temperature. Mobile phase consisted of solvent A (30% methanol/2% acetonitrile/68% H₂O) and solvent B (80% methanol/5% acetonitrile/

15% H₂O). Gradient elution at a flow rate of 1 ml/min was programmed from 50 to 100% of solvent B over 14 min. All analytes and the internal standard were detected at $\lambda = 244$ nm. Data were recorded using Waters Empower Pro (Waters Co., Milford, MA) software. Calibrators ranging from 50 to 800 ng of 6 β -OH TST were spiked in the incubation matrix together with the internal standard 11 α -OH progesterone (50 ng). All calibrators and unknown samples were assayed in duplicate. Peak height ratios of the analyte to that of the internal standard were used to arrive at the calibration line and to estimate the concentrations in the unknown and quality control samples. The amount of 6 β -OH TST in unknown samples was determined by linear regression.

6 β -OH TST Detection by LC/MS. To achieve greater sensitivity than that afforded by HPLC/UV, detection of 6 β -OH TST formation in intestinal S-9 incubations was conducted by mass-spectrometry on a Waters 2695 LC module/Micromass platform LCZ4000 using electrospray-positive ionization mode. The chromatography was performed on an Agilent ZORBAX SB-C18, 2.1 \times 150 mm, 5 μ m column with Phenomenex 2.0 \times 4 mm C18 guard column at room temperature (Phenomenex, Torrance, CA). The mobile phase was comprised of methanol and water with 0.1% formic acid. Linear gradient elution at a flow rate 0.3 ml/min was programmed to decrease from 90 to 20% organic over 5.5 min. 6 β -OH TST was detected as selective ion mode at 305.3 *m/z*, and the internal standard D3-6 β -OH TST (50 ng) was detected at 308.0 *m/z*. The calibration curve ranged from 3 to 800 ng.

Real-Time PCR Assay. Total RNA was isolated using RNeasy mini-isolation kit (Qiagen, Valencia, CA). RNA integrity and purity were verified by gel electrophoresis and UV spectrophotometer. cDNA was synthesized from 2 μ g of total RNA that had an absorbance ratio (at 260 and 280 nm) of 1.8 to 2.0, and reverse transcription was performed using TaqMan reverse transcription reagents from Roche Molecular Systems, Inc. (Branchburg, NJ). The reactions were run as follows: 25°C for 10 min, followed by 50°C for 40 min, then 95°C for 5 min. Real-time PCR assay was carried out with the use of gene-specific 5-carboxyfluorescein-labeled fluorescent MGB probes in an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). β -Actin was used as an endogenous control. Taqman primers and probes for mouse genes Cyp3a16 (Mm00655824_m1), Cyp3a41 (Mm00776855_mH), Cyp3a44 (Mm01703325_mH), Cyp3a11 (Mm00731567_m1), Cyp3a13 (Mm01207107_m1), Cyp3a25 (Mm01221297_m1), mdr1a (Mm00440761_m1), mdr1b (Mm00440736_m1), β -actin (Mm00607939_s1), HNF1 (Mm00493437_g1), HNF3 β (Mm01976556_s1), HNF4 α (Mm00433958_m1), HNF6 (Mm00839394_m1), RAR α (Mm01296311_g1), RXR α (Mm00441185), GhR (Mm01303638), PXR (Mm00803095_m1), CAR (Mm01283980_q1), and transcriptional factors aryl hydrocarbon receptor, mem PR, hypoxia-inducible factor 1 α , and ER (assay IDs are listed in Wang et al., 2006) were assay-on-demand gene expression products purchased from Applied Biosystems. The real-time reaction contained 10 μ l of 2 \times TaqMan Universal PCR Master Mix (Applied Biosystems), 20 ng of RNA-equivalent cDNA, and primers (200 nM) and probes (100 nM) in a final volume of 20 μ l. The reactions were conducted as follows: 95°C hot start for 10 min, followed by 40 cycles at 95°C for 15 s and then 60°C for 60 s. Each sample was analyzed in triplicate or quadruplicate. cDNA synthesized from reference pooled RNA samples (mouse liver and intestine) were used as a calibrator. All data were expressed as relative expression with respect to that of the calibrator using the ABI Sequence Detection System (Applied Biosystems). The mRNA levels of each test gene were normalized to β -actin, according to the following formula: $C_T(\text{test gene}) - C_T(\beta\text{-actin}) = \Delta C_T$. Thereafter, the relative mRNA levels of each gene were calculated using the $\Delta\Delta C_T$ method: $\Delta C_T(\text{test gene}) - \Delta C_T(\text{test gene in the calibrator}) = \Delta\Delta C_T(\text{test gene})$. The -fold changes of mRNA levels were represented as a relative expression $2^{-\Delta\Delta C_T}$.

Luciferase Assay. Luciferase activity was determined in tissue homogenate of the liver, proximal small intestine, and kidney of CYP3A4-tg mice. In brief, 30 to 50 mg of tissue was homogenized in 500 μ l of 1 \times passive lysis buffer using an Eppendorf homogenizer.

Then the homogenate was centrifuged at 4°C for 10 min at 20,000*g*. Protein concentration of the supernatant was determined by standard BCA assay protocol, and 20 μ l (3 mg/ml) was used for the luciferase assay. Luciferase experiments were performed in triplicate using LMaxII 384/LMax II microplate readers (Molecular Devices, Toronto, ON, Canada) following the manufacturer's protocol. Luciferase activity was reported as relative light units normalized per milligram of protein.

Statistical Analysis. All data were analyzed using Excel's one-way analysis of variance followed by unpaired Student's *t* test (Microsoft, Redmond, WA). Correlation between mRNA expression of Cyp3a isoforms and transcription factors was determined by the Spearman rank analysis (STATA version 8.0; Stata Corporation, College Station, TX), and expressed as the corresponding correlation coefficient (*r_s*). A *p* value of <0.05 was considered statistically significant.

