Effect of Gestational Age on mRNA and Protein Expression of Polyspecific Organic Cation Transporters during Pregnancy[§]

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ABSTRACT

Polyspecific organic cation (OC) transporters play important roles in the disposition of clinically used drugs, including drugs used during pregnancy. Pregnancy is known to alter the expression of drug-metabolizing enzymes and transporters, but its specific effect on OC transporters has not been well defined. Using quantitative polymerase chain reaction and liquid chromatography coupled with tandem mass spectrometry targeted proteomics, we determined the effect of pregnancy and gestational age on mRNA and protein expression of major OC transporters in the kidney, liver, and placenta in mice with timed pregnancies. Human organic cation transporter 3 (hOCT3) expression was further investigated in human placentas from the first and second trimesters and at term. Our results showed that pregnancy had a marginal effect on renal mouse organic cation transporter 1/2 (mOct1/2) expression but significantly reduced mouse multidrug and toxin extrusion transporter 1 (mMate1) expression by

20%–40%. Hepatic expression of mOct1 and mMate1 was minimally affected by pregnancy. Human and mouse placentas predominantly expressed OCT3 with little expression of OCT1/2, MATE1/2, and plasma membrane monoamine transporter (PMAT). The hOCT3 protein in first and second trimester and term placentas was quantified to be 0.23 ± 0.033 , 0.38 ± 0.072 , and 0.36 ± 0.099 fmol/ μ g membrane protein, respectively. In contrast with the moderate increase in hOCT3 protein during human pregnancy, mOct3 expression in the mouse placenta was highly dependent on gestational age. Compared with gestational day (gd) 10, placental mOct3 mRNA increased by 37-fold and 46-fold at gd 15 and 19, leading to a 56-fold and 128-fold increase in mOct3 protein, respectively. Our study provides new insights into the effect of pregnancy on the expression of polyspecific OC transporters and supports an important role of OCT3 in OC transport at the placental barrier.

Introduction

Many drugs and toxins, such as metformin, histamine H₂ receptor blockers, and 1-methyl-4-phenylpyridinium (MPP⁺) and paraquat, are hydrophilic organic cations (OCs) that do not readily cross cell membranes by passive diffusion. Polyspecific OC transporters play an important role in the disposition, efficacy, and toxicity of these cationic xenobiotics (Wright and Dantzer, 2004; Koepsell et al., 2007; Giacomini et al., 2010). These transporters are also likely to be involved in various physiologic pathways through their action on endogenous bioactive amine (Wright and Dantzer, 2004; Koepsell et al., 2007; Giacomini et al., 2010). Known polyspecific OC

transporters include the electrogenic organic cation transporters 1–3 (OCT1–3, SLC22A1–3), the proton/cation antiporters termed as multidrug and toxic extrusion proteins 1–2 (MATE1/2, SLC47A1/2), and the plasma membrane monoamine transporter (PMAT, SLC29A4). These transporters have largely overlapping substrate specificities and frequently work in concert to mediate the transepithelial flux of OCs.

In humans, hOCT1 is predominantly expressed in the liver on the sinusoidal membrane of hepatocytes, mediating OC uptake into the hepatocytes (Giacomini et al., 2010). Expressed at the canalicular membrane, hMATE1 further effluxes the OCs into the bile (Giacomini et al., 2010). In the human kidney, hOCT2 is primarily expressed and localized to the basolateral membrane of renal proximal tubular cells (Giacomini et al., 2010). And hOCT2 concentrates OCs into the proximal tubular cells, where OCs can be further excreted into the urine by hMATE1 and hMATE2-K at the apical membrane. The third member of the human OCT family, hOCT3, is broadly distributed in tissues, including the placenta, skeletal muscle, heart, brain, kidney, liver, lung, and intestine (Koepsell et al., 2007). Although hOCT3 has been implicated in the clearance of endogenous monoamines, it could also be involved in transport of xenobiotic OCs. A new polyspecific OC transporter, termed the plasma membrane monoamine transporter

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ABBREVIATIONS: Ct, comparative cycle threshold; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gd, gestational day; GUSB, β glucuronidase; IS, internal standard; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MATE, multidrug and toxin extrusion transporter; MPP⁺, 1-methyl-4-phenylpyridinium; OC, organic cation; OCT, organic cation transporter; P-gp, P-glycoprotein; PMAT, plasma membrane monoamine transporter; qRT-PCR, quantitative real-time polymerase chain reaction; SNP, single-nucleotide polymorphism; T1, first trimester; T2, second trimester.

(PMAT), is widely expressed in various tissues and has largely overlapping substrates and inhibitors with those of OCTs (Engel et al., 2004; Engel and Wang, 2005). Also hPMAT has been implicated in intestinal absorption of OCs (Zhou et al., 2007).

