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Gestational Age-Dependent Changes in Gene Expression of Metabolic Enzymes and Transporters in Pregnant Mice[S]

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ABSTRACT

Pregnancy-induced changes in drug pharmacokinetics can be explained by changes in expression of drug-metabolizing enzymes and transporters and/or normal physiology. In this study, we determined gestational age-dependent expression profiles for all metabolic enzyme and transporter genes in the maternal liver, kidney, small intestine, and placenta of pregnant mice by microarray analysis. We specifically examined the expression of genes important for xenobiotic, bile acid, and steroid hormone metabolism and disposition, namely, cytochrome P450s (*Cyp*), UDP-glucuronosyltransferases (*Ugt*), sulfotransferases (*Sult*), and ATP-binding cassette (*Abc*), solute carrier (*Slc*), and solute carrier organic anion (*Slco*) transporters. Few *Ugt* and *Sult* genes were affected by pregnancy. *Cyp17a1* expression in the maternal liver increased 3- to 10-fold during pregnancy, which was the largest observed change in the maternal tissues. *Cyp1a2*, most *Cyp2* isoforms, *Cyp3a11*, and *Cyp3a13* expression in the liver decreased on gestation days (gd) 15 and 19 compared with nonpregnant controls (gd 0). In contrast, *Cyp2d40*, *Cyp3a16*, *Cyp3a41a*, *Cyp3a41b*, and *Cyp3a44* in the liver were induced throughout pregnancy. In the placenta, *Cyp* expression on gd 10 and 15 was upregulated compared with gd 19. Notable changes were also observed in *Abc* and *Slc* transporters. *Abcc3* expression in the liver and *Abcb1a*, *Abcc4*, and *Slco4c1* expression in the kidney were downregulated on gd 15 and 19. In the placenta, *Slc22a3* (*Oct3*) expression on gd 10 was 90% lower than that on gd 15 and 19. This study demonstrates important gestational age-dependent expression of metabolic enzyme and transporter genes, which may have mechanistic relevance to drug disposition in human pregnancy.

Introduction

Significant physiologic changes during pregnancy are essential to support and protect the developing fetus (Carlin and Alfirevic, 2008). Changes in maternal physiology include, among others, increased renal blood flow and glomerular filtration rate (GFR) (Davison and Hytten, 1975), as well as increased portal vein blood flow (Carlin and Alfirevic, 2008). These physiologic changes alter important drug pharmacokinetics (PK) determinants such as GFR, oral absorption, plasma volume, and plasma protein binding (Anderson, 2005; Anger and Piquette-Miller, 2008; Klieger et al., 2009). Considerable data in the literature also suggest that expression and activities of drug-metabolizing enzymes and transporters are altered during pregnancy (Tracy et al., 2005; Hebert et al., 2008; Feghali and Mattison, 2011). In humans, cytochrome P450 (CYP) enzymes such as CYP2A6, CYP3A4, CYP2D6, and CYP2C9 demonstrate increased activities during pregnancy, whereas the activities of CYP1A2 and CYP2C19 are decreased (Dempsey et al., 2002; Anderson, 2005; Hodge and Tracy, 2007). These changes result in altered drug PK during pregnancy (Unadkat et al., 2007; Hebert et al., 2009).

The trends in human pregnancy regarding expression/activity of metabolic enzymes and drug transporters appear to be similar in pregnant mice. Hepatic CYP3A activity in pregnant mice, for example, is increased to a similar extent as CYP3A in humans (compared with nonpregnant controls) (Mathias et al., 2006; Zhang et al., 2008; Zhou et al., 2010). In addition, hepatic *Cyp1a2* mRNA is decreased during mouse pregnancy, which is consistent with CYP1A2 activity in pregnant women (Tracy et al., 2005; Koh et al., 2011).

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ABBREVIATIONS: ABC, ATP-binding cassette; CYP, cytochrome P450; FDR, false discovery rate; gd, gestation day; GFR, glomerular filtration rate; P-gp, P-glycoprotein; PK, pharmacokinetics; qRT-PCR, quantitative real-time polymerase chain reaction; SLC, solute carrier; UGT, UDP-glucuronosyltranserases.

Fewer studies have been done in humans and animals to investigate changes in activity of drug transporters in the liver, kidney, and small intestine during pregnancy. P-glycoprotein (P-gp) is perhaps the most studied of all transporters, but discrepancies exist between mice and humans. In humans, P-gp–mediated renal secretion of digoxin increased during late gestation (Hebert et al., 2008). However, during mouse pregnancy, not only were P-gp protein levels unaffected in the liver, kidney, and small intestine, but mRNA expression actually decreased in the kidney (Mathias et al., 2006; Zhang et al., 2008). Despite this difference, the role of ATP-binding cassette (ABC) transporters (i.e., P-gp and breast cancer resistance protein) in determining fetal drug exposure has been extensively studied in mouse models because of the similar efflux function of these transporters in mouse and human placenta (Smit et al., 1999; Jonker et al., 2000; Zhang et al., 2007; Zhou et al., 2008).

Current research merely provides snapshots of gene expression or protein activity during pregnancy (Zhou et al., 2008; Zhou et al., 2010). Only in the last several years has there been any attempt to understand gene or protein expression throughout gestation (Wang et al., 2006; Aleksunes et al., 2008; Zhang et al., 2008; Koh et al., 2011). This body of research targeted specific CYP isoforms, as well as important solute carrier (SLC) and ABC transporters. None of these studies, however, offer a comprehensive overview of trends in expression as gestation progresses for *all* mouse isoforms related to drug metabolism and disposition. After considering the similarities between pregnant mice and women, we chose the pregnant mouse as an appropriate animal model to study gestational age-dependent changes in expression of drug disposition genes. Therefore, the goal of this study was to analyze the global gene expression profiles of maternal tissues and placenta at different gestational ages using microarray approaches.

Although the focus of our study was to investigate gestational age-dependent changes in metabolic enzymes and transporters relevant to drug disposition, we could not neglect the fact that changes in *Cyp*, *Abc*, *Slc*, or *Slco* gene expression during pregnancy could potentially impact the homeostasis of endogenous substances such as bile acids and steroid hormones. Characterization of genes involved in bile acid synthesis and distribution during pregnancy may provide a physiologic basis for understanding complications that arise during pregnancy such as intrahepatic cholestasis of pregnancy. Characterization of steroid hormone production during pregnancy supports mechanistic explanations of gestational age-dependent expression of metabolic enzymes and transporters. Thus, we systematically investigated expression of all metabolic enzymes and transporters in the mouse maternal tissues and placenta across gestation, demonstrating significant changes in the expression of many genes including those important for drug, bile acid, and steroid hormone metabolism and transport. These data also provide novel insights into potential changes in drug PK during pregnancy, and support the growing foundation of evidence clinicians need to make decisions regarding dosage selection during pregnancy.

Materials and Methods

Animal Studies. FVB wild-type mice, aged 7–10 weeks, were purchased from Taconic Farms (Hudson, NY), and cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council. The animal protocol for this project was approved by the Institutional Animal Care and Use Committee at the University of Washington (protocol number 4035-01). Briefly, mice were maintained under 12-hour light/dark cycles, and food was provided ad libitum. Female mice, aged 7–10 weeks, were mated with male mice of the same age overnight. Gestation day (gd) 1 was defined as the presence of a sperm plug after overnight housing; gd 0 was defined as nonpregnant mice. Progression of pregnancy was monitored by visual inspection and body weight increase. On gd 0, 7.5, 10, 15, and 19, female mice (n = 5–6 per gestational age) were sacrificed under anesthesia (isoflurane) by cardiac puncture, and the maternal liver, kidney, and small intestine were collected. The placentas were collected on gd 10, 15, and 19. All tissues were rinsed with phosphate-buffered saline, snap-frozen in liquid nitrogen, and stored at −80°C until use.