Results

Changes in Hepatic Cyp3a Activity and Expression (Protein and Transcript) with Gestational Age in WT Mice. Hepatic Cyp3a activity, as measured by testosterone 6 β -hydroxylation, was significantly (*p* < 0.05) increased at gestational days 15 (2.2-fold) and 19 (2.5-fold) compared with nonpregnant animals (Fig. 1A). As expected from the increased activity, hepatic Cyp3a protein expression was also

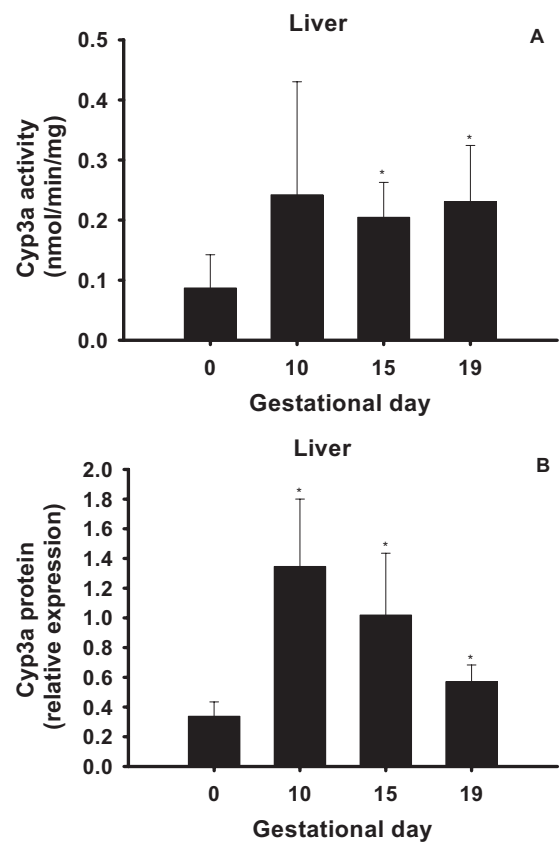


Fig. 1. Hepatic Cyp3a activity and protein expression at different gestational ages in WT mice. A, hepatic S-9 fractions from FVB mice showed significantly greater Cyp3a activity (as measured by testosterone 6 β hydroxylation) at gestational days 15 and 19 compared with that in nonpregnant female mice. B, quantification of the Western blots of hepatic S-9 fractions isolated from FVB mice showed significantly greater expression of Cyp3a at gestational days 10, 15, and 19 compared with that in nonpregnant female mice. Data are shown as mean \pm S.D. (*n* = 5). *, *p* < 0.05.

significantly ($p < 0.05$) increased at gestational days 10, 15, and 19 compared with nonpregnant mice, with the maximum increase (4-fold) occurring at gestational day 10 (Fig. 1B). Similar to findings in our previous report (Mathias et al., 2006), a modest but significant ($p < 0.05$) 2-fold increase in hepatic Cyp3a expression was also observed at gestational day 19.

Because there are multiple mouse Cyp3a isoforms with high sequence similarity (Sakuma et al., 2002), and there are no isoform-specific antibodies for Cyp3a available, it is not possible to differentiate from the Western blot the extent to which pregnancy affects the expression of each isoform. Therefore, we quantified the mRNA expression of each individual isoform of Cyp3a using real-time PCR. The effect of pregnancy on transcript levels of hepatic Cyp3a isoforms was dichotomous. Transcript expression of Cyp3a44 (a female-specific isoform) and Cyp3a16 (a developmentally regulated isoform) is significantly increased 3- and 2-fold and remained constant at all gestational ages (Fig. 2A). However, for another female-specific isoform, Cyp3a41, a significant increase in its hepatic transcript expression was observed at gestational day 10 and 15, but not at day 19 (Fig. 2A). The level of expression of Cyp3a44 transcript was highest at gestational

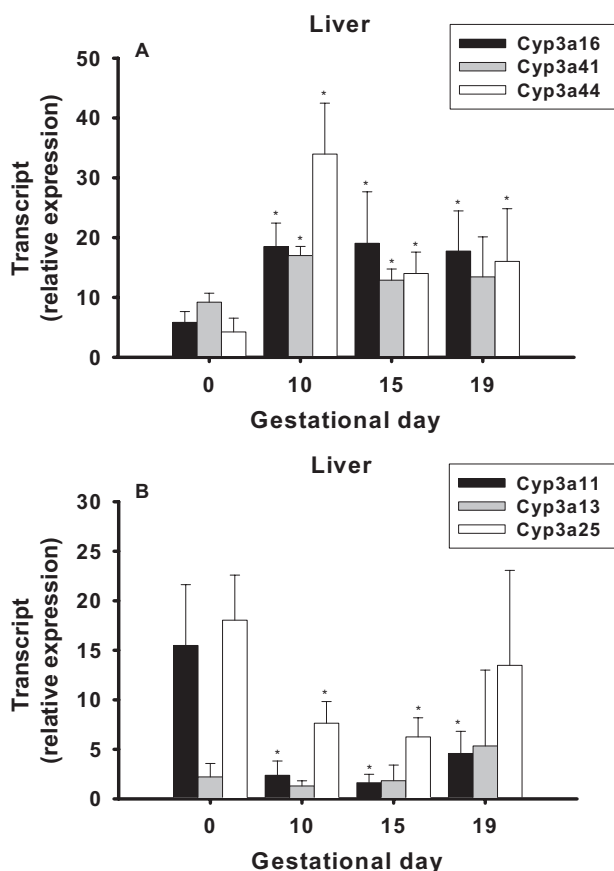


Fig. 2. Hepatic Cyp3a transcript expression by real-time PCR at different gestational ages in WT mice. A, Cyp3a16, Cyp3a41, and Cyp3a44 transcript levels were significantly greater at gestational days 10, 15, and 19 compared with that in nonpregnant female mice. In contrast, Cyp3a11 transcript level was significantly decreased at gestational days 10, 15, and 19, whereas that of Cyp3a25 was significantly decreased at days 10 and 15. B, Cyp3a13 transcript level was not significantly affected by pregnancy. The level of expression here and in subsequent figures is shown as the -fold difference compared with the expression of the same gene in the calibrator. Data are shown as mean \pm S.D. ($n = 4$). *, $p < 0.05$.

