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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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Running title: Cyp3a and P-gp in pregnant mice

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Abbreviations: PI, protease inhibitor; NFV, nelfinavir; Cyp3a, Cytochrome P450 3a; P-gp, P-glycoprotein; GH, growth hormone; TST, testosterone; PXR, pregnane xenobiotic receptor; CAR, constitutive androstane receptor; AhR, aryl hydrocarbon receptor; ER, estrogen receptor; HIF, hypoxia-inducible factor; mem PR, membrane progesterone receptor; GhR, growth hormone receptor; RAR, retinoic acid receptor; RXR, retinoic X receptor; HNF, hepatocyte nuclear factor; HRP, horseradish peroxidase; HPLC/UV: high-performance liquid chromatography-ultraviolet spectrometry; HPLC/MS: high-performance liquid chromatography-mass spectrometry; WT, wild-type; CYP3A4-tg, CYP3A4-promoter-transgenic.

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Abstract

The plasma concentrations of orally administered anti-HIV 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-gp in various tissues. We found hepatic transcripts of Cyp3a16, Cyp3a41 and Cyp3a44 were significantly increased during pregnancy, while 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 Cyp3a41and Cyp3a44 transcripts was positively correlated ($p<0.05$) with HNF6 and ERα 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 expressions were not significantly affected by pregnancy. No change in P-gp protein expression was observed in the liver or kidney during pregnancy, though a significant decrease was observed in the placenta. Since hepatic CYP3A activity also appears to be induced during human pregnancy, the mouse (including CYP3A4-tg mouse) appears to be an excellent animal model to determine the molecular mechanisms for such induction.

Introduction

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 HAART (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, when compared with that of men, non-pregnant 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 (AUC) or mean/median concentration ratio, is in the order of 70% to 90% compared with postpartum or non-pregnant controls. Similarly, a perinatal study (PACTG 358) we have conducted in HIV-1 infected pregnant women has shown that the plasma AUC 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. Similarly, we have found that in pregnant women, hepatic and/or intestinal CYP3A activity is increased 2-3 fold in late pregnancy compared

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with postpartum, as demonstrated by reduced exposure to oral midazolam (Hebert et al., 2006). In the same cohort of women, net 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 have 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. Interestingly, 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 non-pregnant 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.

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Materials and Methods

Chemicals. Methyl-tert-butyl ether (HPLC grade), methanol (HPLC grade), testosterone, 11α-OH-progesterone, phenyl methyl sulfonyl fluoride and NADPH were purchased from Sigma Chemical (St Louis, MO). 6β-OH testosterone was purchased from Steraloids Inc. (Newport, RI). D3-6β-OH testosterone was purchased from Cerilliant Co. (Round Rock, TX). Potassium phosphate monobasic was purchased from J. T. Baker (Phillisburg, 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 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 HRP conjugated anti-mouse, anti-rabbit antibodies were purchased from Bio-Rad Laboratories (Hercules, CA). Hybond™- ECL™ nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ). All other reagents used in experiments were purchased from Fisher Scientific (Fairlawn, NJ).

Animals. Adult (eight to ten weeks) 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 cared 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

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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 day 10, 15 and 19, WT pregnant mice were sacrificed under anesthesia (IP pentobarbital at 100 mg/kg), 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, then the mucosae were removed by gently scrapping the luminal surface using a fine tissue culture scrapper. Tissues were flash frozen in liquid N_2 and stored at −80°C until use. Age and weight matched non-pregnant female mice were used as controls (gestational day 0). Pregnant (day 17) and non-pregnant transgenic CYP3A4-tg mice were sacrificed and tissues were collected as indicated above. As a positive control, tissues from CYP3A4-tg mice (2 males and 1 pregnant female) were collected six hours after administration of a single dose of a Cyp3a inducer, dextramethasone (i.p., 50 mg/kg).

S-9 fraction isolation and protein quantification. Tissue homogenate were prepared as described (Mathias et al., 2006). Briefly, flash frozen tissues were thawed on ice and homogenized in a Wheaton homogenizer with 3-5× volume of homogenization buffer (10 mM KH_2PO_4, 250 mM sucrose, pH7.4) containing protease inhibitors. Liver, kidney and placenta homogenates were centrifuged at 10,000 g for 30 min at 4°C. Intestinal homogenates were briefly spun (600 g for 5 min), and the supernatant was then centrifuged at 10,000 g for 30 min at 4°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.

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SDS-PAGE and Western blot. 2-20 μg (for Cyp3a detection) or 40-80 μg (for P-gp detection) of S-9 fraction were separated by 10% (for Cyp3a) or 4-15% (for P-gp) precast Criterion Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to Hybond™- ECL™ nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ). Western detection followed the ECL procedure according to the manufacturer's instructions (Amersham Bioscience, Piscataway, NJ). Briefly, blots were soaked in PBS buffer for 10 min and then placed in blocking buffer (5% non-fat dry milk, 0.05% Tween-20 in PBS buffer) for 1 hr at room temperature. C219 (1/800), anti-rat 3A2 (1/2000), or anti-β-actin antibody (1/5,000-10,000) were added to the blocking solution and incubation continued for another hour. The blots were then rinsed in wash buffer (0.05% Tween-20 in PBS buffer) and washed twice, 10 min each. The blots were then incubated in blocking buffer with secondary antibodies (1/10,000-20,000 HRP conjugated anti-rabbit or anti-mouse IgG, Bio-Rad Laboratory Inc., Hercules, CA) for one hour. The blots were washed in PBS buffer twice, 20 min each. ECL™ plus reagent (Amersham Biosciences, Piscataway, NJ) was then applied to the membrane following manufacturer's instructions. Chemiluminescent signal was recorded on an X-Omat™ Blue XB-1 film (Perkin Elmer Life Sciences, Rochester, NY). The relative intensity of each protein band was determined using Bio-Rad Chemi-Doc and Quantity One Program (Hercules, CA) following manufacture's instructions.

Cyp3a activity assay. Cyp3a activity was measured by minor modification of testosterone (TST) 6β-hydroxylation assay as described previously (Mathias et al., 2006).

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1 mg/ml to 4 mg/ml of S-9 fraction were pre-incubated at 37°C for 5 min in 100 µl phosphate buffer (100 mM potassium phosphate, pH7.4, 5 mM $MgCl_2$) containing 150 µM substrate TST. Total organic solvent (methanol) content was < 3 %. Reaction was initiated by addition of freshly prepared NADPH (final conc. 1 mM), and terminated at 30 min by addition of 100 µl ice-cold acetonitrile containing 10 µl internal standard 11α-OH progesterone (54 µg/ml, HPLC/UV method) or D3-6β-OH TST (10 µg/ml, LC/MS method). Samples were incubated on ice for 30 min, then were vortex mixed and centrifuged at 15,000 g for 3 min. 20 µl of the supernatant was directly injected onto HPLC/UV or LC/MS.

a. 6β-OH TST detection by HPLC/UV. HPLC/UV was modified from previously described method (Mathias et al., 2006). Briefly, separation of the substrate TST, product 6β-OH TST and internal standard 11α-OH progesterone was carried out on a 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 × 4.6 mm, 5 micron, Alltech) with a guard column (7.5 × 3.2 mm, 5 micron, Alltech) at room temperature. Mobile phase was consisted of Solvent A: 30 % methanol: 2 % acetonitrile : 68 % H_2O; Solvent B: 80 % methanol: 5 % acetonitrile: 15 % H_2O. Gradient elution at a flow rate of 1 ml/min was programmed from 50 % to 100% B over 14 min. All analytes and the internal standard were detected at λ = 244 nm. Data were recorded using Waters Empower™ Pro (Waters Co., Milford, MA) software. Calibrators ranged from 50 ng – 800 ng of 6β-OH TST were spiked in the

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incubation matrix together with the internal standard11α-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 6β-OH TST in unknown samples was determined by linear regression.