A number of medications used by pregnant women are substrates of the polyspecific OC transporters. For example, metformin, a positively charged biguanide transported by hOCT1-3, hMATE1/2, and hPMAT (Kimura et al., 2005; Tanihara et al., 2007; Zhou et al., 2007; Nies et al., 2009), is used to treat gestational diabetes mellitus in pregnant women (Wensel, 2009; Eyal et al., 2010). OCT1-mediated hepatic uptake of metformin is an important determinant of its glucose-lowering effect as the liver is the primary target of metformin action (Shu et al., 2007). Meanwhile, metformin is primarily eliminated by renal secretion via the OCT2/MATE1 pathway (Chen et al., 2009; Tsuda et al., 2009). Metformin has been shown to cross the placenta barrier (Kovo et al., 2008), which may also involve specific OC transporters. Pregnant women undergo extensive physiologic and hormonal changes not experienced by nonpregnant patients, making them unique with respect to drug therapy selection, dosage, efficacy, and safety. Medication used during pregnancy may also cross the placenta and potentially can result in fetal exposure and teratogenicity. Considerable data in the literature suggest that the expression and activity of important drug metabolizing enzymes (e.g., hepatic CYP3A4, CYP2D6, CYP2C9) and transporters (e.g., placental P-glycoprotein [P-gp]) are altered during pregnancy in a gestational age-dependent manner (Mathias et al., 2005; Hodge and Tracy, 2007; Hebert et al., 2008; Isoherranen and Thummel, 2013). In a recent study performed by the Obstetric-Fetal Pharmacology Research Unit Network at the University of Washington, a significant gestational stage-dependent change in metformin pharmacokinetics was seen during pregnancy (Eyal et al., 2010), suggesting that the expression of OC transporters in organs important for metformin disposition may be altered by pregnancy.

Little is known about the effect of pregnancy on the expression of polyspecific OC transporters in various tissues. Although the effect of pregnancy on mRNA expression of drug transporters has been analyzed in large-scale microarray studies and/or in tissue-specific manners in pregnant animal models (Shuster et al., 2013; Yacovino et al., 2013), comprehensive and quantitative mRNA analysis of polyspecific OC transporters has not been performed. Furthermore, few studies have quantified and compared the protein levels of OC transporters between nonpregnant and pregnant states. In this study, we investigated the effect of pregnancy and gestational age on the mRNA expression of polyspecific OC transporters in pregnant mice and human placentas at various gestational stages. Quantitative liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) proteomics was used to quantify the membrane expression of polyspecific OC transporter proteins in both mouse and human tissues.

Materials and Methods

Animals and Tissue Harvest. Adult (8–10 weeks of age) wild-type FVB mice (Taconic Farms, Germantown, NY) were housed in the specific-pathogen-free facility at the University of Washington. The animal studies were approved by the institutional animal care and use committee of the University of Washington. To obtain pregnant mice, we performed timed mating. The date that a vaginal plug was observed was assigned as gestational day 1. Pregnant mice were sacrificed using CO₂. The kidneys, livers, and placentas from pregnant mice at gestational days (gd) 10, 15, and 19 (term in mice is ~20–21 days) were immediately dissected, collected, and flash-frozen in liquid N₂. Tissues were stored at –80°C until use. Tissues from age-matched virgin mice were used as the nonpregnant control.

Human Placenta Source. The use of human placenta as a biologic specimen was approved by the institutional review board at the University of Washington. Human term placentas from normal pregnancies were obtained from the Labor and Delivery Unit at the University of Washington. Normal first trimester (T1, weeks 6–12) and second trimester (T2, weeks 13–25) placentas were provided by the Birth Defects Research Laboratory at the University of Washington, which has institutional review board approval to collect and distribute normal and diseased conceptual tissues for research use. All placenta tissues were from healthy, uncomplicated pregnancies obtained from women (70% Caucasians and 30% other ethnicity) aged 16–38 years. Upon collection, the placentas were immediately snap frozen and stored at –80°C until use. To minimize RNA degradation, the time interval from surgery or delivery to tissue preparation did not exceed 60–90 minutes.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time Polymerase Chain Reaction Assays. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA) or Qiagen Mini RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA integrity and purity were verified by gel electrophoresis and ultraviolet spectrophotometry. Total RNA (2 µg) was reverse transcribed to first-strand cDNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions, and all cDNA samples were prepared in a final volume of 100 µl. Taqman real-time polymerase chain reaction (PCR) reagents, assay primers, and probes for human hOCT1-3 (SLC22A1-3), hMATE1 (SLC47A1), hPMAT (SLC29A4), hGUSB (β glucuronidase), hGAPDH (glyceraldehyde-3-phosphate dehydrogenase), hβ-actin, mouse mOCT1-3 (Slc22a1-3), mMate1-2 (Slc47a1-2), mPmat (Slc29a4), mGusb, mGapdh, and mβ-actin were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA). Taqman real-time PCR assays were performed on an Applied Biosystems 7900HT fast real-time PCR system as described previously elsewhere (Duan and Wang, 2010). To ensure the same amount of cDNA was loaded to the 96-well plates for the quantitative real-time PCR (qRT-PCR) analysis, 5 µl from each cDNA sample, which contains 100 ng of RNA-equivalent cDNA, was added to the real-time reaction that contains 10 µl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems) and 1 µl of 20× primer/probe mix in a final volume of 20 µl. Each sample was analyzed in duplicate or triplicate.