day 10 (8-fold) and then declined to approximately 5-fold higher expression at gestational day 15 compared with nonpregnant mice (Fig. 2A). Contrary to the increased expression of Cyp3a16, Cyp3a41, and Cyp3a44, the hepatic expression of Cyp3a25 and Cyp3a11 transcripts decreased with gestational age, and Cyp3a13 mRNA levels remained constant (Fig. 2B). Cyp3a11 transcript level decreased dramatically (~ 9 -fold) from gestational day 10 compared with nonpregnant animals and remained relatively constant for the remainder of the pregnancy, whereas Cyp3a25 transcript levels decreased significantly (~ 2 -fold) at gestational days 10 and 15, and at gestational day 19, there was no significant decrease compared with nonpregnant animals (Fig. 2B). In addition, each isoform of Cyp3a showed different abundance at transcript levels in nonpregnant state. Based on absolute C_T values, the expression of hepatic Cyp3a isoform transcripts was $3a41 > 3a11 > 3a25 > 3a16 > 3a44 > 3a13$.

Human CYP3A4 Promoter Activity and Endogenous Cyp3a Activity in Pregnant CYP3A4-tg Mice. Hepatic human CYP3A promoter activity was approximately four times higher in pregnant (day 17) mice than that in nonpregnant CYP3A4-tg mice ($p < 0.001$, Fig. 3A). As a positive control, dexamethasone treatment dramatically increased (>230 -fold) hepatic luciferase activity in both male and pregnant mice (data not shown). In contrast, CYP3A promoter activities in the kidney and the proximal small intestine (Fig. 3B) were not significantly increased by pregnancy. Similar to previous reports (Zhang et al., 2003), male mice ($n = 2$) had higher basal luciferase signal than female mice (data not shown). Consistent with the WT mouse data (Fig. 1A), hepatic mouse Cyp3a activity, as measured by testosterone 6β -hydroxylation, was significantly higher at day 17 pregnancy (~ 3 -fold, $p < 0.05$) compared with nonpregnant animals (Fig. 3C). Likewise, hepatic Cyp3a41 transcript expression was also significantly increased (~ 2 -fold, $p < 0.05$) during pregnancy (Fig. 3D).

Changes in the mRNA Expression of Hepatic Transcription Factors with Gestational Age in WT Mice. Various transcription factors are involved in the regulation of Cyp3a (Sakuma et al., 2002, 2004; Yamada et al., 2002; Tirona and Kim, 2005; Cheung et al., 2006; Jarukamjorn et al., 2006). To investigate whether pregnancy has an effect on the expression of these modulators, we examined the expression profiles of various transcription factors at the transcript level. The hepatic expression of HNF3 β , HNF6, and GhR transcripts was significantly increased during pregnancy, though the time course of the increase was different (Fig. 4A). The expression of GhR gradually increased during the course of pregnancy and peaked (~ 10 -fold higher) at day 19 of gestation. The expression of HNF6 was increased at gestational days 10 and 15 (~ 2 -fold), but at day 19, it was not significantly different from that in nonpregnant animals. The expression of HNF3 β was significantly higher (~ 1.5 -fold) only in early pregnancy (day 10). In contrast, hepatic CAR transcript expression was decreased by approximately 50% at midgestational ages (day 10 and 15) compared with that in nonpregnant animals (Fig. 4B). The hepatic expression of transcription factors PXR, HNF4 α , HNF1 α , RAR α , and RXR α was essentially unchanged during pregnancy (Fig. 4, B and C). The results for the other transcription factors were similar to those detailed in our previous report (Wang et al., 2006).

Correlation of Transcript Expression of Cyp3a with Transcription Factors in WT Mice. To gain insight into the mechanisms by which pregnancy altered the expression of Cyp3a isoforms, we investigated whether the expression of transcripts of different Cyp3a isoforms was correlated with that of the transcription factors affected by pregnancy. Data obtained from Spearman rank analysis are summarized in Table 1. Significant positive correlations at the transcript level were observed between Cyp3a41 and ER α , HNF6; between Cyp3a44 and ER α , HNF6; between Cyp3a11 and CAR, mem PR2; and between Cyp3a25 and CAR, mem PR1 and mem PR2. In contrast, significant negative correlation at the transcript level was observed between Cyp3a44 and CAR, and between Cyp3a11, Cyp3a25, and HNF6. No significant correlations were observed between Cyp3a isoforms and other transcription factors.

Changes in Intestinal Cyp3a Activity and Expression (Protein and Transcripts) with Gestational Age in WT Mice. In our previous study, we showed that intestinal Cyp3a protein expression at near term (days 18–19) was not different from nonpregnant animals (Mathias et al., 2006). In addition, we could not detect any Cyp3a activity (testosterone hydroxylation by HPLC/UV) in intestinal S-9 fractions. To increase our ability to measure intestinal Cyp3a activity, we made S-9 fractions of the intestinal mucosae (rather than of the entire intestinal tissue) and developed a highly sensitive

high-performance LC/MS assay to measure Cyp3a activity by testosterone 6 β -hydroxylation. The calibrators of this assay ranged from 3 to 800 ng, and the assay had an error of less than 8.5%, with a standard deviation of less than 12.3%. The detection limit was 1.3 ng (~4 pmol) of 6 β -OH testosterone loaded on-column, which is approximately 10-fold more sensitive than the HPLC/UV method. Despite these modifications, we could not detect Cyp3a activity in the S-9 fractions of the scraped mucosae isolated from either the proximal or the distal small intestine. However, using the same assay, we could easily detect Cyp3a activity in the mouse liver S-9 fractions. It is interesting that we were able to detect Cyp3a protein expression in the scraped mucosae of the proximal intestine, but the expression was not affected by pregnancy (Fig. 5, A and B). Consistent with this observation, transcript levels of the Cyp3a isoforms Cyp3a16, Cyp3a41, Cyp3a44, Cyp3a11, Cyp3a13, and Cyp3a25 in either the proximal or distal small intestine were not significantly different between pregnant and nonpregnant animals (Fig. 5C).