b. 6β-OH TST detection by LC/MS. To achieve greater sensitivity than that afforded by HPLC/UV, detection of 6β-OH TST formation in intestinal S-9 incubations was conducted by mass-spectrometry on a Waters 2695 LC module/Micromass platform LCZ4000 using electro spray positive ionization mode. The chromatography was performed on an Agilent ZORBAX SB-C18, 2.1 × 150 mm, 5 μm column with Phenomnex 2.0 × 4 mm C18 guard column at room temperature. 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. 6β-OH TST was detected as selective ion mode at 305.3 m/z, and the internal standard D3-6β-OH TST (50 ng) was detected at 308.0 m/z. The calibration curve ranged from 3 ng 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 2μg of total RNA that had a A260/A280 ratio of 1.8-2.0, and reverse transcription was performed using TagMan® reverse transcription reagents from Roche Molecular Systems, Inc. (Branchburg, New

Jersey). 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 FAM-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), HNF3β (Mm01976556_s1), HNF4α (Mm00433958_m1), HNF6 (Mm00839394_m1), RARα (Mm01296311_g1), RXRα (Mm00441185), GhR (Mm01303638), PXR (Mm00803095_m1), CAR (Mm01283980_q1), and transcriptional factors AhR, mem PR, HIF1α, 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 μl 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 20 ng of RNA equivalent cDNA, and primers (200 nM) and probes (100 nM) in a final volume of 20 μl. The reactions were conducted as follows: 95°C hot start for 10 min, followed by 40 cycles at 95°C for 15 sec and then 60°C for 60 sec. 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, Foster City, CA). The mRNA levels of each test gene were normalized to β-actin, according to the following formula: C_T (test gene) - C_T (β-actin) =

ΔC_T. Thereafter, the relative mRNA levels of each gene were calculated using the $\Delta\Delta C_T$ method: ΔC_T (test gene) - ΔC_T (test gene in the calibrator) = $\Delta\Delta C_T$ (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. Briefly, 30-50 mg of tissue was homogenized in 500 µl 1× 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 µl (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, Canada) following the manufacture's protocol. Luciferase activity was reported as relative light units normalized per mg protein (RLU/mg).

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

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Results

Changes in hepatic Cyp3a activity and expression (protein and transcript) with gestational age in WT-mice Hepatic Cyp3a activity, as measured by testosterone 6β-hydroxylation, was significantly ($p < 0.05$) increased at gestational day 15 (2.2-fold) and 19 (2.5-fold), when compared with non-pregnant animals (Fig. 1A). As expected from the increased activity, hepatic Cyp3a protein expression was also significantly ($p < 0.05$) increased at gestational day 10, 15 and 19 when compared with non-pregnant mice, with the maximum increase (4-fold) occurring at gestational day 10 (Fig. 1B). Similar to 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 day 10 (8-fold), then declined to about 5-fold higher expression at gestational day 15

compared with non-pregnant 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 non-pregnant 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 non-pregnant animals (Fig. 2B). In addition, each isoform of Cyp3a showed different abundance at transcript levels in non-pregnant state. Based on absolute Ct 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 ~ 4 times higher in pregnant (day 17) mice than that in non-pregnant 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, $p < 0.05$) when compared with non-pregnant animals (Fig. 3C). Similarly,

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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; Sakuma et al., 2004; Cheung et al., 2006; Yamada et al., 2002; Jarukamjorn et al., 2006; Tirona and Kim 2005). 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 HNF3β, 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 day 10 and 15 (~ 2-fold), but at day 19, it was not significantly different from that in non-pregnant animals. The expression of HNF3β was significantly higher (~ 1.5-fold) only in early pregnancy (day 10). In contrast, hepatic CAR transcript expression was decreased by about 50 % at mid-gestational ages (day 10 and 15), when compared with that in non-pregnant animals (Fig. 4B). The hepatic expression of transcription factors, PXR, HNF4α, HNF1α, RARα, and RXRα, was essentially unchanged during pregnancy (Fig. 4B, 4C). The results for the other transcription factors were similar to those detailed in our previous report (Wang et al., 2006).

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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 ERα, HNF6; between Cyp3a44 and ERα, HNF6; between Cyp3a11 and CAR, mem PR2; between Cyp3a25 and CAR, mem PR1, mem PR2. In contrast, significant negative correlation at the transcript level was observed between Cyp3a44 and CAR; 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 had shown that intestinal Cyp3a protein expression at near term (day 18-19) was not different from non-pregnant 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 HPLC/MS assay to measure Cyp3a activity by testosterone 6β-hydroxylation. The calibrators of this assay ranged from 3 - 800 ng, and the assay had 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 6β-OH testosterone loaded on-column, which is about 10-fold more sensitive than the HPLC/UV

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method. Despite these modifications, we could not detect Cyp3a activity in the S-9 fractions of the scrapped 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. Interestingly, we were able to detect Cyp3a protein expression in the scrapped mucosae of the proximal intestine, but the expression was not affected by pregnancy (Fig. 5A, 5B). Consistent with this observation, transcript levels of the Cyp3a isoforms Cyp3a16, Cyp3a41, Cyp3a44, Cyp3a11, Cyp 3a13, and Cyp3a25 in either the proximal or distal small intestine, were not significantly different between pregnant and non-pregnant 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 was about 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. 6B, 6C). In the kidney, P-gp protein expression was not changed throughout pregnancy, though expression of both P-gp transcript *mdr1a* and *mdr1b* was significantly reduced during pregnancy ($p < 0.05$, Fig. 6). Interestingly, in the kidney, the expression level (based on the absolute Ct values) of *mdr1b* was about 9-fold greater than that of *mdr1a*, whereas in the placenta, the expression level of *mdr1a* was about 7-fold greater than that of *mdr1b* (data not shown).

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Discussion

Consistent with our previous study conducted on gestational day 18~19 (Mathias et al., 2006), mouse hepatic Cyp3a activity, as measured by testosterone 6β-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 day 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 commercially available polyclonal anti-rat CYP3A2 antibody. Due to 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 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 gestation 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, as the changes in the latter also occurred at gestational day 15. Moreover, the transcript expression of some Cyp3a isoforms increased while those of others decreased (Fig. 2).

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The transcript expression data suggest that pregnancy increases hepatic Cyp3a activity by transcriptional up-regulation of selective Cyp3a isoforms. Next, we asked if this pregnancy-factor also transcriptionally up-regulates the human CYP3A promoter in the transgenic mouse model expressing 13 kb 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 when compared with that in non-pregnant transgenic mice. As expected and consistent with the non-transgenic mice, the transcript expression of a representative isoform Cyp3a41, and the endogenous hepatic Cyp3a activity on gestational day 17 was about 2-fold higher than that in non-pregnant transgenic mice (Fig. 3C, 3D). Additionally, 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. Remarkably, the magnitude of this activation, the increase in Cyp3a activity and CYP3A4 promoter activity was similar. Based on these data, we conclude that pregnancy-induced increase in hepatic Cyp3a activity is, at least in part, due 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; Sakuma et al., 2004; Cheung et al., 2006; Yamada et al., 2002; Jarukamjorn et al., 2006). During pregnancy, placental growth hormone (PGH) plasma concentrations rise continuously, gradually replacing the pituitary growth hormone (GH) which remains at a constant low concentration from

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midgestation onwards (Caufriez et al., 1993). PGH differs from GH only by 13 amino acid residues (Frankenne et al., 1990), but they both bind to the same receptor, the growth hormone 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 down-stream transcription factors in the GH signaling cascade, such as HNF3β 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 (Fig. 1, 2, 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 day of gestation (McCormack and Greenwald 1974). It also has been reported in the mouse that estradiol induces the expression of Cyp3a41 and Cyp3a44 transcripts (Sakuma et al., 2004), while it suppresses Cyp3a11 expression (Yamada et al., 2002). Interestingly, we found that hepatic mRNA expression of ERα was significantly increased during

pregnancy (Wang et al., 2006). Moreover, significant positive correlation was found between the expression of hepatic Cyp3a41, Cyp3a44 and ERα transcripts. Therefore, it is possible that an increase in circulating estrogens as well as an increase in the hepatic expression of its receptor ERα, results in increased hepatic expression of Cyp3a41, Cyp3a44 transcripts and a decrease in Cyp3a11 transcripts. Collectively, increased PGH and estrogen plasma concentrations likely 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 as 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 increases 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 if 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, though 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. Amongst several possible explanations are changes in the stability of the protein, post translational regulation of the protein and the semi-quantitative nature of Westerns 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

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expression in mice and human is similar, indicates that the mouse can be used as a model to investigate regulation of placenta 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 of these CYP3A cleared drugs will need to be increased to achieve plasma concentrations achieved in non-pregnant 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 (http://aidsinfo.nih.gov/ContentFiles/PerinatalGL.pdf).