To quantify the transcript numbers of genes of interest, comparative cycle threshold (Ct) method was used (Zhang et al., 2008). The mRNA levels of each tested gene were normalized to a housekeeping gene according to the following formula: Ct (test gene) – Ct (housekeeping genes) = ΔCt. Thereafter, the relative mRNA levels of each gene were calculated using the ΔΔCt method: ΔCt (test gene) – ΔCt (test gene in the calibrator) = ΔΔCt (test gene). The fold changes of mRNA levels were represented as a relative expression 2^{–ΔΔCt}. For greater precision of the mRNA quantification by qRT-PCR, the expression of the commonly used housekeeping genes (hGUSB/mGusb, hβ-actin/mβ-actin, and hGAPDH/mGapdh) was first analyzed in human and mouse tissues at various gestational stages. The Ct values of the housekeeping genes in the tissues were determined and compared across the pregnancy stages using Student's *t* test. The housekeeping gene that showed the least variation was chosen for normalization of the target genes.

Membrane Protein Preparation and Quantification of Transporters by LC-MS/MS Analysis. Total membrane proteins were prepared from mouse (kidney, liver, and placenta) and human (placenta) tissues using the Proteo Extract native membrane protein extraction kit (Calbiochem/EMD Millipore, San Diego, CA) according to the manufacturer's instructions. The total membrane protein concentration was determined by a BCA (bicinchoninic acid) protein assay kit (Pierce/Thermo Scientific, Rockford, IL). The membrane fraction was digested by trypsin as per conditions described elsewhere (Prasad et al., 2013). Briefly, the isolated membrane proteins were denatured at 95°C, reduced with dithiothreitol, and alkylated with iodoacetamide in ammonium bicarbonate buffer. The protein samples were digested at 37°C for 24 hours by trypsin, and the reaction was quenched and spiked with the internal standard (IS) solution and centrifuged at 5000g for 5 minutes before analysis.

Protein quantification was based on unique signature peptides as surrogates for quantification of these transporters and the corresponding isotopically [¹³C₆,¹⁵N₄]-arginine or [¹³C₆,¹⁵N₂] lysine labeled peptides as IS. Selected unique signature peptides for these transporters are shown in Table 1. These peptides were selected based on criteria previously described elsewhere (Kamiie et al., 2008). Peptides with predicted transmembrane regions, single-nucleotide

TABLE 1

Optimized MS/MS parameters of proteotypic peptides selected for targeted analysis of mOct1, mOct2, mOct3, mMate1, and hOCT3

Residues labeled with arginine-¹³C₆, ¹⁵N₄ and lysine-¹³C₆, ¹⁵N₂ are shown in bold and italic. Because mouse and human Oct3/OCT3 surrogate peptides showed similar characteristics, single IS was used for both transporters.

Transporter	Peptide	Precursor Ion	Product Ions	Fragmentor	Collision Energy
mOct1	GVALPETIEEAENLGR	849.5	1357.7	160	20
		849.5	679.3	160	20
mOct1 (IS)	GVALPETIEEAENLGR	854.5	1367.7	160	20
		854.5	684.3	160	20
mOct2	LNPSFLDLVR	587.3	228.13	140	15
		587.3	946.54	140	15
mOct2 (IS)	LNPSFLDLVR	592.34	956.54	140	15
		592.34	228.13	140	15
mMate1	TEESAPGPGGADAASER	801.4	1084.5	160	18
		801.4	542.7	160	18
mMate1 (IS)	TEESAPGPGGADAASER	806.4	1094.5	160	18
		806.4	547.8	160	18
mOct3	GIALPETVEDVEK	700.4	1158.6	140	16
		700.4	1045.5	140	16
		700.4	579.8	140	18
mOct3 (IS)	GIALPETVDDVEK	697.5	1039.3	140	16
hOCT3	GIALPETVDDVEK	693.5	1031.3	140	16
		693.5	516.1	140	18
		693.5	355.1	140	13
hOCT3 (IS)	GIALPETVDDVEK	697.5	1039.3	140	16

IS, internal standard.

polymorphisms (SNPs), posttranslational modifications, or those susceptible to degradation were excluded. Continuous R and K sequences (i.e., RR, RK, KR, and KK) were excluded to avoid the miscleavages. Other characteristics, such as stability and LC retention, were also taken into consideration during peptide selection.