Changes in Tissue P-gp Expression (Protein and Transcripts) with Gestational Age in WT Mice. Expression of P-gp protein and transcripts (*mdr1a*, *mdr1b*) was not changed during pregnancy in the liver (Fig. 6). In the placenta, P-gp protein expression was highest at early gestational age and decreased with the progression of pregnancy (Fig. 6A). At near term (day 19), placental P-gp expression

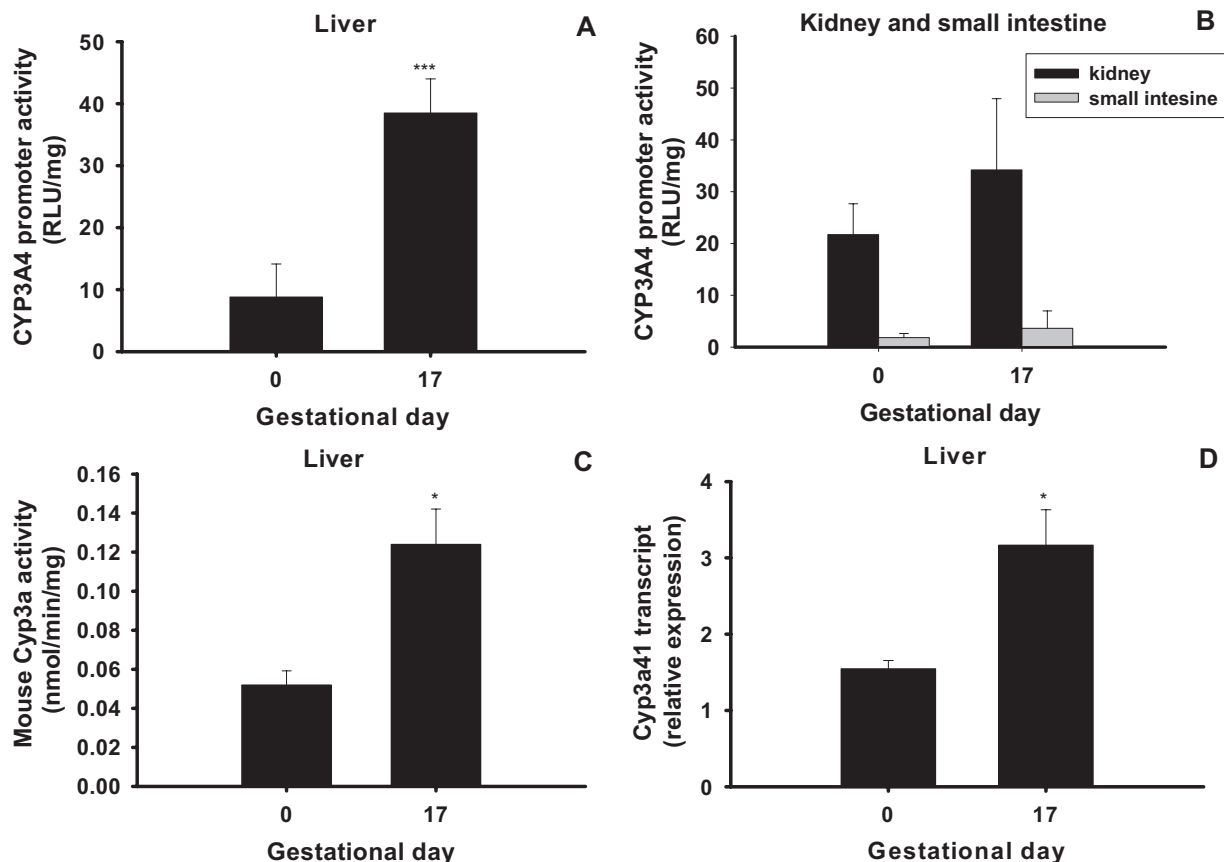


Fig. 3. CYP3A4 promoter activity, endogenous Cyp3a activity, and endogenous Cyp3a41 expression in tissues from pregnant and nonpregnant CYP3A4-tg mice. Compared with nonpregnant CYP3A4-tg mice ($n = 4$), tissue homogenate from day 17-pregnant CYP3A4-tg mice ($n = 5$) showed significantly greater luciferase activity in the liver (A) but not in the proximal small intestine or kidney (B). C, liver S-9 fractions from pregnant CYP3A4-tg mice showed significantly higher Cyp3a activity (as measured by testosterone 6 β -hydroxylation) than nonpregnant CYP3A4-tg mice. D, hepatic mRNA expression of Cyp3a41 is significantly higher in pregnant than in nonpregnant CYP3A4-tg mice. Data are shown as mean \pm S.D. ***, $p < 0.001$; *, $p < 0.05$.

was approximately one third of that at gestational day 10 ($p < 0.05$). However, the expression of placental P-gp transcripts *mdr1a* and *mdr1b* was not changed by gestational ages (Fig. 6, B and C). In the kidney, P-gp protein expression

was not changed throughout pregnancy, although expression of both P-gp transcripts *mdr1a* and *mdr1b* was significantly reduced during pregnancy ($p < 0.05$; Fig. 6). It is interesting that in the kidney, the expression level (based on the absolute C_T values) of *mdr1b* was approximately 9-fold greater than that of *mdr1a*, whereas in the placenta, the expression level of *mdr1a* was approximately 7-fold greater than that of *mdr1b* (data not shown).