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References

Acosta EP, Bardeguez A, Zorrilla CD, Van Dyke R, Hughes MD, Huang S, Pompeo L, Stek AM, Pitt J, Watts DH, Smith E, Jimenez E and Mofenson L (2004) Pharmacokinetics of saquinavir plus low-dose ritonavir in human immunodeficiency virus-infected pregnant women. *Antimicrob Agents Chemother* **48**:430-436.

Anakk S, Kalsotra A, Shen Q, Vu MT, Staudinger JL, Davies PJ and Strobel HW (2003) Genomic characterization and regulation of Cyp3a13: role of xenobiotics and nuclear receptors. *FASEB J* **17**:1736-1738.

Angel JB, Khaliq Y, Monpetit ML, Cameron DW and Gallicano K (2001) An argument for routine therapeutic drug monitoring of HIV-1 protease inhibitors during pregnancy. *AIDS* **15**:417-419.

Baumann G, Davila N, Shaw MA, Ray J, Liebhaber SA, and Cooke NE (1991) Binding of human growth hormone (GH)-variant (placental GH) to GH-binding proteins in human plasma. *J Clin Endocrinol Metab* **73**:1175-1179.

Camarillo IG, Thordarson G, Ilkbahar YN and Talamantes F (1998) Development of a homologous radioimmunoassay for mouse growth hormone receptor. *Endocrinology* **139**:3585-3589.

Caufriez A, Frankenne F, Hennen G, and Copinschi G (1993) Regulation of maternal IGF-I by placental GH in normal and abnormal human pregnancies. *Am J Physiol* **265**:E572-577.

Cheung C, Yu AM, Chen CS, Krausz KW, Byrd LG, Feigenbaum L, Edwards RJ, Waxman DJ and Gonzalez FJ (2006) Growth hormone determines sexual

dimorphism of hepatic cytochrome P450 3A4 expression in transgenic mice. *J Pharmacol Exp Ther* **316**:1328-1334.

Contreras B and Talamantes F (1999) Growth hormone (GH) and 17beta-estradiol regulation of the expression of mouse GH receptor and GH-binding protein in cultured mouse hepatocytes. *Endocrinology* **140**:4725-4731.

Dai D, Bai R, Hodgson E and Rose RL (2001) Cloning, sequencing, heterologous expression, and characterization of murine cytochrome P450 3a25*(Cyp3a25), a testosterone 6beta-hydroxylase. *J Biochem Mol Toxicol* **15**:90-99.

Emoto C, Yamazaki H, Yamazaki S, Shimada N, Nakajima M and Yokoi T (2000) Characterization of cytochrome P450 enzymes involved in drug oxidations in mouse intestinal microsomes. *Xenobiotica **30***:943-953.

Frankenne F, Scippo ML, Van Beeumen J, Igout A, and Hennen G (1990) Identification of placental human growth hormone as the growth hormone-V gen expression product. *J Clin Endocrinol Metab* **71**:15-18.

Hebert M, Unadkat J, Carr DB, Kirby B, Eckert L and Easterling TR (2006) The effects of pregnancy on cytochrome P450 3A (CYP3A) and P-glycoprotein (P-gp) activity. SGI annual meeting. March 22-25. Toronto, Canada.

Jarukamjorn K, Sakuma T, Jaruchotikamol A, Ishino Y, Oguro M, and Nemoto N (2006) Modified expression of cytochrome P450 mRNAs by growth hormone in mouse liver. *Toxicology* **219**:97-105.

Kim AE, Dintaman JM, Waddell DS and Silverman JA (1998) Saquinavir, an HIV protease inhibitor, is transported by P-glycoprotein. *J Pharmacol Exp Ther* **286**:1439-1445.

Lahuna O, Fernandez L, Karlsson H, Maiter D, Lemaigre P, Rousseau G, Gustafsson J and Mode A (1997) Expression of hepatocyte nuclear factor 6 in rat liver is sex-dependent and regulated by growth hormone. *Proc Natl Acad Sci USA* **94**:12309-12313.

Legraverend C, Eguchi H, Strom A, Lahuna O, Mode A, Tollet P, Westin S, and Gustafsson JA (1994) Transcriptional activation of the rat CYP2C13 gene promoter involves HNF-1, HNF-3, and members of the orphan receptor subfamily. *Biochemistry* **33**:9889-9897.

Liddle C, Goodwin BJ, George J, Tapner M, and Farrell GC (1998) Separate and interactive regulation of cytochrome P450 3A4 by triiodothyronine, dexamethasone, and growth hormone in cultured hepatocytes. *J Clin Endocrinol Metab* **83**: 2411-2416.

Lown KS, Kolars JC, Thummel KE, Barnett JL, Kunze KL, Wrighton SA and Watkins PB (1994) Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. Lack of prediction by the erythromycin breath test. *Drug Metab Dispos* **22**:947-955.

MacFarland A, Abramovich DR, Ewen SW and Pearson CK (1994) Stage-specific distribution of P-glycoprotein in first-trimester and full-term human placenta. *Histochem J* **26**:417-423.

Mathias AA, Hitti J and Unadkat JD (2005) P-glycoprotein and breast cancer resistance protein expression in human placentae of various gestational ages. *Am J Physiol Regul Integr Comp Physiol* **289**:R963-969.

Mathias AA, Maggio-Price L, Lai Y, Gupta A and Unadkat JD (2006) Changes in Pharmacokinetics of Anti-Hiv Protease Inhibitors During Pregnancy: The Role of Cyp3a and P-Glycoprotein. *J Pharmacol Exp Ther* **316**:1202-1209.

McCormack JT and Greenwald GS (1974) Progesterone and oestradiol-17beta concentrations in the peripheral plasma during pregnancy in the mouse. *J Endocrinol* **62**:101-107.

Mofenson LM (2003) Advances in the prevention of vertical transmission of human immunodeficiency virus. *Semin Pediatr Infect Dis* **14**:295-308.

Nellen JF, Schillevoort I, Wit FW, Bergshoeff AS, Godfried MH, Boer K, Lange JM, Burger DM and Prins JM (2004) Nelfinavir plasma concentrations are low during pregnancy. *Clin Infect Dis* **39**:736-740.

Sakuma T, Endo Y, Mashino M, Kuroiwa M, Ohara A, Jarukamjorn K and Nemoto N (2002) Regulation of the expression of two female-predominant CYP3A mRNAs (CYP3A41 and CYP3A44) in mouse liver by sex and growth hormones. *Arch Biochem Biophys* **404**:234-242.

Sakuma T, Kitajima K, Nishiyama M, Endo Y, Miyauchi K, Jarukamjorn K and Nemoto N (2004) Collaborated regulation of female-specific murine Cyp3a41 gene expression by growth and glucocorticoid hormones. *Biochem Biophys Res Commun* **314**:495-500.

Tirona RG and Kim RB (2005) Nuclear receptors and drug disposition gene regulation. *J Pharm Sic* **94**:1169-1186

Tracy TS, Venkataramanan R, Glover DD, Caritis SN; National Institute for Child Health and Human Development Network of Maternal-Fetal-Medicine Units (2005)

Temporal changes in drug metabolism (CYP1A2, CYP2D6 and CYP3A activity) during pregnancy. *Am J Obstet Gynecol* **192**:633-639.