The LC-MS/MS parameters were optimized to quantify selected peptides in the tissues samples. The analysis was performed using Agilent 6460A triple-quadrupole mass spectrometer coupled to Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA) operated in electrospray ionization (ESI) positive ionization mode. Approximately 2 μ g of the trypsin digest (5 μ l of LC injection volume) was injected onto the column (Kinetex 2.6 μ m, C18, 100 $\times$ 3 mm; Phenomenex, Torrance, CA). The LC flow rate was 0.4 ml/min, and two different mobile phase gradient programs were used to quantify the transporters. For mOct1, mOct2, and mMate1, the gradient program was 97% A (water containing 0.1% v/v formic acid) and 3% B (acetonitrile containing 0.1% v/v formic acid) held for 4 minutes, followed by three steps of linear gradient of mobile phase B concentration of 3% to 12%, 12% to 25%, and 25% to 30% over 4–8 minutes, 8–10 minutes, and 10–14 minutes, respectively. This was followed by the washing step (90% mobile phase B) for 0.9 minutes, and re-equilibration for 4.5 minutes.

Similarly, for mOct3 and hOCT3, the gradient program was 97% A and 3% B held for 4.5 minutes, followed by two steps of linear gradient of mobile phase B concentration of 3% to 18% and 18% to 24% over 4.5–7.5 minutes and 7.5–11.5 minutes, respectively. This was followed by the washing step (80% mobile phase B) for 1 minute and re-equilibration for 5 minutes. The doubly charged parent to singly charged product transitions for the analyte peptides and their respective stable isotope labeled peptides were monitored using optimized MS/MS parameters (Table 1).

The data were processed by integration of the peak areas generated from the reconstructed ion chromatograms for the analyte peptides and their respective IS using the MassHunter software (Agilent Technologies). For data analysis, the peak response of multiple reaction monitoring (MRM) transitions from each peptide was averaged and the area ratio of analyte peptide versus IS peptide was obtained. Relative protein expression of individual OC transporters was presented as the area ratio of analyte peptide over IS peptide normalized by the total membrane protein amount in the injected trypsin digests. For absolute quantification of hOCT3 in human placenta, the nonlabeled signature peptide was synthesized as a calibrator. The amount of hOCT3 protein was calculated from the standard curve obtained from the quantitative data generated with the calibrator peptide.

Data Analysis. For each mRNA or protein expression data point, data were obtained from mouse tissues from 3–6 animals and expressed as mean $\pm$ S.D. Statistical significance in the expression in various mouse tissues was determined by unpaired Student's *t* test (GraphPad Prism 5.04, La Jolla, CA). The mRNA and protein expression were correlated using a linear regression, and the corresponding *r*² and *P* values were calculated. Expression data in human placentas were obtained from 6–16 placenta tissues per gestational stage. Because of the small sample size for each group, the difference in the human placental expression was determined by a nonparametric method, the Mann-Whitney *U* test (GraphPad Prism 5.04). *P* < 0.05 was considered statistically significant.

Results

Fluctuation of Housekeeping Genes in Various Tissues during Pregnancy. For greater precision of the mRNA quantification by qRT-PCR, we first determined the absolute Ct values for the housekeeping genes in the mouse kidney, liver, and placenta from nonpregnant or pregnant mice at gd 10, 15, and 19 (Supplemental Fig. 1). The data showed that in the kidney mGusb expression was not affected by pregnancy and therefore was used for normalization for kidney expression. In mouse liver and placenta, mGapdh and m β -actin were relatively stable and we used mGapdh for normalization. The *C_t* values for the housekeeping genes hGUSB, hGAPDH and h β -actin in human placentas were shown in Supplemental Fig. 2. All three genes showed a similar expression pattern across different gestational stages. Because hGUSB showed least variability across all samples, we used hGUSB for data normalization in human placenta.