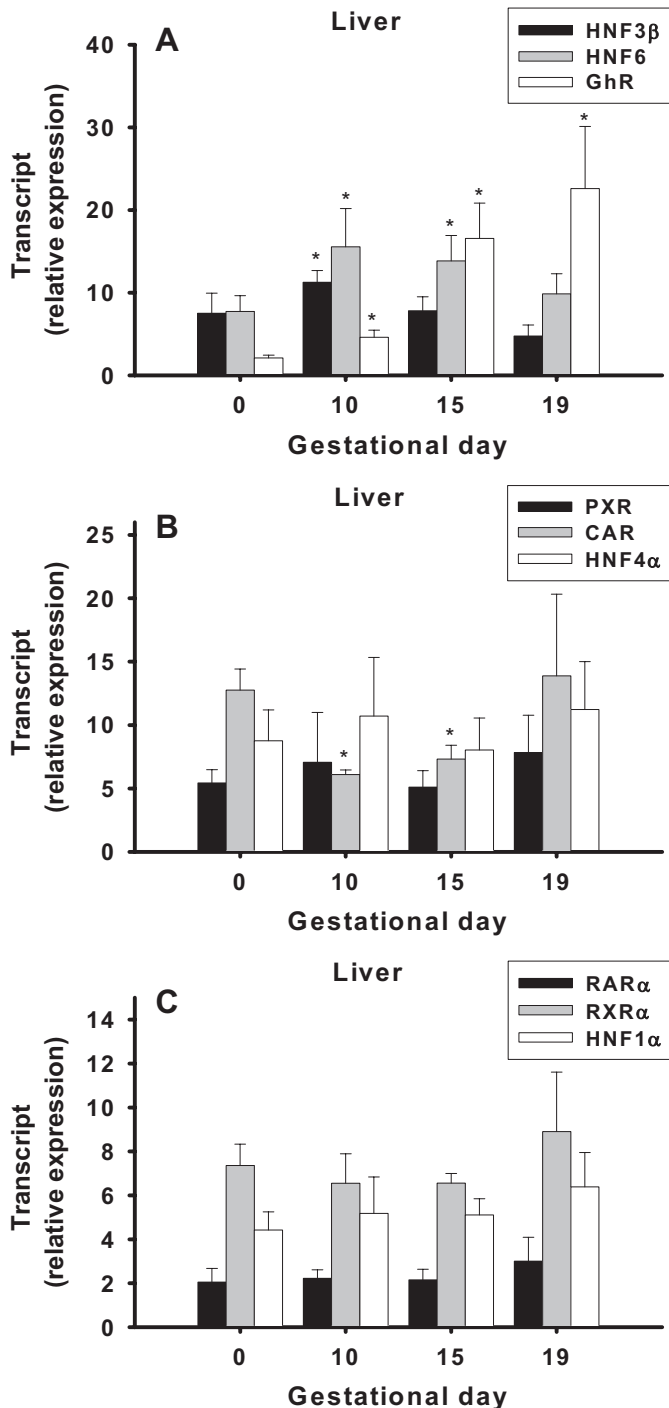


Fig. 4. Transcript expression of hepatic nuclear transcription factors by real-time PCR at different gestational ages in WT mice. A, compared with that in nonpregnant female mice, hepatic HNF3 β transcripts were significantly greater at gestational day 10, HNF6 transcripts were significantly greater at gestational days 10 and 15, and GhR transcript levels were significantly greater at gestational days 10, 15 and 19. B, in contrast, hepatic CAR transcripts were significantly decreased at gestational days 10 and 15. No change in the hepatic expression of PXR and HNF4 α (B) or RAR α , RXR α , and HNF1 α (C) was observed during pregnancy. Data are shown as mean \pm S.D. ($n = 4$). *, $p < 0.05$.

Discussion

Consistent with our previous study conducted on gestational days 18 to 19 (Mathias et al., 2006), mouse hepatic Cyp3a activity, as measured by testosterone 6 β -hydroxylation, was also increased at gestational day 15 (Fig. 1A). We also found that hepatic Cyp3a protein expression was significantly increased from earlier gestational day 10 to later gestational days 15 and 19 (Fig. 1B). There are eight different Cyp3a isoforms coded by the mouse genome (Zaphiropoulos 2003), of which only six have been cloned. The antibody used here to measure mouse Cyp3a expression was the com-

TABLE 1

Correlations (r_s) of hepatic mRNA expression between Cyp3a isoforms and transcriptional factors in pregnant ($n = 4$) and nonpregnant ($n = 4$) WT mice

	Cyp3a41	Cyp3a44	Cyp3a16	Cyp3a13	Cyp3a11	Cyp3a25
PXR						
r_s	0.04	0.21	-0.06	0.41	0.40	0.30
p	0.90	0.44	0.81	0.12	0.13	0.26
CAR						
r_s	-0.47	-0.54	-0.40	0.45	0.70	0.69
p	0.06	0.03*	0.13	0.08	<0.01*	<0.01*
AhR						
r_s	-0.05	-0.12	0.22	0.05	-0.10	0.16
p	0.85	0.66	0.42	0.85	0.72	0.55
ERα						
r_s	0.57	0.50	0.24	-0.10	-0.29	-0.01
p	0.02*	0.05*	0.37	0.72	0.27	0.97
mem PR1						
r_s	0.17	0.18	0.11	0.14	0.24	0.54
p	0.52	0.51	0.69	0.59	0.38	0.03*
mem PR2						
r_s	-0.29	-0.09	-0.55	-0.01	0.52	0.54
p	0.27	0.75	0.03*	0.97	0.04*	0.03*
GhR						
r_s	0.15	0.21	0.49	-0.08	-0.48	-0.39
p	0.57	0.44	0.05*	0.77	0.06	0.13
RARα						
r_s	0.33	0.20	0.41	0.21	-0.04	0.24
p	0.21	0.45	0.11	0.44	0.87	0.36
RXRα						
r_s	-0.19	-0.24	-0.17	0.27	0.45	0.59
p	0.49	0.36	0.53	0.31	0.08	0.02*
HIF1α						
r_s	-0.09	-0.05	0.23	0.07	-0.15	-0.06
p	0.73	0.85	0.39	0.80	0.57	0.84
HNF1α						
r_s	0.11	0.19	0.21	-0.17	-0.23	-0.03
p	0.68	0.49	0.43	0.53	0.39	0.91
HNF3β						
r_s	0.44	0.35	0.15	-0.35	-0.30	-0.32
p	0.08	0.19	0.57	0.18	0.26	0.22
HNF4α						
r_s	0.04	0.28	-0.16	0.32	0.36	0.30
p	0.88	0.29	0.55	0.23	0.17	0.39
HNF6						
r_s	0.56	0.61	0.44	-0.21	-0.56	-0.57
p	0.02*	0.01*	0.09	0.44	0.02*	0.02*