Total RNA Extraction. Total RNA was isolated from the snap-frozen mouse tissues using the miRNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions for purification of total RNA from animal tissues. The integrity of RNA samples was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA),which is the recognized standard in the field. RNA integrity was evaluated using the RNA integrity number and by observing distinct and sharp 18s and 28s ribosomal RNA peaks. The RNA integrity number of all samples was greater than 9. RNA quantity and purity were determined with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) by measuring optical density (OD_{260}), as well as $OD_{260/280}$ and $OD_{260/230}$ ratios, respectively. Samples with $OD_{260/280}$ ratios greater than 1.9 and $OD_{260/230}$ ratios greater than 1.6 were considered acceptable for further processing. Five RNA samples per gestation day that passed these stringent quality control measures were selected from each tissue group for further processing.

Microarray Hybridization and Data Analysis. Processing of the RNA samples was carried out according to the AffymetrixGeneChip Whole Transcript Sense Target labeling protocol (http://www.affymetrix.com/index.affx) as reported previously (Anderson et al., 2011). The arrays were scanned with an AffymetrixGeneChip 3000 scanner. Image generation and feature extraction were performed using the AffymetrixGeneChip Command Console software. Affymetrix Mouse Gene 1.0 ST Arrays were used for this study. The output files from AffymetrixGeneChip Command Console were further processed using the Bioconductor oligo package (Carvalho and Irizarry, 2010). Raw data were normalized using quantile normalization and then summarized at the transcript level using a robust multi-array average method (Irizarry et al., 2003). For the maternal tissues, we made univariate comparisons of gd 7.5, 10, 15, and 19 to gd 0 using the Bioconductor limma package, which fits an analysis of variance model to the data and then computes individual contrasts. For the placenta, univariate comparisons of gd 10 and 15 to gd 19 were also made using Bioconductor. The limma package uses an empirical Bayes adjustment on the variance estimate (based on all genes on the array) to increase power to detect true differences (Smyth, 2004). P values were adjusted for multiplicity with the program q-value, which allows the selection of statistically significant genes while controlling the estimated false discovery rate (FDR) (Tusher et al., 2001; Dabney and Storey, 2006).

The adjusted P values or the FDR values are only interpretable when genes are ranked by unadjusted P values, from the smallest to the largest. The corresponding FDR value for an unadjusted P value of 0.05 varies for each gestational age and tissue. A given FDR value states that the group of genes listed above the corresponding unadjusted P value cut-off has a particular FDR associated with it. This FDR value represents a percentage of genes within the group list whose fold changes in expression are there by chance alone (i.e., false positive results). FDR values are therefore displayed as supplemental material for each gestation day comparison with gd 0 for maternal tissues or gd 19 for placenta (Supplemental Tables 1–14). Microarray data presented in this study were deposited in the National Center for Biotechnology Information Gene Expression Omnibus data repository under accession number GSE41438.

Filtration Criteria for Differentially Expressed Genes. Expression patterns of differentially expressed genes were visualized by creating heatmaps of relevant genes. Ordering of genes in these plots was determined by hierarchical clustering of the gene expression values.

For the overall gene expression trends shown in heatmaps, genes with a larger than 2-fold or less than 0.5-fold change in expression compared with gd 0, an unadjusted P value less than 0.05, and a $\log_2$ average fluorescent intensity greater than 4, for at least one gestation day, were considered to be differentially expressed for the maternal liver, kidney, and small intestine. Because the placenta is an organ undergoing development as gestation progresses, the number of differentially expressed genes in this tissue, as determined using the above criteria, was much larger than the number of genes

in the maternal tissues. To limit the number of differentially expressed genes in the placenta to something comparable to the maternal tissues, we implemented a more stringent statistical criteria, namely, genes with a larger than 2-fold or less than 0.5-fold change on gd 10 or 15 compared with gd 19, an unadjusted *P* value less than 0.00005, and a $\log_2$ average fluorescent intensity greater than 4, for at least one gestation day. Fluorescent intensity is an indication of signal strength and data integrity. Higher fluorescent intensity ensures that a detected change is not background noise and is therefore an important factor to consider before further examination of gene expression data.

For changes in gene expression shown in relevant tables, expression of metabolic enzyme and transporter genes, namely, *Cyp*, *Ugt*, and *Sult* enzymes as well as *Abc*, *Slc*, and *Slco* transporters, were filtered based on a lower fold change criteria that included genes demonstrating larger than 1.5-fold or less than 0.65-fold changes in expression, as well as unadjusted *P* values less than 0.05 for maternal tissues and placenta, and average $\log_2$ fluorescent intensities greater than 4, for at least one gestation day compared with gd 0 (maternal tissues) or gd 19 (placenta).

To examine *Cyp*, *Abc*, and *Slc* genes that are important for drug metabolism and transport, but do not meet previous filtration criteria needed for inclusion in tables, we selected specific genes known to be involved in drug metabolism and disposition (e.g., *Cyp1*, *Cyp2*, and *Cyp3* isoforms) and filtered them according to unadjusted *P* values less than 0.01 and $\log_2$ average fluorescent intensities greater than 4, for at least one gestation day compared with gd 0 for maternal tissues and gd 19 for placenta. Fold changes in expression of these genes were smaller, but are meaningful to understand drug PK during pregnancy and were therefore presented in relevant figures.

Quantitative Real-Time Polymerase Chain Reaction Validation. To validate microarray gene expression data, quantitative real-time polymerase chain reaction (qRT-PCR) was used. We selected three genes from the 25 maternal liver tissues ($n = 5$ for gd 0, 7.5, 10, 15, and 19) and seven genes from the 15 placental tissues ($n = 5$ for gd 10, 15, and 19), which totaled 180 microarray gene expression data for validation. The three genes selected for RNA quantification in the maternal liver were *Abcc3*, *Cyp17a1*, and *Cyp2d40*. The seven genes selected for RNA quantification in the placenta were *Abcb1a*, *Abcb1b*, *Abcg2*, *Cyp2s1*, *Slc22a3*, *Slco1a4*, and *Slco2b1*. These genes were selected because they either demonstrated large changes in the microarray data or are important for xenobiotic metabolism and transport. Fluorogenic 5′ nuclease-based assays were employed as previously described (Anderson et al., 2011; Cole et al., 2011; Stamper et al., 2011). *Actb* (*β-actin*) amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula to calculate expression levels. Variations in *β-actin* gene expression throughout gestation were small (<25% in the maternal liver and <18% in the placenta) (data not shown), and *β-actin* was therefore selected as an internal control to normalize the data for the respective tissue.

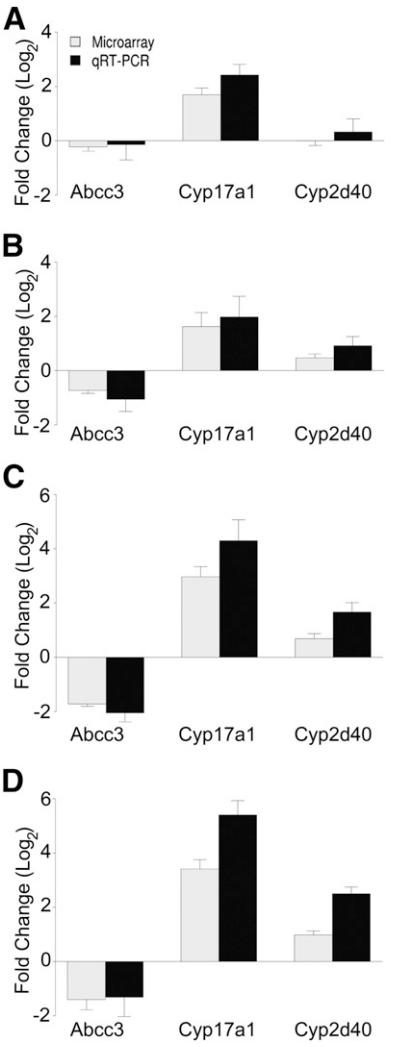

Fig. 1. Comparison of microarray and qRT-PCR gene expression data in the maternal liver. Shown are means ± S.D. of gene expression data from five maternal liver samples on gd 7.5 (A), gd 10 (B), gd 15 (C), and gd 19 (D) compared with those on gd 0 and expressed as $\log_2$ fold changes.