mRNA Quantification of Renal and Hepatic mOct and mMate Transporters in Nonpregnant and Pregnant Mice at Different Gestational Ages. Mice with timed pregnancies were used to investigate the influence of pregnancy and gestational age on OC transporter expression in the kidney and liver. This information is logistically unavailable from human pregnancy. Although many of the functional characteristics and tissue-distribution patterns of OC transporters are conserved between humans and rodents, there are also some species differences. Most notably, the human kidney predominantly expresses

hOCT2 whereas both mOct1 and mOct2 are expressed in the rodent kidneys (Koepsell et al., 2007). In addition, the human kidney expresses both hMATE1 and hMATE2-K whereas mouse kidney expresses mMate1 only (Lickteig et al., 2008). As expected, qRT-PCR results showed that mOct1, mOct2 and mMate1 were highly expressed in the kidneys from nonpregnant and pregnant mice (Fig. 1A). In contrast, no significant expression was observed for mOct3, mMate2 or mPmat. There was no significant difference in renal mOct1 mRNA expression between nonpregnant and pregnant mice at gd 10, 15, and 19 (Fig. 1A). A trend of slight decrease in renal mOct2 mRNA expression was observed during pregnancy, but the decrease only became statistically significant ($P < 0.05$) at gd 10. A small but significant decrease ($\sim 30\%$) in renal mMate1 mRNA expression was observed at gd 10 and 15 (Fig. 1A).

In the mouse liver, mOct1 and mMate1 mRNA was highly expressed, whereas mRNA expression of other transporters (mOct2, mOct3, mMate2, and mPmat) was minimal or undetectable. Compared with nonpregnant mice, there were slight decreases in hepatic expression of mOct1 mRNA at all three pregnancy stages, but only the decrease at gd 15 was statistically significant (Fig. 1B). No significant change was observed in hepatic mMate1 mRNA expression from the pregnant mice as compared with the nonpregnant mice.

Protein Quantification of Renal and Hepatic mOct and mMate Transporters in Nonpregnant and Pregnant Mice at Different Gestational Ages. Next, we used a novel LC-MS/MS targeted proteomics approach to determine the influence of pregnancy on renal and hepatic expression of mOct1, mOct2, and mMate1 proteins. The relative protein expression of each individual mouse OC transporters was measured across

gestational age using transporter unique signature peptides (Table 1) and LC-MS/MS, as detailed in the *Materials and Methods* section. As shown in Fig. 1C, a marginal reduction in mOct1 and mOct2 proteins was observed in pregnant mouse kidneys, with gd 15 showing the most significant decrease. Notably, renal mMate1 protein expression showed 22%, 36%, and 29% decrease at gd 10, 15, and 19, respectively. In the mouse liver, expression of Oct2 protein was negligible (Fig. 1D). The mOct1 protein showed a minimal (10%–15%) decrease at gd 15 and gd 19 whereas Mate1 protein expression was not affected by pregnancy (Fig. 1D). The overall impact of pregnancy on the expression of mOct and mMate proteins was consistent with the mRNA expression of individual transporters during pregnancy (Fig. 1).

mRNA Expression Pattern of OC Transporters in Human and Mouse Placenta. Previous studies suggested a high expression of hOCT3/mOct3 in the placenta (Kekuda et al., 1998; Sata et al., 2005). However, the quantitative expression of other OC transporters in the placenta has not been thoroughly investigated. Here, our qRT-PCR data showed that hOCT3 is expressed at a much greater level (>30 -fold) than any other OC transporter (Fig. 2A). A similar expression pattern was also observed in late-stage (gd 19) mouse placenta (Fig. 2B). Together, these data clearly demonstrated that in both humans and mice hOCT3/mOct3 is the predominant polyspecific OC transporter expressed in term or near-term placentas.

mOct3 mRNA and Protein Expression in Mouse Placenta at Various Gestational Stages. Our study had confirmed that mOct3 is the predominant polyspecific OC transporter in the mouse placenta. We next investigated whether the placental mRNA expression levels

Fig. 1. mRNA and protein expression of mouse OC transporters in kidney (A and C) and liver (B and D) at different gestational ages. Total RNA isolated from the kidneys and livers of nonpregnant (virgin) and pregnant mice at gd 10, 15, and 19 was reverse transcribed, and the mRNA levels of six OC transporters were determined by qRT-PCR. The relative protein expression of individual transporters across different gestational age was measured in the membrane protein prepared from kidney and liver using LC-MS/MS. The mRNA expression is normalized to mGusb in kidney and to mGapdh in liver. Data are expressed as mean $\pm$ S.D. from 3–6 mice at each gestational age. *Statistically significantly different from expression at nonpregnant state ($P < 0.05$).