r_s , Spearman rank correlation coefficient; AhR, aryl hydrocarbon receptor; HIF, hypoxia-inducible factor.

* Significant correlation.

mercially available polyclonal anti-rat CYP3A2 antibody. Because of the high amino acid sequence similarity between mouse and rat CYP3A (Dai et al., 2001; Anakk et al., 2003; Sakuma et al., 2004) and the polyclonal heterogeneity of anti-rat CYP3A2 antibody, it is reasonable to assume that

this antibody will recognize most, if not all, of the mouse isoforms.

To determine the mechanisms of increased protein expression and activity of hepatic Cyp3a during pregnancy, we examined the relative transcript expression of six hepatic

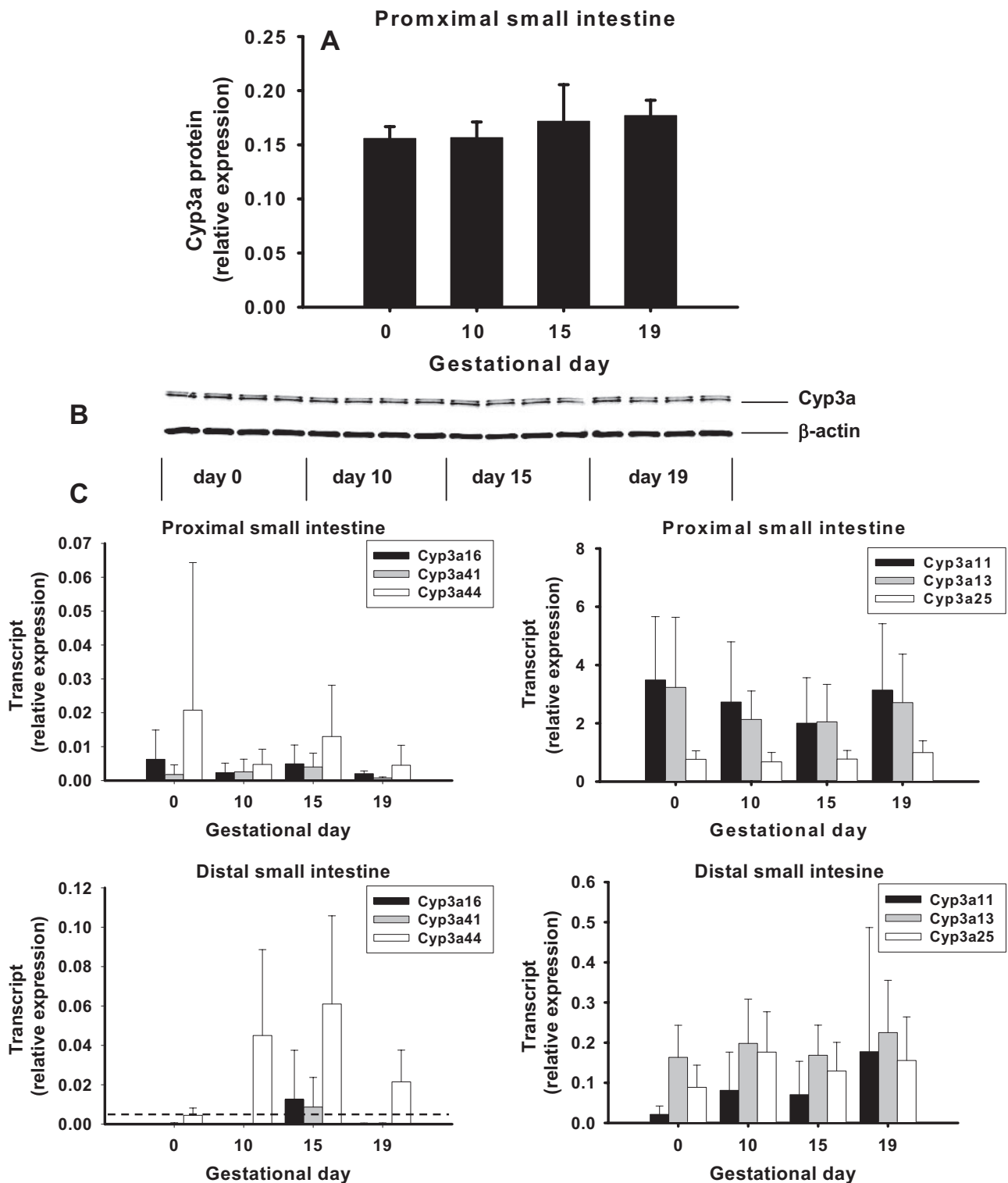


Fig. 5. Intestinal Cyp3a protein and transcript expression at different gestational ages ($n = 4$, mean \pm S.D.) in WT mice. Quantification (A) of Western blots (B) of proximal intestinal mucosae S-9 fractions showed no effect of pregnancy on intestinal Cyp3a protein expression. Likewise, expression of Cyp3a isoform transcripts in the proximal or distal small intestine was not affected by pregnancy (C). Broken line indicates the detection limit of the real-time PCR, which is defined as the C_T value of calibrator mRNA without reverse transcription, which is usually approximately 37 under our experimental conditions. Data are shown as mean \pm S.D. ($n = 4$).