Results

Validation of Microarray Data by qRT-PCR. Gene expression levels determined by microarray analysis correlated well with mRNA levels determined by qRT-PCR (Pearson's *R* coefficients of 0.99 and 0.95 for the liver and placenta, respectively) (Supplemental Fig. 1). A direct comparison of the approaches is shown in Figs. 1 and 2. *Cyp17a1*, *Cyp2d40*, and *Abcc3* (*Mrp3*) expression in the maternal liver is shown in Fig. 1; placental expression of *Cyp2s1*, *Abcb1a* (*Mdr1a*), *Abcb1b* (*Mdr1b*), *Abcg2* (*Bcrp1*), *Slc22a3* (*Oct3*), *Slco1a4* (*Oatp4c1*), and *Slco2b1* (*Oatp2b1*) is shown in Fig. 2. In both tissues, the absolute magnitude of change in expression was consistently greater for the qRT-PCR data compared with the microarray data. Both approaches also agreed on the direction of change (upregulation or downregulation), except for *Cyp2d40* on gd 7.5 in the maternal liver as well as *Abcg2* and *Slco1a4* on gd 15 in the placenta where changes in gene expression were small. Taken together, these results establish the accuracy and reliability of our microarray analysis.

Overall Gestational Age-Dependent Trends in Gene Expression. Hierarchical one-dimensional clustering of differentially expressed genes for each tissue is shown in Fig. 3. Gene expression levels in the liver and small intestine were similar between replicate samples within the same gestational age group. Changes in expression in the liver and small intestine (upregulation or downregulation) were more robust as gestation progressed, peaking on gd 15 and/or gd 19 (Fig. 3, A and C). Interestingly, the kidney exhibited a bimodal pattern of gene expression that peaked on gd 10 and gd 19 (Fig. 3B). This apparent trend is the result of replicate samples gd 10_3 and gd 10_6. In addition, the expression on gd 15 in the placenta was not very different from term placenta (gd 19); however, large differences were visible on gd 10 compared with gd 19 (Fig. 3D). Figure 3D also shows that the pattern of expression between placenta sample replicates was very similar, except for gd 19_1, which appeared to be more robust in comparison with the other replicates in the gd 19 group. Although expression in the placenta was lower on gd 10, this is in comparison with gd 19 placenta levels, indicating that gene expression in the placenta is actually increasing throughout the course of pregnancy. All tissues demonstrated a preference for upregulation during pregnancy; however, the pattern of changes in gene expression throughout gestation was uniquely different for the kidney.

The filtration criteria used to generate heatmaps in Fig. 3 were also used to count the number of differentially expressed genes for each

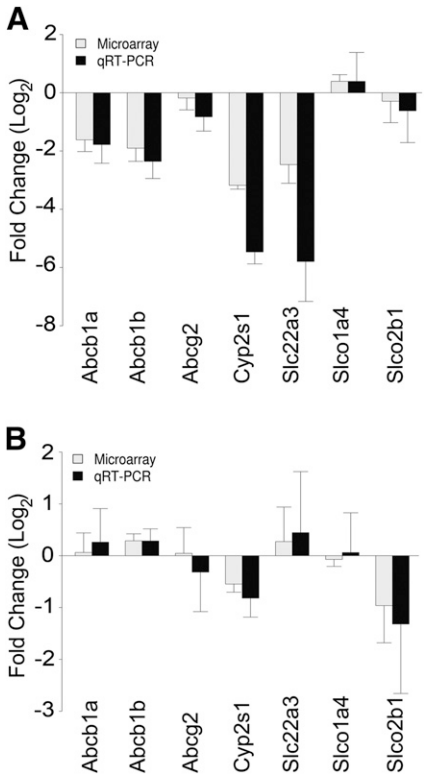

Fig. 2. Comparison of microarray and qRT-PCR gene expression data in the placenta. Shown are means ± S.D. of gene expression data from five placenta samples on gd 10 (A) and gd 15 (B) compared with those on gd 19 and expressed as $\log_2$ fold changes.

gestation day. In the liver, 56, 103, 216, and 138 genes were differentially expressed on gd 7.5, 10, 15, and 19, respectively. In the kidney, there were 17, 163, 38, and 74 genes differentially expressed on gd 7.5, 10, 15, and 19, respectively. The small intestine had 37, 55, 47, and 75 differentially expressed genes on gd 7.5, 10, 15, and 19, respectively. In the placenta, 856 genes were differentially expressed on gd 10, but only 8 genes were differentially expressed on gd 15. As a whole, this study identified 1883 differentially expressed genes in the maternal tissues and placenta.

Drug Metabolism during Pregnancy. Major gene families involved in drug metabolism (*Cyp*, *Ugt*, and *Sult*) were analyzed for gestational age-dependent changes in expression. Raw microarray data from these gene families were filtered based on three criteria. First, on at least one gestation day, fold changes in expression (compared with gd 0 for maternal tissues and gd 19 for placenta) had to be greater than 1.5 or less than 0.65. Second, these fold changes had to have a corresponding unadjusted *P* value that was less than 0.05. Third, for a particular fold-change that was significant, the associated average $\log_2$ fluorescent intensity had to be larger than 4. The resulting genes are shown in Table 1 and Supplemental Tables 15–17 for genes in the maternal liver, kidney, small intestine, and placenta, respectively.

In the maternal liver, the *Cyp* and *Slc* gene families were most significantly altered during pregnancy; however, only a few genes had larger than 2-fold changes in expression. By far the largest upregulation occurred for *Cyp17a1*, which was increased 3.3-fold, 8.2-fold, and 10.4-fold on gd 7.5/10, gd 15, and gd 19, respectively. *Cyp4a12a/b* was decreased by 70%–80% as gestation progressed, which was the largest downregulation in *Cyp* gene expression. *Cyp2b13*, *Cyp2c50*, *Cyp2c54*, *Cyp2c55*, *Cyp2d9*, and *Cyp4a12* expression were also decreased throughout gestation. Many of the *Cyp* isoforms thought to be involved in drug metabolism, such as the *Cyp1*, *Cyp2*, and *Cyp3* families, did not demonstrate large enough changes in gene expression to be included in Table 1. Therefore, time-dependent trends in the expression of *Cyp1*, *Cyp2*, and *Cyp3* families are shown in Fig. 4, which was generated with only two filtration criteria, namely, the unadjusted *P* value associated with a fold-change had to be less than 0.01 and the average $\log_2$ fluorescent intensity had to be greater than 4 for at least one gestational age. These criteria allowed us to examine with confidence smaller, but significant, changes in gene expression related to drug metabolism. The data in Fig. 4 indicate that many genes from the *Cyp1*, *Cyp2b*, *Cyp2c*, and *Cyp2d* families are downregulated as gestation progresses (Fig. 4, A–C). This pattern is also true for *Cyp3a11* (Fig. 4A). Whereas the majority of *Cyp* genes were downregulated during pregnancy, *Cyp2d40*, *Cyp3a16*, *Cyp3a41a*, *Cyp3a41b*, and *Cyp3a44* were upregulated (Fig. 4D). Overall, changes in hepatic gene expression of *Cyp* isoforms important for drug metabolism were rather small (downregulated by less than 50% or upregulated less than 1.5-fold).