Thorne C and Newell ML (2005) The safety of antiretroviral drugs in pregnancy. *Expert Opin Drug Saf* **4**:323-335.

Unadkat JD, Wara DW, Hughes MD, Mathias AA, Holland DT, Paul ME, Connor J, Huang S, Nguyen BY, Watts DH, Mofenson LM, Smith E, Deutsch P, Kaiser KA, Tuomala RE (2007) Pharmacokinetics and safety of indinavir in human immunodeficiency virus-infected pregnant women. *Antimicrob Agents Chemother* **51**:783-786.

van Heeswijk RP, Veldkamp A, Mulder JW, Meenhorst PL, Lange JM, Beijnen JH and Hoetelmans RM (2001) Combination of protease inhibitors for the treatment of HIV-1-infected patients: a review of pharmacokinetics and clinical experience. *Antivir Ther* **6**:201-229.

Wang H, Wu X, Hudkins K, Mikheev A, Zhang H, Gupta A,Unadkat JD and Mao Q (2006) Expression of the breast cancer resistance protein (BCRP1/Abcg2) in tissues from pregnant mice: effects of pregnancy and correlations with nuclear receptors. *Am J Physiol Endocrinol Metab* **291**:E1295-1304.

Yamada H, Gohyama N, Honda S, Hara T, Harada N and Oguri K (2002) Estrogen-dependent regulation of the expression of hepatic Cyp2b and 3a isoforms: assessment using aromatase-deficient mice. *Toxicol Appl Pharmacol* **180**:1-10.

Zaphiropoulos PG (2003) A map of the mouse Cyp3a locus. *DNA Seq* **14**:155-162.

Zhang W, Purchio AF, Chen K, Wu J, Lu L, Coffee R, Contag PR, and West DB (2003) A transgenic mouse model with a luciferase reporter for studying in vivo

transcriptional regulation of the human CYP3A4 gene. *Drug Metab Dispos* **31**:1054-1064.

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Footnotes

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Figure legends

Fig. 1. Hepatic Cyp3a activity and protein expression at different gestational ages in WT-mouse.

Hepatic S-9 fractions from FVB mice showed significantly greater Cyp3a activity (as measured by testosterone 6β hydroxylation) at gestational day 15 and 19 when compared with that in non-pregnant females (A). Quantification of the Western blots of hepatic S-9 fractions isolated from FVB mice showed significantly greater expression of Cyp3a at gestational day 10, 15 and 19 compared with that in non-pregnant females (B). Data are shown as mean ± SD (n = 5). * $p < 0.05$

Fig. 2. Hepatic Cyp3a transcript expression by real-time PCR at different gestational ages in WT-mouse.

Cyp3a16, Cyp3a41 and Cyp3a44 transcript levels were significantly greater at gestational day 10, 15 and 19 compared with that in non-pregnant females (A). In contrast, Cyp3a11 transcript level was significantly decreased at gestational day 10, 15, and 19, while that of Cyp3a25 was significantly decreased at day 10 and 15. Cyp3a13 transcript level was not significantly affected by pregnancy (B). Level of expression here and in subsequent figures is shown as fold-difference compared to the expression of the same gene in the calibrator. Data are shown as mean ± SD (n = 4). * $p < 0.05$

Fig. 3. CYP3A4 promoter activity, endogenous Cyp3a activity and endogenous Cyp3a41 expression in tissues from pregnant and non-pregnant CYP3A4-tg mice.

Compared with non-pregnant CYP3A4-tg mice (n=4), tissue homogenate from day 17-pregnant CYP3A4-tg mice (n=5) showed significantly greater luciferase

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activity in the liver (A), but not in the proximal small intestine or kidney (B). Liver S-9 fractions from pregnant CYP3A4-tg mice showed significantly higher Cyp3a activity (as measured by testosterone 6β-hydroxylation) than non-pregnant CYP3A4-tg mice (C). Hepatic mRNA expression of Cyp3a41 is significantly higher in pregnant than in non-pregnant CYPA4-tg mice (D). Data are shown as mean±SD. *** $p < 0.001$, * $p < 0.05$

Fig. 4. Transcript expression of hepatic nuclear transcription factors by real-time PCR at different gestational ages in WT-mouse.

When compared with that in non-pregnant females, hepatic HNF3β transcripts were significantly greater at gestational day 10, HNF6 transcripts were significantly greater at gestational day 10 and day 15, and GhR transcript levels were significantly greater at gestational day 10, 15 and 19 (A). In contrast, hepatic CAR transcripts were significantly decreased at gestational day 10 and day 15 (B). No change in the hepatic expression of PXR and HNF4α (B) or RARα, RXRα and HNF1α (C) was observed during pregnancy. Data are shown as mean±SD (n=4). * $p < 0.05$

Fig. 5. Intestinal Cyp3a protein and transcript expression at different gestational ages (n = 4, mean ± SD) in WT-mouse.

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). Dashed line indicates the detection

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limit of the real-time PCR, which is defined as the Ct value of calibrator mRNA without reverse transcription, which is usually around 37 under our experimental conditions. Data are shown as mean±SD (n=4).

Fig. 6. P-gp expression in the liver, kidney and placenta in WT-mouse.

Expression of P-gp protein in the live and kidney was not affected by pregnancy, however, placental P-gp expression significantly decreased as pregnancy proceeded (A). *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 (B, C). Data are shown as mean±SD (n=4). * $p < 0.05$

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Table 1. Correlations (r_s) of hepatic mRNA expression between Cyp3a isoforms and transcriptional factors in pregnant (n=4) and non-pregnant (n=4) WT-mice.

		Cyp3a41	Cyp3a44	Cyp3a16	Cyp3a13	Cyp3a11	Cyp3a25
PXR	r_s[1]	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 PR 2	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