Cyp3a isoforms. We found that pregnancy differentially affects the transcript expression of each isoform (Fig. 2). The expression of the internal standard, β -actin, was relatively stable during pregnancy, with a trend of decreased expression at gestational day 19 ($0.05 < p < 0.1$; data not shown). However, this decrease cannot completely explain the changes observed in the expression of the various Cyp3a isoforms, because the changes in the latter also occurred at gestational day 15. Moreover, the transcript expression of

some Cyp3a isoforms increased, whereas those of others decreased (Fig. 2).

The transcript expression data suggest that pregnancy increases hepatic Cyp3a activity by transcriptional up-regulation of selective Cyp3a isoforms. Next, we asked whether this pregnancy factor also transcriptionally up-regulates the human CYP3A promoter in the transgenic mouse model expressing 13 kilobases of the CYP3A4 promoter fused to the luciferase gene (Zhang et al., 2003). Hepatic CYP3A4 promoter activity, determined by the luciferase assay, was significantly increased (~ 4 -fold) at gestational day 17 compared with that in nonpregnant transgenic mice. As expected and consistent with the nontransgenic mice, the transcript expression of a representative isoform Cyp3a41 and the endogenous hepatic Cyp3a activity on gestational day 17 was approximately 2-fold higher than that in nonpregnant transgenic mice (Fig. 3, C and D). In addition, the human CYP3A4 promoter activity and endogenous Cyp3a activity were highly correlated ($r_s = 0.75$, $p < 0.05$), strongly supporting the conclusion that pregnancy simultaneously and transcriptionally activates the human CYP3A4 promoter as well as some of the mouse Cyp3a isoforms. It is remarkable that the magnitude of this activation and the increase in Cyp3a activity and CYP3A4 promoter activity were similar. Based on these data, we conclude that pregnancy-induced increase in hepatic Cyp3a activity is due, at least in part, to transcriptional-activation of hepatic Cyp3a16, Cyp3a41, and Cyp3a44 enzymes.

Many physiological hormones such as growth hormones and estrogens are known to regulate cytochrome P450 expression (Sakuma et al., 2002, 2004; Yamada et al., 2002; Cheung et al., 2006; Jarukamjorn et al., 2006). During pregnancy, placental growth hormone (PGH) plasma concentrations increase continuously, gradually replacing the pituitary growth hormone (GH), which remains at a constant low concentration from midgestation onward (Caufriez et al., 1993). PGH differs from GH by only 13 amino acid residues (Frankenne et al., 1990), but they both bind to the same receptor, GhR, with similar affinity (Baumann et al., 1991). Previous findings have shown that increased GH concentrations enhance the expression of not only its own receptor (Camarillo et al., 1998) but also of downstream transcription factors in the GH signaling cascade, such as HNF3 β and HNF6 (Legraverend et al., 1994; Lahuna et al., 1997). In addition, it also increases the expression of Cyp3a41 in the mouse (Jarukamjorn et al., 2006) and human CYP3A4 expression and activity in hepatocytes (Liddle et al., 1998). Our findings are consistent with these observations (Figs. 1, 2, and 4). Moreover, the hepatic expression of HNF6 transcripts was positively correlated with that of Cyp3a44 and Cyp3a41 and negatively correlated with that of Cyp3a11 and Cyp3a25 in the WT mice (Table 1). In addition, GhR transcript expression significantly correlated with both CYP3A4 promoter activity ($r_s = 0.783$, $p = 0.013$) and endogenous Cyp3a activity ($r_s = 0.733$, $p = 0.025$) in the CYP3A4-tg mice. These data strongly suggest that, during pregnancy, the growth hormone signaling pathway is activated and involved in the up-regulation of the female-specific isoforms in the mouse.

During pregnancy, mouse plasma estradiol concentration is high at the beginning, reaches a nadir at 2 to 3 days of gestation, and increases to a maximum at 18 to 19 days of gestation (McCormack and Greenwald, 1974). It also has