Cyp expression in the maternal kidney was very dynamic during pregnancy (Supplemental Table 15). A few genes, namely *Cyp2d9*, *Cyp2d12*, and *Cyp3a41a/b*, were altered in a gestational age-dependent manner. *Cyp3a41a/b* was highly upregulated by approximately 12-fold on gd 19. In the small intestine, only *Cyp1a1*, *Cyp2a5*, *Cyp2c55*, and *Cyp2j6* displayed larger than 1.5-fold changes in expression during pregnancy (Supplemental Table 16). The *Cyp* isoforms that were most notably changed in the placenta during pregnancy are likely responsible for xenobiotic detoxification (*Cyp2s1*), arachidonic acid metabolism (*Cyp4a14*), bile acid synthesis (*Cyp7b1*), steroid biosynthesis (*Cyp11a1*, *Cyp11b1*, and *Cyp17a1*), and retinoic acid and vitamin D regulation (*Cyp26a1* and *Cyp2r1*) (Supplemental Table 17). In contrast to the liver, all *Cyp* enzymes in the placenta, except for *Cyp2s1* and *Cyp7b1*, were upregulated during mid-gestation (gd 10) and decreased to term expression levels by mid to late gestation (gd 15). Placental *Cyp1*, *Cyp2*, and *Cyp3* gene families were further evaluated for gestational age-dependent trends in expression (Fig. 4E). All *Cyp* isoforms in the placenta were upregulated on gd 10 compared with gd 19, with two exceptions. *Cyp1a1* peaked on gd 15 rather than gd 10, and *Cyp3a57* was downregulated on gd 10 (rather than upregulated) compared with gd 19.

Across tissues, *Sult* and *Ugt* expression were relatively unaffected by pregnancy. In the maternal liver, *Sult1d1* was decreased by as much as 60% on gd 15 (Table 1). In the kidney and small intestine, only a few *Sult* and *Ugt* isoforms were altered during pregnancy, but their relevance to drug metabolism is not well understood (Supplemental Tables 15–17). In the placenta, *Sult1a1*, *Sult5a1*, and *Ugt1a9* were only affected on gd 10 during pregnancy (Supplemental Table 17). Specifically, *Sult1a1* was downregulated by 50% on gd 10 compared with gd 19, and *Sult5a1* and *Ugt1a9* were upregulated more than 2-fold. Overall, the number of *Sult* and *Ugt* genes in the maternal tissues and placenta affected by pregnancy was small, but the magnitude of changes in expression for these genes was quite large.

Drug Transport during Pregnancy. Gestational age-dependent expression of important drug transporter genes such as *Abc*, *Slc*, and *Slco* transporters were characterized using the same fold change, unadjusted *P* value, and fluorescent intensity filtration criteria as the metabolic enzymes. *Abc* transporter expression in maternal tissues appeared relatively unaffected by pregnancy compared with non-pregnant controls (gd 0), except for *Abcc3* (*Mrp3*) and *Abcb1a/1b* (mouse *P-gp* or *Mdr1a/1b*) (Table 1; Supplemental Tables 15 and 16). In the maternal liver, expression of *Abcc3* and *Abcb1a* was decreased by approximately 70% and 40%, respectively, on gd 15. When the

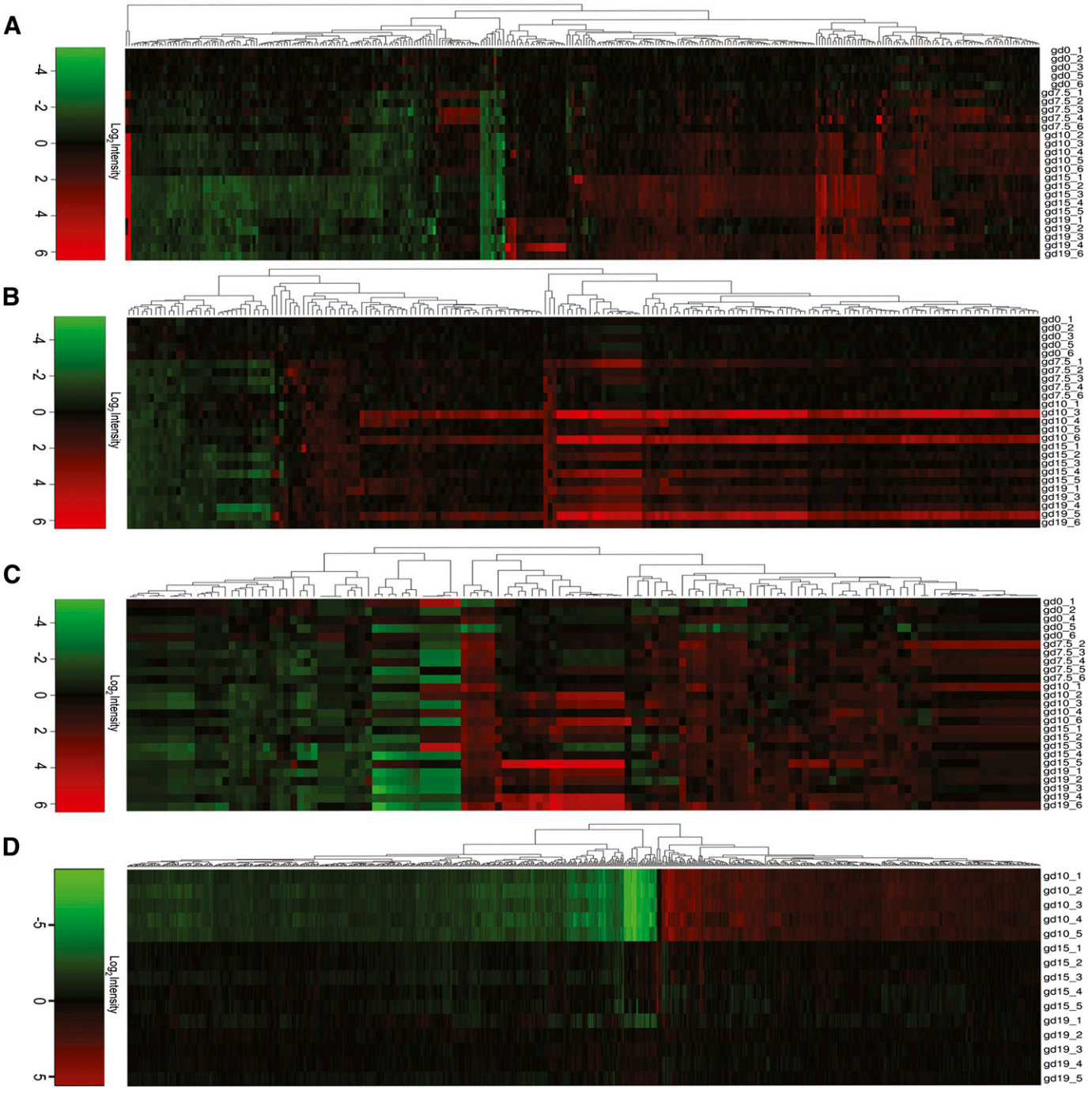

Fig. 3. One-dimensional heatmaps of differentially expressed genes. Liver (A), kidney (B), small intestine (C), and placenta (D). Each individual replicate (n = 5 per gestation day) was compared with the average expression on gd 0 (A–C) or gd 19 (D). Differentially expressed gene lists were generated based on the following inclusion criteria: fold change >2 or <0.5 with an unadjusted P value < 0.05 (unadjusted $P < 0.00005$ for placenta), and the $\log_2$ average fluorescent intensity >4. Red indicates an increase, and green indicates a decrease in gene expression. Color intensity is displayed on a $\log_2$ scale.

fold change filtration criteria was removed and the unadjusted P value criteria was lowered to less than 0.01, *Abcc6* (*Mrp6*) was down-regulated by 20–30% on gd 10 and 15 (Fig. 5A). In the maternal kidney, *Abcb1a* expression was constantly decreased by 30–40% throughout mid to late gestation (Fig. 5C; Supplemental Table 15). *Abcc4* expression in the maternal kidney was also decreased by 20%–30% on gd 10 and 15 and returned to nonpregnant levels by term (Fig. 5C). Changes in transporter expression in the small intestine were minimal compared with the liver and kidney (Supplemental Table 16). There were many changes, however, in placental *Abc* transporters (Fig. 5D; Supplemental Table 17). Specifically, *Abcb1a* and *Abcb1b* were 70% and 80% lower, respectively, on gd 10 compared with gd19. *Abcc5* (*Mrp5*) expression was 3-fold higher on gd 10 compared with gd 19. It appears that most *Abc* transporters in the placenta, with a few exceptions, tend to be suppressed during mid to late gestation (gd 10 and 15) and then return to the control levels on gd 19.