Fig. 2. mRNA expression of various OC transporters in (A) human term placenta and (B) mouse placenta at gd 19. Total RNA was isolated from human term placentas ($n = 6$) and mouse placentas at gd 19 ($n = 3$). cDNA was synthesized by reverse transcriptase, and mRNA levels of hOCT1-3/mOCT1-3, hMATE1/2/mMATE1/2, and hPMAT/mPmat were determined by qRT-PCR. The results were normalized to hGUSB or mGapdh and represent mean $\pm$ S.D.

of mOCT3 change with gestational stages. The mRNA expression of placental mOCT3 at gd 15 and 19 was respectively 37-fold and 46-fold greater than that at gd 10 (Fig. 3A). Consistently, much stronger bands were observed at gd 15 and 19 when mOCT3 transcripts were amplified from the mouse placentas by semiquantitative PCR (Fig. 3A). We then quantified the relative expression of mOCT3 protein using LC-MS/MS. Consistent with the mRNA expression results, the protein expression of mOCT3 at gd 15 and 19 was increased by 56-fold and 128-fold, respectively (Fig. 3B). The mOCT3 mRNA expression at gd 19 appeared slightly higher than that at gd 15, but the difference was not statistically significant (Fig. 3A). However, mOCT3 protein expression at gd 19 was significantly higher than that at gd 15 (Fig. 3B).

These data suggest that expression of the mOCT3 gene in the mouse placenta is gestational-age dependent, and the expression of mOCT3 mRNA and protein is greatly increased after gd 10 and reaches maximum at mid-to-late pregnancy. In addition to mOCT3, we also examined the protein expression of mMate1 in the mouse placentas at gd 10, 15, and 19. Consistent with the mMate1 mRNA expression in mouse placenta at gd 19 (Fig. 2B), there was no detectable mMate1 protein expression in any of the placenta samples examined (data not shown), further supporting that OCT3 protein is the major OC transporter in the placenta.

hOCT3 mRNA and Protein Expression in Human Placenta at Different Trimesters. The influence of gestational age on hOCT3 mRNA and protein expression in human placentas was measured in first trimester (T1, $n = 11$), second trimester (T2, $n = 16$), and term ($n = 6$) placentas from normal, uncomplicated pregnancies. To eliminate any potential regional difference in hOCT3 mRNA expression, tissues from the outer surface (villus parenchyma) of the placenta were used in the study as preliminary analysis showed little regional variation in hOCT3 mRNA expression in this area (data not shown).

Different from the mouse, hOCT3 mRNA expression in human placentas did not show a significant increase in middle and late pregnancy (Fig. 4A), although there appeared a slight increase in hOCT3 mRNA expression in T2 placentas as compared with T1. A large interindividual variability (>20 -fold) of hOCT3 mRNA expression was found in the placentas, especially those from T1 and T2 (Table 2).

We then quantified the absolute amount of hOCT3 protein by LC-MS/MS in six randomly chosen placenta samples at each gestational age. The mean hOCT3 protein in T1, T2, and term placentas was quantified to be 0.23 ± 0.033 , 0.38 ± 0.072 , and 0.36 ± 0.099 fmol/ μ g membrane protein, respectively (Fig. 4B; Table 2). Compared with T1, the hOCT3 protein levels showed $\sim 65\%$ and 56% increase at T2 and term, respectively. In contrast to the large interindividual variability in hOCT3 mRNA levels, a much smaller variability (<3 -fold) was observed for hOCT3 protein in the human placenta samples. When the correlative

analysis was performed, the protein levels showed a general trend of increase with mRNA levels (Fig. 4C). The correlation between hOCT3 mRNA and protein levels was weak, with an r^2 value of 0.21. The slope statistically significantly deviated from zero with a P value of 0.049.

Fig. 3. mOCT3 mRNA and protein expression in mouse placenta at different gestational ages. (A) Total RNA isolated from the placenta of nonpregnant (virgin) and pregnant mice at gd 10, 15, and 19 was reverse transcribed, and the mRNA levels of Oct3 were determined by semiquantitative reverse-transcriptase PCR and qRT-PCR. (B) Relative protein expression of mOCT3 in isolated membrane protein of mouse placentas was measured using LC-MS/MS. The mRNA levels determined by qRT-PCR were normalized to mGapdh. Data are mean $\pm$ S.D. ($n = 3$ per group). *Statistically significantly different from the expression at gd 10 ($P < 0.05$).

Fig. 4. Quantification of hOCT3 mRNA (A) and protein (B) in human placentas at different gestational stages and correlation analysis of mRNA and protein expression (C). Total RNA was isolated from first trimester (T1, $n = 11$), second trimester (T2, $n = 16$), and term ($n = 6$) placentas, and the mRNA levels were determined by qRT-PCR and normalized to hGUSB. Membrane protein was isolated from T1, T2, and term placentas ($n = 6$ for each gestational age placenta). The hOCT3 protein levels were quantified using a LC-MS/MS method. Each data point represents an individual placenta. The mRNA and protein expressions of hOCT3 were correlated using linear regression.