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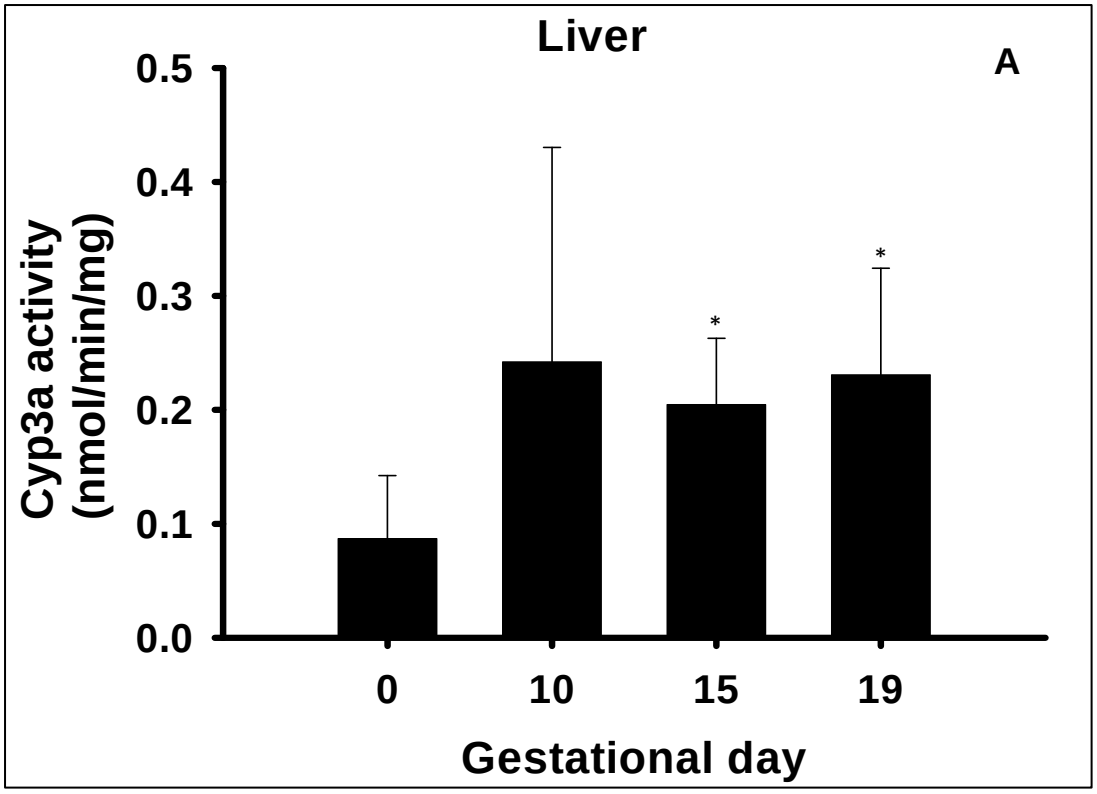
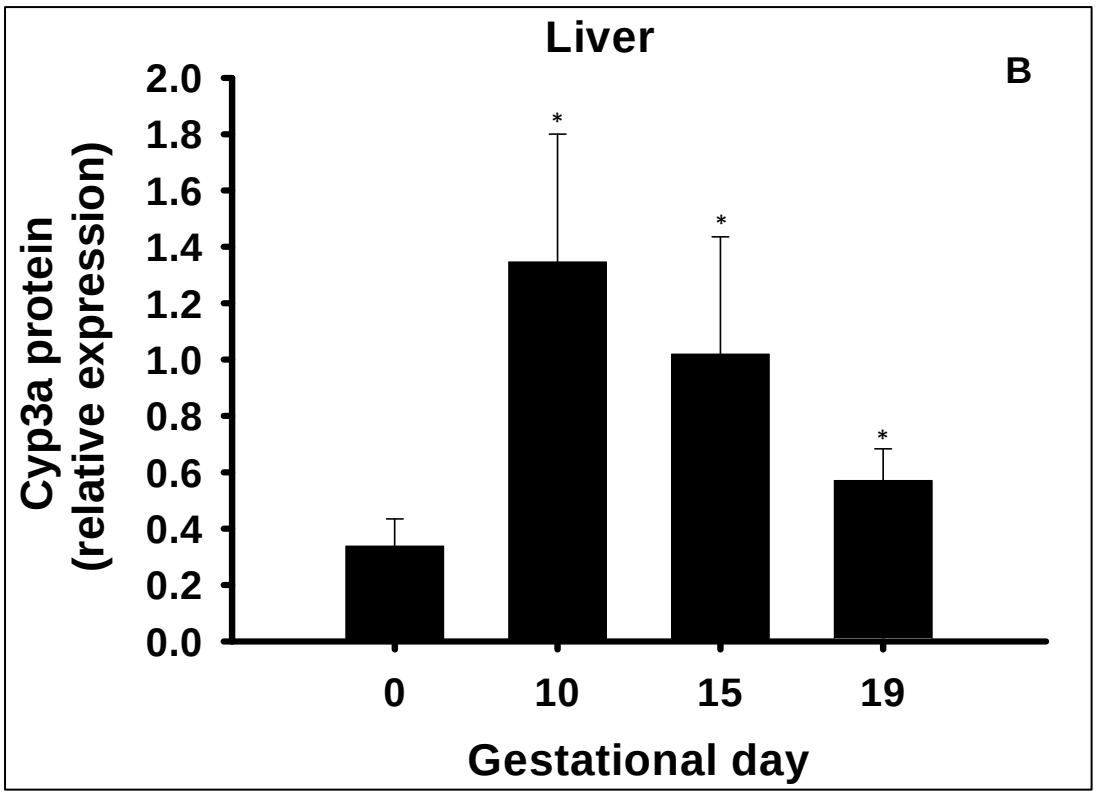
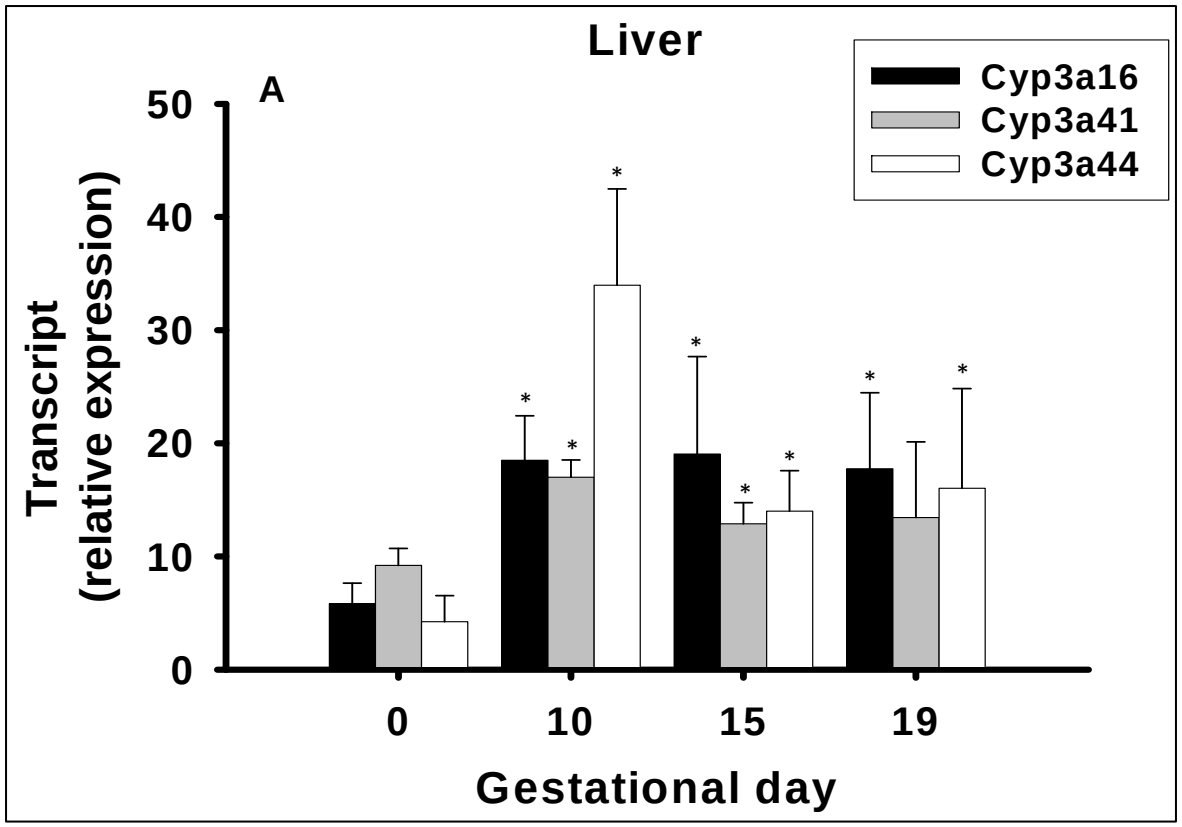
	p	0.73	0.85	0.39	0.80	0.57	0.84
HNF1α	r_s	0.11	0.19	0.21	-0.17	-0.23	-0.03
	p	0.68	0.49	0.43	0.53	0.39	0.91
HNF3β	r_s	0.44	0.35	0.15	-0.35	-0.30	-0.32
	p	0.08	0.19	0.57	0.18	0.26	0.22
HNF4α	r_s	0.04	0.28	-0.16	0.32	0.36	0.30
	p	0.88	0.29	0.55	0.23	0.17	0.39
HNF6	r_s	0.56	0.61	0.44	-0.21	-0.56	-0.57
	p	0.02*	0.01*	0.09	0.44	0.02*	0.02*