Expression of many *Slc* and *Slco* transporter genes in the maternal liver and kidney was significantly altered throughout gestation

TABLE 1

Gestational age-dependent changes of metabolic enzyme and transporter genes in the maternal liver

This list of *Cyp*, *Sult*, *Ugt*, *Abc*, *Slc*, and *Slco* genes was generated using the following filtration criteria: fold change >1.5 or <0.65, unadjusted $P < 0.05$, and $\log_2$ average fluorescent intensity >4 on at least one gestation day (gd). Shown are fold changes compared with gd 0. Fold change is defined as the ratio of fluorescent intensity of the gene on a gestation day to that on gd 0. *P* values are unadjusted.

Gene Symbol	gd 7.5		gd 10		gd 15		gd 19	
	Fold Change	*P*	Fold Change	*P*	Fold Change	*P*	Fold Change	*P*
Cyp2b13	0.7	0.024	0.5	<0.001	0.3	<0.001	0.3	<0.001
Cyp2c50	0.9	0.133	0.6	<0.001	0.4	<0.001	0.5	<0.001
Cyp2c54	0.9	0.047	0.7	<0.001	0.6	<0.001	0.7	<0.001
Cyp2c55	0.8	0.302	0.8	0.284	0.5	0.001	0.7	0.033
Cyp2d9	0.7	<0.001	0.5	<0.001	0.5	<0.001	0.6	<0.001
Cyp2d40	1.0	0.808	1.5	0.002	1.7	<0.001	1.9	<0.001
Cyp2g1	1.0	0.66	1.2	0.094	1.6	<0.001	1.2	0.027
Cyp3a16	1.4	<0.001	1.4	<0.001	1.6	<0.001	1.4	<0.001
Cyp4a12a	0.2	<0.001	0.3	<0.001	0.2	<0.001	0.2	<0.001
Cyp4a12b	0.3	<0.001	0.3	<0.001	0.3	<0.001	0.3	<0.001
Cyp4a14	1.7	0.24	1.3	0.598	0.3	0.021	0.6	0.237
Cyp4a31	1.5	0.004	1.6	0.002	1.8	<0.001	1.9	<0.001
Cyp4f15	0.9	0.242	0.7	<0.001	0.6	<0.001	0.8	0.066
Cyp7b1	1.5	0.003	1.3	0.046	1.5	0.002	1.5	0.006
Cyp8b1	0.9	0.322	1.1	0.598	1.4	0.064	1.6	0.006
Cyp17a1	3.3	<0.001	3.3	<0.001	8.2	<0.001	10.4	<0.001
Cyp26a1	1.0	0.962	2.1	0.082	2.9	0.015	1.1	0.752
Cyp39a1	0.8	0.103	0.5	<0.001	0.3	<0.001	0.4	<0.001
Cyp51	1.0	0.84	0.8	0.176	0.8	0.069	0.6	0.001
Sult1d1	0.9	0.195	0.6	<0.001	0.4	<0.001	0.7	0.021
Ugt2b37	1.5	0.004	1.5	0.005	0.9	0.354	1.0	0.836
Abca8a	0.9	0.249	0.7	0.018	0.5	<0.001	0.8	0.103
Abcb1a	1.1	0.431	0.9	0.573	0.6	0.008	0.9	0.356
Abcb6	0.9	0.032	0.8	0.006	0.6	<0.001	0.8	<0.001
Abcc3	0.9	0.412	0.7	0.01	0.3	<0.001	0.4	<0.001
Abcg5	0.8	0.157	0.7	0.013	0.6	<0.001	0.8	0.092
Abcg8	0.8	0.259	0.6	0.002	0.8	0.134	1.0	0.815
Slc1a2	0.9	0.362	1.1	0.329	1.7	<0.001	2.1	<0.001
Slc3a1	1.7	0.009	1.7	0.007	1.8	0.003	2.1	0.001
Slc6a9	1.1	0.612	2.1	<0.001	4.3	<0.001	2.8	<0.001
Slc7a2	0.7	0.014	0.5	<0.001	0.6	0.001	0.7	0.068
Slc7a8	1.3	0.005	1.5	<0.001	1.2	0.023	1.0	0.979
Slc9a3r1	1.0	0.843	0.9	0.419	0.6	<0.001	0.8	0.009
Slc13a2	1.0	0.663	1.2	0.162	1.5	<0.001	1.1	0.564
Slc15a1	1.0	0.832	0.9	0.542	1.7	0.018	1.0	0.897
Slc15a4	0.7	0.006	0.6	<0.001	0.6	<0.001	0.8	0.029
Slc16a1	1.1	0.422	1.4	0.002	1.7	<0.001	1.3	0.018
Slc16a12	1.0	0.932	0.7	0.002	0.4	<0.001	0.7	0.009
Slc16a13	0.9	0.237	1.2	0.011	1.6	<0.001	1.3	0.001
Slc16a6	1.2	0.363	3.4	<0.001	8.9	<0.001	2.7	<0.001
Slc17a1	1.0	0.658	0.8	0.057	0.5	<0.001	0.7	<0.001
Slc17a2	1.0	0.872	0.7	0.002	0.4	<0.001	0.6	<0.001
Slc17a4	0.8	0.036	1.4	0.003	1.6	<0.001	1.3	0.01
Slc17a8	0.7	0.009	0.6	0.001	0.6	<0.001	0.7	0.012
Slc19a2	0.8	0.103	0.7	0.003	0.6	<0.001	0.9	0.181
Slc22a15	0.9	0.131	0.8	0.024	0.6	<0.001	0.9	0.083
Slc22a2	1.1	0.677	1.0	0.978	3.6	<0.001	1.9	<0.001
Slc22a23	1.1	0.484	1.1	0.389	1.6	<0.001	1.2	0.008
Slc24a3	0.9	0.426	0.9	0.314	1.7	<0.001	1.3	0.009
Slc24a6	1.1	0.579	0.5	<0.001	0.5	<0.001	1.2	0.295
Slc25a4	0.6	0.005	0.8	0.116	0.7	0.008	0.6	0.004
Slc25a25	0.7	0.223	1.0	0.898	1.3	0.31	0.6	0.026
Slc25a30	1.5	0.011	1.3	0.092	1.8	<0.001	1.4	0.019
Slc25a32	0.7	0.006	0.7	0.002	0.6	<0.001	0.8	0.057
Slc25a47	0.8	0.063	0.6	0.001	0.5	<0.001	0.8	0.226
Slc30a10	1.2	0.118	1.5	0.001	1.1	0.362	0.9	0.288
Slc34a2	1.3	0.211	2.6	<0.001	1.7	0.034	1.1	0.657
Slc35c2	0.9	0.215	1.2	0.013	1.7	<0.001	1.5	<0.001
Slc36a1	1.1	0.161	1.5	<0.001	2.1	<0.001	1.5	<0.001
Slc37a1	1.5	0.018	2.5	<0.001	4.0	<0.001	1.9	<0.001
Slc37a4	0.8	0.028	0.8	0.052	0.6	<0.001	0.9	0.452
Slc38a4	0.9	0.216	0.7	0.001	0.4	<0.001	0.6	<0.001
Slc39a14	1.1	0.213	1.4	0.001	1.7	<0.001	1.3	0.022
Slc39a4	0.8	0.011	0.8	0.002	0.6	<0.001	0.8	<0.001
Slc40a1	1.0	0.732	1.1	0.054	1.5	<0.001	1.0	0.988
Slc41a1	1.3	0.011	1.6	<0.001	1.6	<0.001	1.3	0.01
Slc41a2	1.8	0.073	5.2	<0.001	8.3	<0.001	3.7	<0.001
Slc41a3	1.1	0.159	1.2	0.06	1.6	<0.001	2.2	<0.001
Slc43a1	1.0	0.813	1.3	0.008	1.7	<0.001	1.7	<0.001
Slc43a3	1.1	0.375	1.0	0.69	0.6	<0.001	0.7	<0.001
Slc46a3	0.9	0.123	0.8	0.02	0.5	<0.001	0.8	0.006
Slco1a4	1.0	0.901	0.8	0.074	0.5	<0.001	0.9	0.367