Discussion

This study evaluated the effects of pregnancy on mRNA and protein expression of polyspecific OC transporters in the kidney, liver, and placenta, organs that are important for maternal disposition and fetal exposure to OC drugs. Because the livers and kidneys of pregnant women are unavailable for gene analysis, we used a mouse model with timed pregnancies for our study. The gestational ages (gd 10, 15, 19) selected in this study represent critical times for placental and fetal development and have been previously used to mimic the early, middle, and late stages in human pregnancy (Wang et al., 2006; Zhang et al., 2008). For placenta, both human and mouse tissues at various gestational stages were analyzed and compared.

Our study showed that mRNA and protein expressions of mOCT1 and mOCT2 in the mouse kidney are marginally affected by pregnancy

(Fig. 1, A and C). Similarly, hepatic expression of mOCT1 and mMate1 mRNA and protein is also minimally affected by pregnancy (Fig. 1, B and D). However, in the kidney, mMate1 mRNA and protein expressions significantly declined by 20%–40% throughout pregnancy, with maximum down-regulation of mMate1 membrane protein observed at mid-pregnancy (Fig. 1C).

Our results are consistent with a recent report that also showed a down-regulation of renal apical drug efflux transporters (Mrp2, Mrp4, Mate1) in pregnant mice (Yacovino et al., 2013). The down-regulation of apical drug efflux transporters is anticipated to result in a reduced tubular secretory capacity during pregnancy. In addition, the significant down-regulation of apical efflux transporter (i.e., Mate1) accompanied by fewer changes in the basolateral uptake transporters (i.e., OCT1/2) during pregnancy may, theoretically, lead to renal drug

TABLE 2

hOCT3 mRNA and protein quantification in human placentas from first and second trimesters and at term

Data are expressed as mean $\pm$ S.D. The difference in the human placental expression was determined by nonparametric Mann-Whitney U test.

Gestational Age	hOCT3 Transcript Expression mRNA/GUSB		hOCT3 Protein Quantification	
	Mean $\pm$ S.D.	Range	Mean $\pm$ S.D.	Range
	<i>Arbitrary Unit</i>		<i>fmol/μg Membrane Protein</i>	
T1	36.79 $\pm$ 17.82 ($n = 11$)	5.81–63.41	0.23 $\pm$ 0.033 ($n = 6$)	0.18–0.26
T2	58.93 $\pm$ 33.22 ($n = 16$)	21.20–137.40	0.38 $\pm$ 0.072 ($n = 6$)*	0.25–0.48
Term	38.57 $\pm$ 13.49 ($n = 6$)	26.23–64.32	0.36 $\pm$ 0.099 ($n = 6$)*	0.18–0.47

* Significantly different from the expression at T1 ($P < 0.05$).

accumulation and an increased risk of nephrotoxicity. These findings seem counterintuitive, as urinary excretion of xenobiotics is generally enhanced during pregnancy in humans and rodents.

We previously had reported that metformin renal clearance increased significantly in middle and late pregnancy as compared with the postpartum period (Eyal et al., 2010). Because we did not see an increase in Oct or Mate renal expression, altered renal expression of OC transporters may not be the reason underlying the observed increase in renal clearance of metformin during pregnancy. Other pregnancy-associated changes, such as elevation in renal blood flow and glomerular filtration, may contribute to the increased total renal clearance of metformin in pregnancy. However, it should be noted that our study was conducted in mice, so we cannot exclude the possibility that expression of hOCT2 and hMATE1/2-K in the kidney is differently affected by human pregnancy.

Although OCT3/Oct3 was recognized for its high expression in placenta (Kekuda et al., 1998; Sata et al., 2005), the expression of other OC transporters in the placenta was less studied. Previous Northern analysis found no expression of hMATE1 in the human placenta (Otsuka et al., 2005), but a high level of rMate1 expression was reported in the rat placenta (Terada et al., 2006; Ahmadimoghaddam et al., 2012). Here, we comprehensively analyzed the expression profiles of OCT1-3, MATE1-2, and PMAT in the same placental tissues obtained from healthy term human placenta and in mouse placenta at gd 19. Our data clearly showed that OCT3 is the predominant OC transporter expressed in term or near-term human and mouse placentas (Fig. 2).