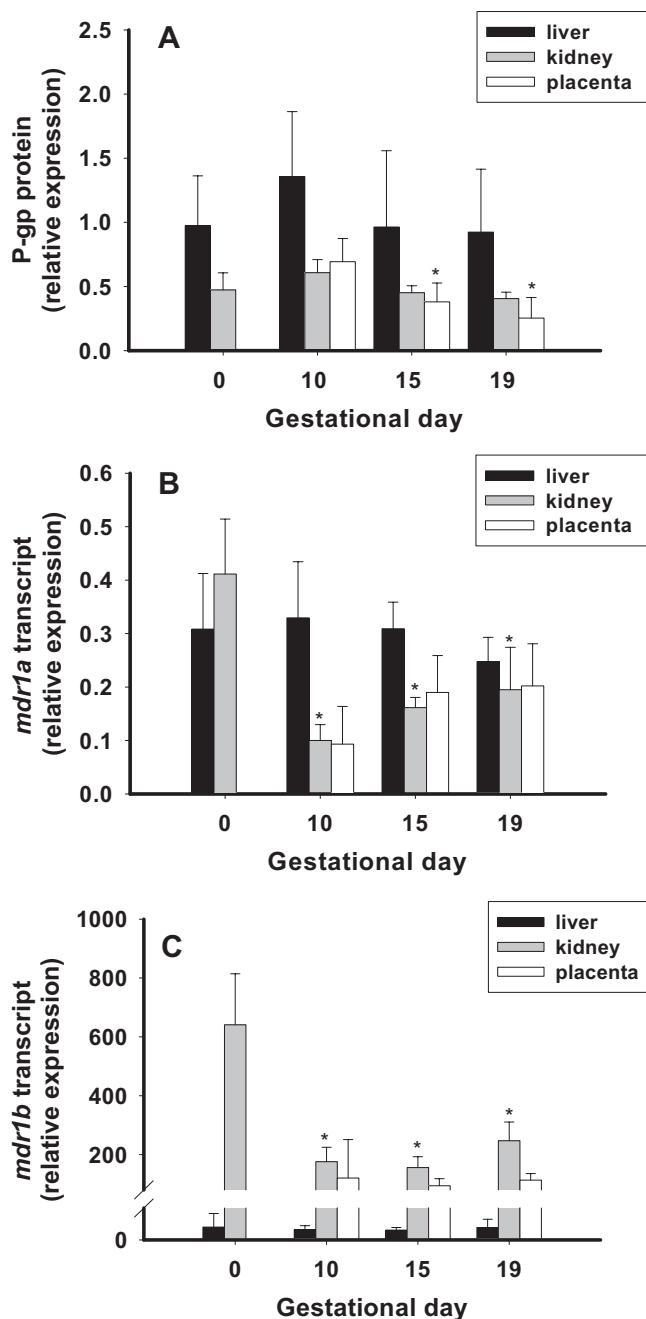


Fig. 6. P-gp expression in the liver, kidney, and placenta in WT mice. A, expression of P-gp protein in the liver and kidney was not affected by pregnancy; however, placental P-gp expression significantly decreased as pregnancy proceeded. B and C, *mdr1a* or *mdr1b* transcript expression was not affected by pregnancy in the liver or placenta; however, the expression of *mdr1a* and *mdr1b* transcripts was significantly reduced by pregnancy in the kidney. Data are shown as mean \pm S.D. ($n = 4$). *, $p < 0.05$.

been reported in the mouse that estradiol induces the expression of Cyp3a41 and Cyp3a44 transcripts (Sakuma et al., 2004), although it suppresses Cyp3a11 expression (Yamada et al., 2002). It is interesting that we found that hepatic mRNA expression of ER α was significantly increased during pregnancy (Wang et al., 2006). Moreover, significant positive correlation was found between the expression of hepatic Cyp3a41, Cyp3a44, and ER α transcripts. Therefore, it is possible that an increase in circulating estrogens and an increase in the hepatic expression of its receptor ER α result in increased hepatic expression of Cyp3a41, Cyp3a44 transcripts, and a decrease in Cyp3a11 transcripts. Together, increased PGH and estrogen plasma concentrations probably activate a cascade of events that lead to increased expression of various transcriptional factors and receptors, which ultimately results in enhanced expression and activity of Cyp3a proteins. Consistent with this hypothesis, studies in mouse hepatocytes have shown that GH or estradiol treatment alone does not increase GhR expression (Contreras and Talamantes, 1999) or transcript expression of Cyp3a44 or Cyp3a41 (Sakuma et al., 2002). However, combined treatment of these two hormones dramatically increases (5–6-fold) hepatic expression of both Cyp3a41 and Cyp3a44 transcripts (Sakuma et al., 2002).

In contrast to the liver, intestinal Cyp3a activity in the WT mice was not detectable by HPLC/UV (Mathias et al., 2006) or by a more sensitive LC/MS method at any of the gestational ages, indicating low Cyp3a activity in the small intestine. This is consistent with our previous observations (Mathias et al., 2006) and those of others (Emoto et al., 2000). Similar to our previous observations (Mathias et al., 2006), pregnancy did not affect Cyp3a protein or transcript expression in the intestine of the WT mice (Fig. 5). This is not surprising because tissue-specific expression and activity of CYP3A4 enzymes has also been reported in humans (Lown et al., 1994). Consistent with these results, no transcriptional activation of human CYP3A4 promoter was observed either in the small intestine or kidney of the CYP3A4-tg mice (Fig. 3). These data suggest a consistent pattern of tissue-specific regulation of both mouse and human CYP3A4 genes. Based on these data, we predict that pregnancy will increase hepatic but not intestinal activity of CYP3A. Indeed, we have observed increased CYP3A activity in human pregnancy as measured by increased oral clearance of midazolam (Hebert et al., 2006). However, because midazolam was administered orally, we are unable to determine whether this effect is hepatic, intestinal, or both.

In contrast to hepatic Cyp3a expression, and consistent with our previous study (Mathias et al., 2006), the expression of P-gp protein in the liver or kidney in the WT mice was not affected by pregnancy. Moreover, expression of *mdr1* transcripts in the liver was not affected by pregnancy, although renal *mdr1* transcripts were decreased during pregnancy. It is not clear why changes in the expression of renal *mdr1* transcripts and protein do not match. Among several possible explanations are changes in the stability of the protein, post-translational regulation of the protein, and the semiquantitative nature of Western blots and their inability to differentiate between plasma membrane and intracellular protein. We also observed that mouse placental P-gp protein expression, but not *mdr1* transcripts, decreased significantly with gestational age. The reasons listed above for the kidney can

also be offered to explain this discrepancy. Nevertheless, this change in mouse placental protein expression with gestational age reproduces our previous finding in the human placenta (Mathias et al., 2005). Others have also observed this change using the whole placenta (MacFarland et al., 1994). Our observations that dynamics of placental P-gp expression in mice and human are similar indicates that the mouse can be used as a model to investigate the regulation of placental P-gp during pregnancy.

Based on these data, we propose that the mouse (including the CYP3A4-tg mouse) is an excellent animal model to determine the molecular mechanisms by which pregnancy induces CYP3A activity and regulates placental P-gp expression. Induction of CYP3A enzyme by pregnancy has considerable clinical significance in designing appropriate dosing regimens for pregnant women of narrow therapeutic-window drugs cleared extensively by CYP3A enzymes. For such drugs, the dose will need to be increased to achieve plasma concentrations achieved in nonpregnant women and men. Indeed, in the treatment of HIV-1 infection during pregnancy, regimens that include ritonavir are now recommended to counteract the lower plasma concentrations of the protease inhibitors observed during pregnancy (information available at <http://aidsinfo.nih.gov/contentfiles/perinatalgl.pdf>).

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