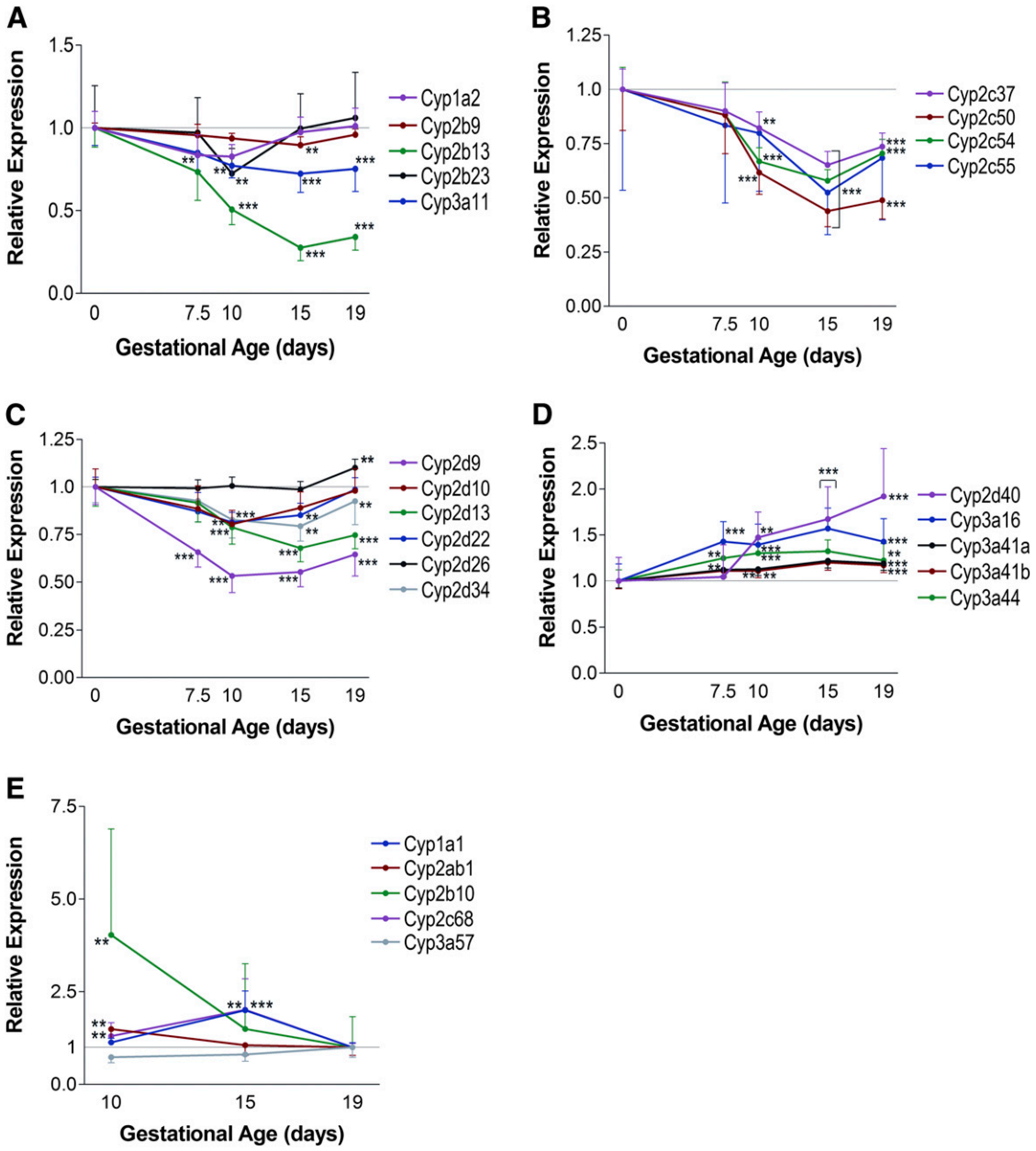

Fig. 4. Gestational age-dependent trends in cytochrome P450 gene expression in the maternal liver and placenta. Shown are expression means ± S.E. for selected genes in the liver (A–D) and placenta (E) from five animals. All panels are expressed as relative fold changes compared with gd 0 (liver) or gd 19 (placenta). Data points in brackets share the same statistical significance. **Unadjusted P value < 0.01; ***unadjusted P value < 0.001.

(Table 1; Supplemental Table 15). In the liver, a large portion of these *Slc* transporters were nutrient transporters, rather than xenobiotic transporters. Gestational age-dependent trends were evident, however, for *Slco1b2* (*Oatp1b2*, ortholog of human *OATP1B3*), *Slc10a1* (*Ntcp*), and *Slc47a1* (*Mate1*), which were constantly downregulated by approximately 25% on gd 15 and gd 19 (Fig. 5B). In the maternal kidney, many *Slc* and *Slco* transporters demonstrated only moderate gestational age-dependent changes in expression (Supplemental Table 15). *Slco4c1* (*Oatp4c1*), on the other hand, showed decreased expression (approximately 60–70%) across gestation (Fig. 5C). In the small intestine, several *Slc* transporter genes demonstrated gestational age-dependent changes in expression; however, their role in xenobiotic transport is not known. For example, *Slc6a9*, *Slc10a5*, and *Slc12a6* were downregulated by 20–30% on gd 10 and/or gd 15, whereas *Slc25a43*, *Slc31a1*, *Slc35c2*, and *Slc36a1* expression increased as much as 2.3-fold during pregnancy (Supplemental Table 16). In the placenta, *Slc22a3* (*Oct3*) expression was 80% lower on gd 10 compared with gd 19 (Supplemental Table 17). In contrast, *Slc22a5* (*Octn2*) and *Slc6a2* (*Net*) were both upregulated more than 2.5-fold on gd 10 (Fig. 5F). Overall, relatively few *Slc* and *Slco* transporter genes known to be involved in drug transport were affected during pregnancy. Changes in expression of *Slco1b2* in the liver, *Slco4c1* in the kidney, and *Slc22a3* in the placenta, however, may affect drug pharmacokinetics during pregnancy.