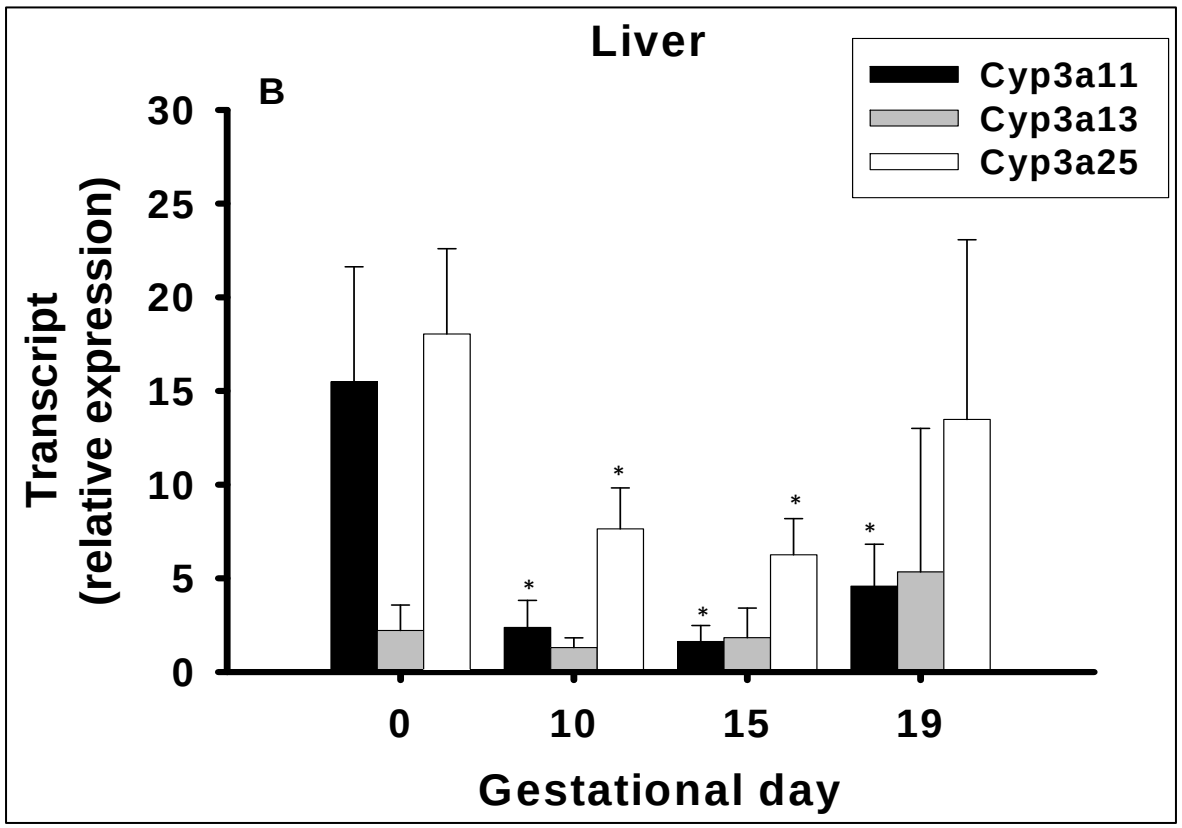
PXR, pregnane X receptor; CAR, constitutive androstane receptor; AhR, aryl hydrocarbon receptor; ER, estrogen receptor; HIF, hypoxia-inducible factor; mem PR, membrane progesterone receptor; GhR, growth hormone receptor; RAR, retinoic acid receptor; RXR, retinoic X receptor; HNF, hepatocyte nuclear factor.

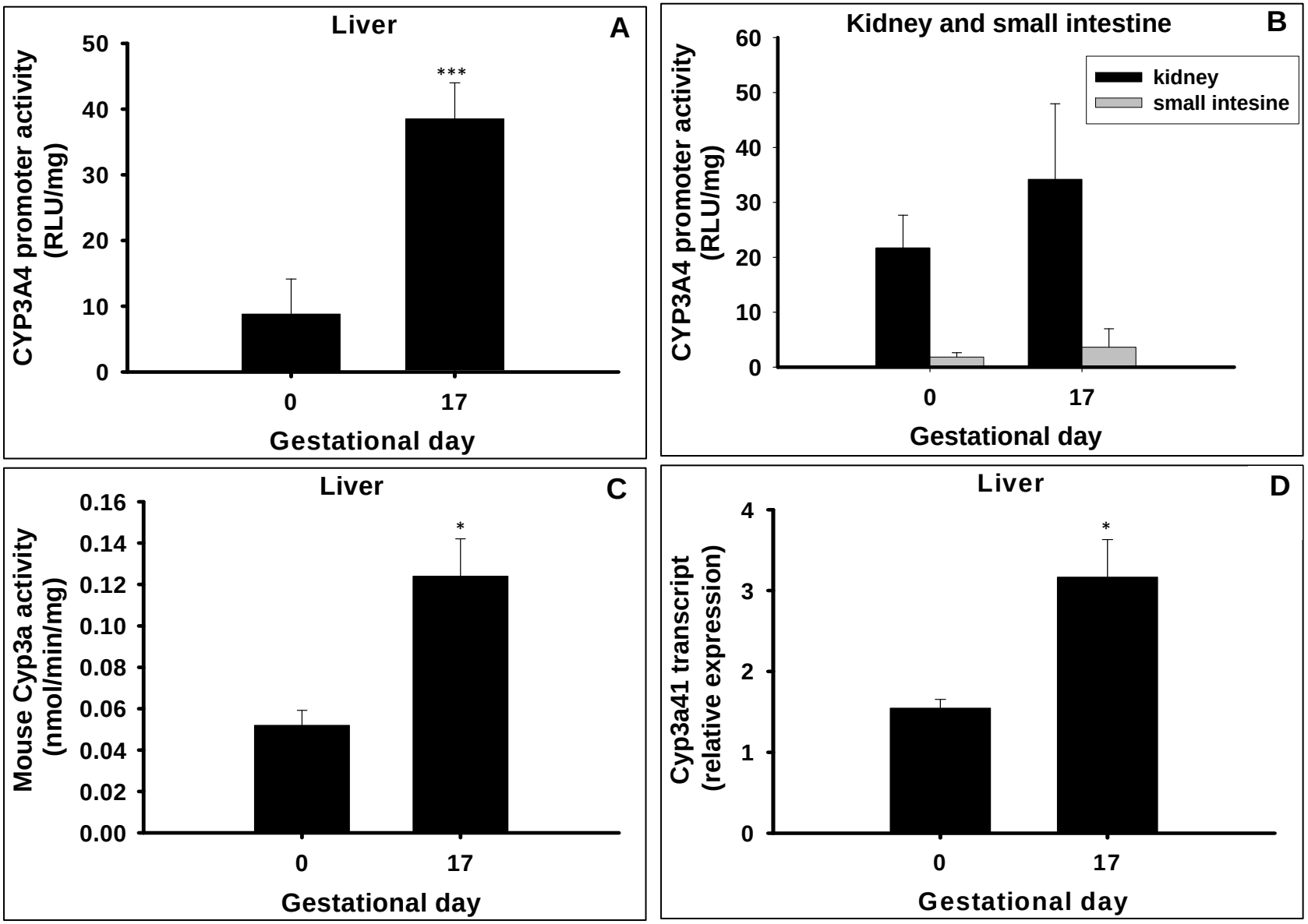
[1], Spearman rank correlation coefficient; * significant correlation.