Metabolism and Transport of Endogenous Substances during Pregnancy. Gestational age-dependent trends were also observed for genes involved in the metabolism and transport of endogenous substances such as bile acids and steroid hormones. According to our data, bile acid production may be altered during pregnancy, particularly in late gestation. For example, *Cyp7a1* and *Cyp7b1*, the

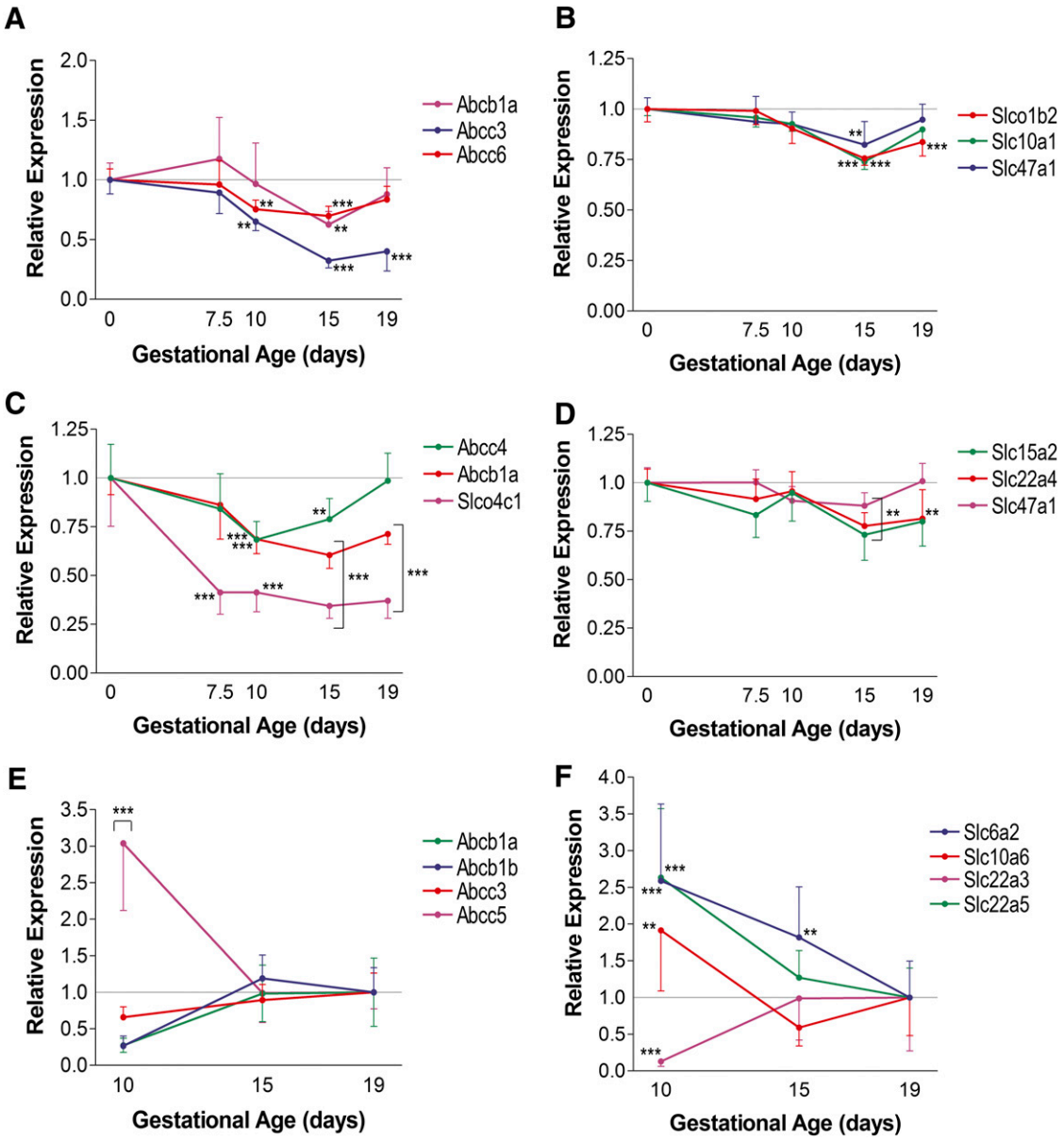

Fig. 5. Gestational age-dependent trends in ABC and SLC transporter gene expression in the maternal liver, kidney, and placenta. Shown are expression means ± S.E. for selected genes in the liver (A and B), kidney (C and D), and placenta (E and F) from five animals. All panels are expressed as relative fold changes compared with gd 0 (liver and kidney) or gd 19 (placenta). Data points in brackets share the same statistical significance. **Unadjusted P value < 0.01; ***unadjusted P value < 0.001.

hepatic enzymes responsible for the rate-limiting step in bile acid synthesis, were significantly induced as much as 1.5-fold across gestation. The expression of bile acid phase I metabolic enzyme, *Cyp3a11*, decreased by approximately 30% in a gestational age-dependent manner (Table 1; Supplemental Table 18). The expression of bile acid transporters also changed. *Abcb1a* (*Mdr1a*), a canalicular bile acid efflux transporter, decreased by 40% on gd 15 (Supplemental Table 18), and *Abcc3* (*Mrp3*), the primary transporter responsible for basolateral efflux of bile acids from hepatocytes into the systemic circulation, decreased by 30%, 70%, and 60% on gd 10, 15, and 19, respectively (Fig. 5A; Supplemental Table 18). Expression of bile acid hepatic uptake transporters *Slc10a1* (*Ntcp*) and *Slco1b2* (*Oatp1b2*) was decreased slightly by approximately 20–30% on gd 15 and 19 (Supplemental Table 18).

Fold changes in steroidogenic enzyme expression were quite large in the maternal liver and placenta; however, the number of genes that changed throughout gestation was low. In the maternal liver, *Cyp17a1*, which oxidizes progesterone and pregnenolone to their 17α-OH metabolites, was induced 3- to 10-fold across gestation (Table 1). In the placenta, expression of *Cyp11a1*, which converts cholesterol to pregnenolone, was 2.8-fold higher on gd 10 compared with gd 19, yet 30% lower than term levels on gd 15 (Supplemental Table 17). Likewise, *Cyp17a1* was 2.7-fold higher on gd 10 compared with gd 19 (Supplemental Table 17). Therefore, both *Cyp11a1* and *Cyp17a1* in the placenta were downregulated during pregnancy, but their expression returns approximately to control levels by gd 15. The effects of pregnancy on hepatic and placental expression of aromatase (*Cyp19a1*), an enzyme that converts androstenedione to estrogens, were very small (data not shown). Murine orthologs of other important human steroidogenic enzymes, such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD), are not known. Several mouse isoforms of *Hsd* genes were

affected in a statistically significant way during pregnancy (Supplemental Tables 13 and 14).

Discussion

In the present study, we have performed the first comprehensive gestational age-dependent analysis of metabolic enzyme and transporter gene expression in the maternal liver, kidney, small intestine, and placenta of pregnant mice using microarray analysis. Ten genes were selected for validation by qRT-PCR based on their robust changes in expression and/or involvement in drug metabolism or transport. Fold changes in the qRT-PCR data were consistently higher than those in the microarray data, but the direction of changes was similar between the two approaches (Figs. 2 and 3). These differences in magnitude are possibly due to higher detection sensitivity of qRT-PCR (Evans et al., 2002). We did not expect differences in the reliability/accuracy of the microarray data across tissues because sample preparation was the same for all tissues; therefore, qRT-PCR validation for the liver and placenta was considered adequate. As a secondary validation, we confirmed our results with previously well characterized genes affected during pregnancy. These included induction of *Cyp3a16* (Zhang et al., 2008) and *Cyp8b1* (Aleksunes et al., 2012) in the liver and downregulation of *Abcc3* in the liver (Aleksunes et al., 2012), *Abcb1a* in the kidney (Zhang et al., 2008), and *Abcc5* in the placenta (Aleksunes et al., 2008) (Table 1; Supplemental Tables 15–17).

In the kidney, a bimodal pattern of gene expression on gd 10 and 19 was produced by two kidney samples, gd 10_3 and gd 10_6. This interanimal variability appears to be specific for the kidney because the liver, small intestine, and placenta collected from the same mice did not exhibit such a pattern. The reason for such an observation is not known; however, recent computational studies do predict that cooperative binding of transcription factors to DNA promotes a bimodal gene expression response in eukaryotic systems (Gutierrez et al., 2012). Thus, the same might explain the bimodal pattern of gene expression in the mouse kidney observed in this study.

The data from the small intestine and placenta should be interpreted with caution for the following reasons. The entire small intestine was used to isolate total RNA, resulting in a mixing of RNA from smooth muscle tissues and mucosal epithelial cells. Our data therefore do not reflect changes in gene expression in the more functionally relevant enterocytes (from a drug disposition perspective), nor do they reflect changes in specific sections along the small intestine. Consequently, we placed less of an emphasis on the small intestine data. The formation of placenta is different between mice and humans, which complicates translation of the placental data to human pregnancy. For example, development of the prominent chorionic villus structure occurs quickly in human pregnancy (by day 21 of 270), but not until gd 11.5 in the mouse. This may explain why the mouse placenta had more differentially expressed genes on gd 10 versus gd 15, and challenges whether it is best to analyze gene expression before gd 11.5. Interpretation of drug transport across the mouse placenta on gd 15 and 19 is still relevant to human placenta, because both mice and humans use syncytiotrophoblasts to modulate the exchange of nutrients, hormones, and xenobiotics between the mother and fetus.

Our study is the first to characterize gestational age-dependent changes in gene expression for all metabolic enzymes and transporters. Zhang et al. (2008) reported that although *Cyp3a16*, *Cyp3a41*, and *Cyp3a44* were induced in the maternal liver during pregnancy, *Cyp3a11*, *Cyp3a13*, and *Cyp3a25* were downregulated. This is consistent with our results (Fig. 4). Koh et al. (2011) examined gestational age-dependent expression of hepatic *Cyp1a2*, *Cyp2a5*, *Cyp2b10*, *Cyp2c37*, *Cyp2d22*, *Cyp2e1*, *Cyp3a11*, and *Cyp3a41* using qRT-PCR. Again, their observations mirror our data (Fig. 4). In the placenta, *Cyp* upregulation during mid-gestation is also consistent with previous studies (Hakkola et al., 1996). We are the first, however, to examine *Cyp* gene expression in the maternal kidney. Interestingly, *Cyp3a41a* and *Cyp3a41b* were markedly induced in late gestation. Implications of *Cyp3a41a/b* induction are not known, but might include altered xenobiotic metabolism, increased bioactivation of nephrotoxic pathways, or increased sodium retention to control blood pressure (Clore et al., 1992).

We also used our microarray data to explore CYP-mediated steroid hormone biosynthesis during pregnancy. CYP17A1 is a critical enzyme for steroid hormone biosynthesis. The large induction of hepatic *Cyp17a1* (approximately 3- to 10-fold) suggests that production of 17α-hydroxyprogesterone and 17α-hydroxypregnenolone may increase during mouse pregnancy, which is consistent with 17α-OH progesterone increases in humans during pregnancy (Dörr et al., 1989). Increased levels of 17α-OH metabolites could ultimately increase estrogen production in the maternal liver; however, downregulation of placental *Cyp17a1* throughout gestation favors progesterone formation, not estrogen. Therefore, our microarray data support the general pattern of steroid hormone biosynthesis during pregnancy, that is, although progesterone and estrogen are increasingly produced as gestation progresses, the majority of progesterone is made by the placenta in late gestation (Raunig et al., 2011). Nevertheless, interpretation of placental steroidogenesis in mice should be done with caution because one study has shown that the pattern of gestational age-dependent expression of some steroidogenic enzymes can be opposite between mice and humans (Malassiné et al., 2003).

Our results revealed that *Abc* transporter gene expression was decreased during pregnancy, particularly *Abcc3* in the liver, *Abcb1a* in the kidney, and *Abcc5* in the placenta. Similarly, several *Slc* or *Slco* transporters important for drug disposition were moderately decreased during pregnancy (such as *Slco1b2* in the liver and *Slco4c1* in the kidney). *Slc22a3* (*Oct3*) and *Slc6a2* (*Net*) in the placenta were downregulated and upregulated, respectively, to a larger extent. The small number of transporters affected by pregnancy may still be enough, however, to explain reported increases in hepatic bile acid concentrations in late pregnancy in mice (Milona et al., 2010; Aleksunes et al., 2012). Consistent with these previous studies, our data support increased bile acid synthesis (*Cyp7b1*) and decreased basolateral and canalicular bile acid efflux (*Abcc3* and *Abcb1a*) during mouse pregnancy. Decreased expression of hepatic uptake transporters in the maternal liver may therefore be an adaptive response to manage increased intracellular bile acid concentrations.

Translation of our findings to human pregnancy is not straightforward. One reason is that gene expression and protein levels are also not always correlative (Schwanhäusser et al., 2011). In the placenta, *Abcb1a/1b* expression increases; however, its protein expression has been shown to decrease as gestation progresses (Zhang et al., 2008). Microarray data therefore may not entirely reflect pregnancy's effect on protein expression/activity. Clinical studies indicate that activity of CYP2C9, CYP2D6, and CYP3A4 increases during pregnancy, but activity of CYP1A2 and CYP2C19 decreases (Dempsey et al., 2002; Anderson, 2005; Hodge and Tracy, 2007). In addition, the number of *Cyp1*, *Cyp2*, and *Cyp3* isoforms in mice is greater than that in humans, and there are potential species differences in substrate specificity and/or enzymatic activity. All of these factors make it challenging to predict drug metabolism during human pregnancy using the mouse data. If our microarray data do predict CYP activity, then xenobiotic

substrates of CYP1 or CYP2 isoforms may need to be given at smaller doses during pregnancy. Prediction for CYP3A substrates is less certain, because *Cyp3a16*, *Cyp3a41a/b*, and *Cyp3a44* are upregulated during pregnancy, yet *Cyp3a11* and *Cyp3a13* are downregulated. In addition, fold changes in *Cyp3a* gene expression do not reflect the magnitude of increase that we and others have shown for in vivo CYP3A activity in pregnant mice and humans (Mathias et al., 2006; Hebert et al., 2009; Zhou et al., 2010). Therefore, the *Cyp* isoform(s) that best represent in vivo CYP3A activity should be carefully evaluated.

Mouse *Cyp3a11* is most closely related to human *CYP3A4* with 76% amino acid identity and may be the dominant contributor to CYP3A activity in mouse liver (Martignoni et al., 2006). However, CYP3A11 is likely not the mouse CYP3A isoform responsible for increased CYP3A activity in pregnant mice, because its gene expression was downregulated over gestation. *Cyp3a13* and *Cyp3a16* appear less important because hepatic *Cyp3a13* levels are 5- to 10-fold lower than *Cyp3a11* in nonpregnant female mice, and *Cyp3a16* is considered a fetal isoform (Martignoni et al., 2006). On the other hand, *Cyp3a41a/b* and *Cyp3a44* are female-specific isoforms expressed solely in the liver, and they are known to be induced by growth hormone, glucocorticoids, and estradiol (Sakuma et al., 2002; Sakuma et al., 2004; Jarukamjorn et al., 2006). Indeed, maternal estradiol plasma concentrations triple from gd 10 to gd 17 (Barkley et al., 1979), which parallels the trend of hepatic induction of *Cyp3a41a/b* and *Cyp3a44* during gestation (Fig. 4D). Clearly, isoform-specific quantitation of protein levels and activity is needed to further evaluate which isoforms are responsible for the in vivo CYP3A activity in pregnant mice.

In summary, we have determined global gene expression profiles in the maternal tissues and placenta of pregnant mice, providing a genetic basis for understanding gestational age-dependent physiologic and pharmacological changes during pregnancy. These data also offer a means to study the mechanisms of regulation behind such changes. On the basis of our findings, mid to late gestation (gd 10–15) may be the best time to study the full impact of mouse pregnancy on drug metabolism and disposition.

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Authorship Contributions

Participated in research design: Mao, Thummel, Hebert, Farin.

Conducted experiments: Shuster, Tsai.

Contributed new reagents or analytic tools: MacDonald, Beyer, Bammler.

Performed data analysis: Shuster, MacDonald, Beyer, Bammler.

Wrote or contributed to the writing of the manuscript: Shuster, Mao, Tsai, Farin, Bammler, Beyer, Thummel, Hebert.

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