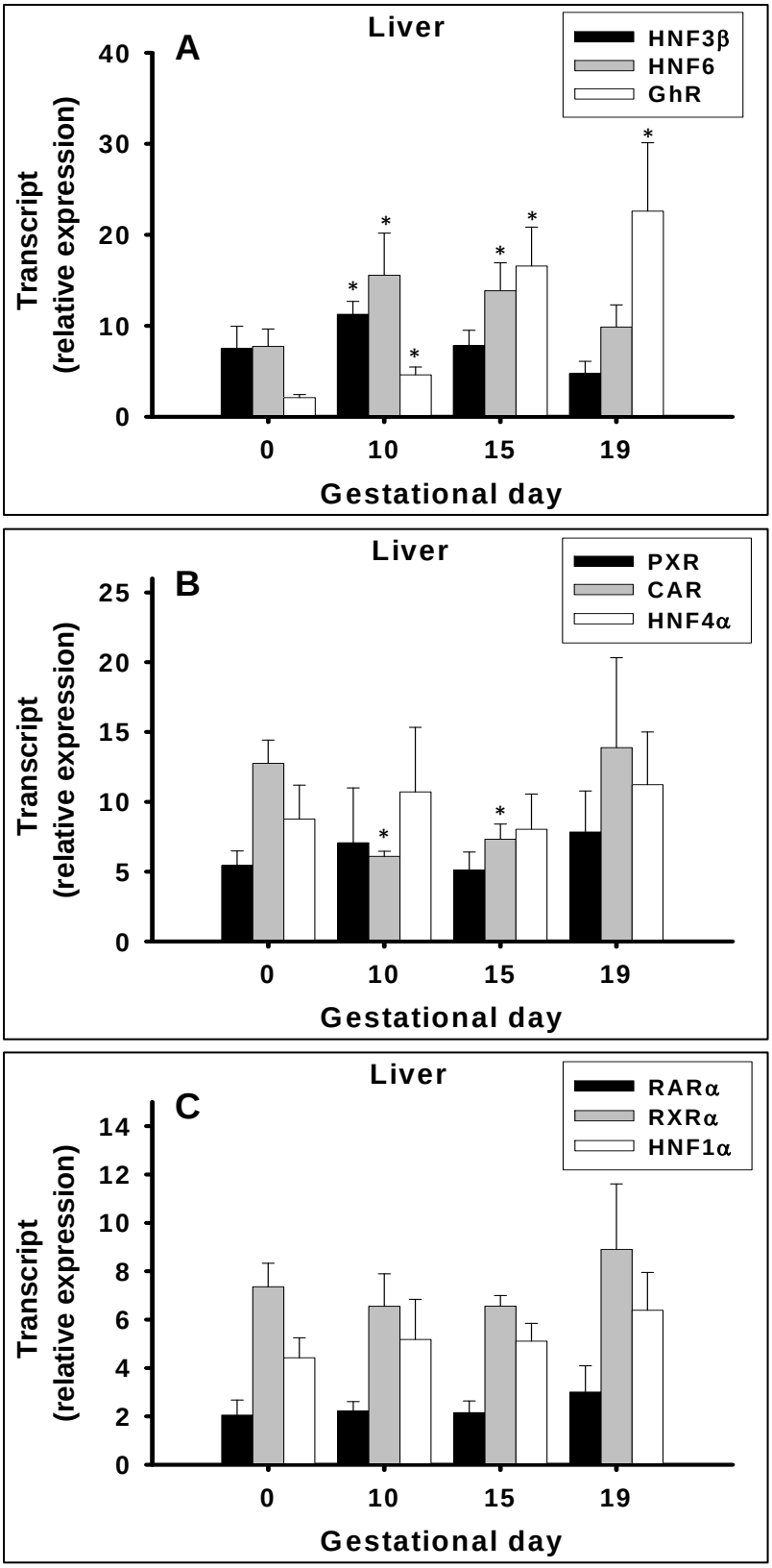

Liver
A
Cyp3a activity
(nmol/min/mg)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
0
10
15
19
Gestational day

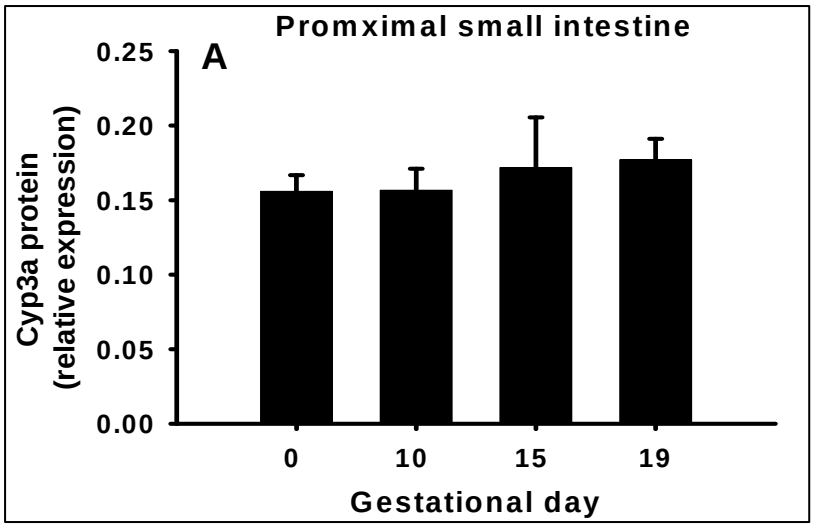

Liver
B
Cyp3a protein
(relative expression)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
0
10
15
19
Gestational day

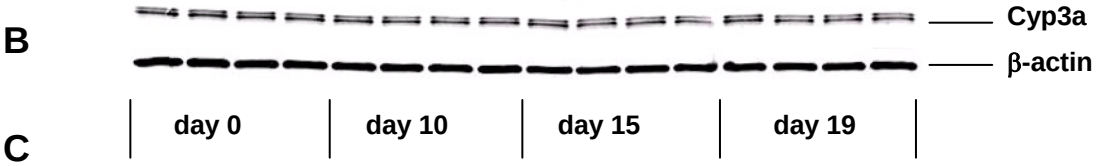

Liver
A
Cyp3a16
Cyp3a41
Cyp3a44
Transcript
(relative expression)
50
40
30
20
10
0
0
10
15
19
Gestational day


Liver
B
Cyp3a11
Cyp3a13
Cyp3a25
Transcript
(relative expression)
30
25
20
15
10
5
0
0
10
15
19
Gestational day

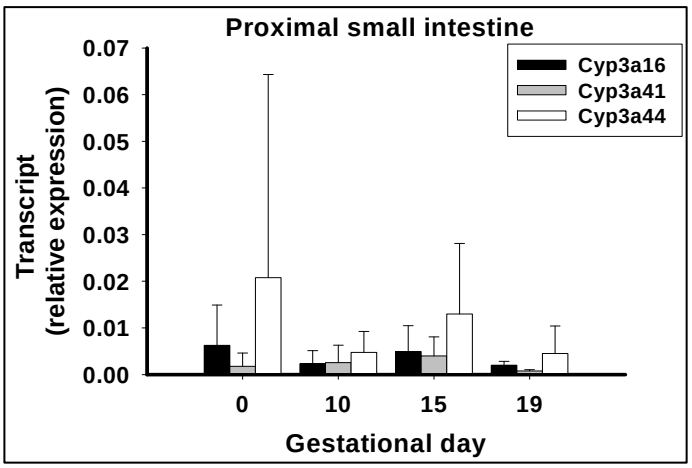

Liver
A
CYP3A4 promoter activity (RLU/mg)
50
40
30
20
10
0
***
0
17
Gestational day
Kidney and small intestine
B
CYP3A4 promoter activity (RLU/mg)
60
50
40
30
20
10
0
kidney
small intesine
0
17
Gestational day
Liver
C
Mouse Cyp3a activity (nmol/min/mg)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
0
17
Gestational day
Liver
D
Cyp3a41 transcript (relative expression)
4
3
2
1
0
*
0
17
Gestational day

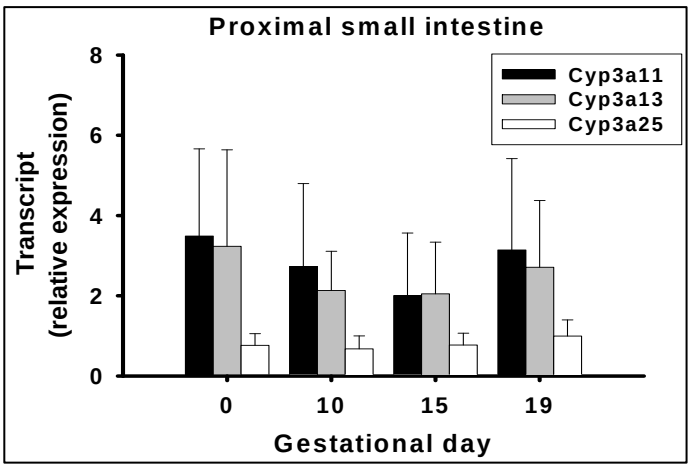

A
Liver
HNF3β
HNF6
GhR
Transcript (relative expression)
40
30
20
10
0
0
10
15
19
Gestational day
B
Liver
PXR
CAR
HNF4α
Transcript (relative expression)
25
20
15
10
5
0
0
10
15
19
Gestational day
C
Liver
RARα
RXRα
HNF1α
Transcript (relative expression)
14
12
10
8
6
4
2
0
0
10
15
19
Gestational day

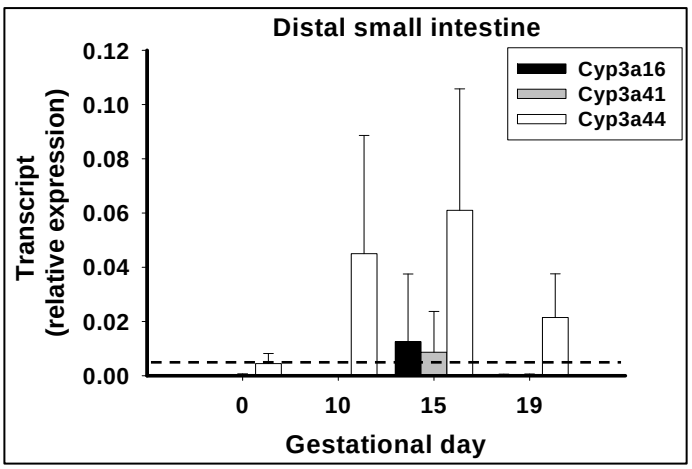

Promximal small intestine
A
Cyp3a protein
(relative expression)
0.25
0.20
0.15
0.10
0.05
0.00
0
10
15
19
Gestational day

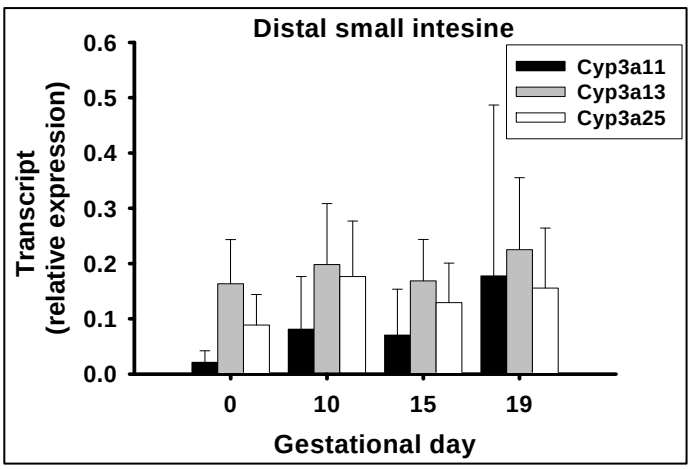

B
Cyp3a
β-actin
day 0
day 10
day 15
day 19

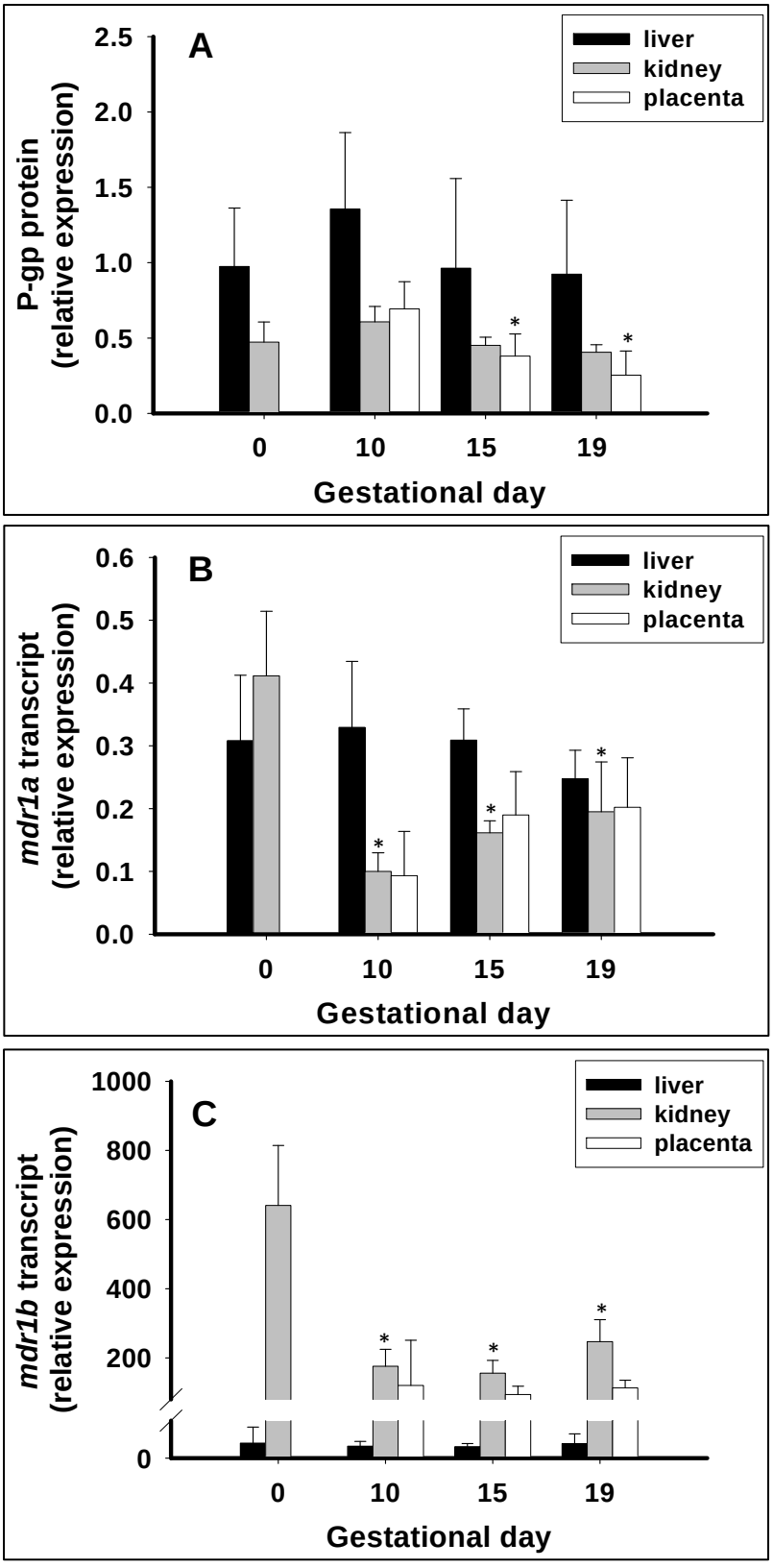
C


Proximal small intestine
Transcript
(relative expression)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Cyp3a16
Cyp3a41
Cyp3a44
0
10
15
19
Gestational day


Proximal small intestine
Transcript
(relative expression)
8
6
4
2
0
Cyp3a11
Cyp3a13
Cyp3a25
0
10
15
19
Gestational day


Distal small intestine
Transcript
(relative expression)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Cyp3a16
Cyp3a41
Cyp3a44
0
10
15
19
Gestational day


Distal small intesine
Transcript
(relative expression)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Cyp3a11
Cyp3a13
Cyp3a25
0
10
15
19
Gestational day


A
liver
kidney
placenta
P-gp protein
(relative expression)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
0
10
15
19
Gestational day
B
liver
kidney
placenta
mdr1a transcript
(relative expression)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
0
10
15
19
Gestational day
C
liver
kidney
placenta
mdr1b transcript
(relative expression)
1000
800
600
400
200
0
*
*
*
0
10
15
19
Gestational day