Previous membrane vesicle and immunostaining studies suggested a basal membrane localization of OCT3/Oct3 proteins in human and rat placentas (Sata et al., 2005; Ahmadimoghaddam et al., 2012). The precise role of OCT3 in OC transport at the placental barrier is presently unclear. When pregnant Oct3-null mice were given intravenous MPP⁺, a model substrate for all OC transporters, a significant reduction in MPP⁺ accumulation was observed in homozygous Oct3^{-/-} embryos (Zwart et al., 2001), suggesting that mOct3 mediates maternal-to-fetal OC transport. In contrast, Ahmadimoghaddam et al. (2012) showed high expression of rMate1 in rat placenta and suggested that rOCT3 and rMate1 work together to sequentially mediate fetal-to-maternal transport of MPP⁺. Here, we found that MATE1/Mate1 mRNA and protein are minimally expressed in mouse and human placentas (Fig. 2), which challenges an OCT3/MATE tandem-transport model in these species. More studies are needed to elucidate the OC transport mechanisms at the human placental barrier.

Recent studies have shown that the placental expression of drug efflux transporters (e.g., P-gp, breast cancer resistance protein [BCRP]) change with gestational age, which may influence fetal drug exposure in a gestational-stage-dependent manner (Zhang et al., 2008; Zhou et al., 2008). Our data showed that mOct3 mRNA in mouse placenta at gd 15 and 19 showed a >35-fold increase over that at gd 10 (Fig. 3A). This activation of mOct3 mRNA in mouse placenta between early and middle pregnancy was also previously noticed by other investigators (Zwart et al., 2001; Shuster et al., 2013). Using LC-MS/MS, we further showed that mOct3 protein levels in the mouse placenta also increased dramatically at gd 15 and 19 (Fig. 3B). Interestingly, such a dramatic change was not observed for hOCT3 in human placenta (Fig. 4). Despite a large interindividual variation of mRNA levels, the mean hOCT3 mRNA expression did not show a gestational age-dependent change (Fig. 4A). When the absolute amount of protein was quantified, T2 and term human placenta only showed a moderate increase in hOCT3 protein (Fig. 4B). Taken together, our data suggest that although human and mouse term placentas share a similar expression pattern of polyspecific OC transporters and predominantly

express OCT3/Oct3, placental expression of the mouse mOct3 gene is regulated by gestational age and is transcriptionally activated after gd 10.

Recently, the absolute membrane protein levels of drug transporters have been determined in several human tissues, especially the liver (Uchida et al., 2011; Ohtsuki et al., 2012; Prasad et al., 2013). The protein levels for the well-established drug transporters hOCT1, hMATE1, P-gp, and BCRP in the human liver ranged from 0.14 to 7.35 fmol/ μ g by the LC-MS/MS method (Ohtsuki et al., 2012; Prasad et al., 2013). The hOCT3 protein amount in the human placenta determined in our study was 0.2–0.4 fmol/ μ g membrane protein. This value is comparable to those reported for the hepatic drug transporters. However, our absolute quantification of hOCT3 protein was performed using a single target peptide. We acknowledge that protein quantification by the LC-MS/MS method can be peptide-dependent because variability in trypsin digestion might result in different protein yields when quantified using different target peptides. Nevertheless, to the best of our knowledge, ours is the first report for absolute protein quantification for a human placental drug transporter by LC-MS/MS. Because it is more costly and time-consuming to measure the absolute protein amount, only relative protein expression measurement was performed for mOct1, mOct2, mOct3, and mMate1. Although it is informative on pregnancy-induced changes in protein expression of a specific transporter, this value may not be used quantitatively for cross-comparison between different transporters due to the peptide-dependent nature of the LC-MS/MS method.

Levels of mRNA expression have been widely used as a surrogate for protein expression or activity in biologic samples. In our study, we observed that the overall patterns of OC transporter mRNA expression are generally consistent with their protein expression, especially in various mouse tissues that are collected and processed under highly controlled conditions. Transporters that were not detectable at mRNA levels were also not detected at protein levels (Figs. 1 and 3).

In human placenta, we observed a trend of increase of hOCT3 protein with its mRNA levels. However, the correlation between hOCT3 mRNA and protein levels was relatively weak (Fig. 4C). Poor correlation was also reported for other drug transporters in the human liver (Ohtsuki et al., 2012). A closer examination of Fig. 4 reveals that the weak correlation can be traced down to a large variability (>20-fold) in mRNA levels but a relatively small (<3-fold) variability in hOCT3 protein levels in the human placenta samples. This may reflect the fact that proteins are more stable whereas mRNAs are more prone to degradation during human sample preparation. The mRNA instability could lead to permutations in the correlation analysis. In addition, poor correlations might be due to the fact that mRNA is extracted from total lysate of tissue whereas protein is isolated plasma membrane fraction, as previously suggested elsewhere (Ohtsuki et al., 2012).

In summary, we have determined the impact of pregnancy on mRNA and protein expression of polyspecific OC transporters in kidney, liver, and placenta. In mice, pregnancy has a marginal effect on renal expression of mOct1/2 but reduces mMate1 mRNA and protein expression by 20%–40%. Hepatic expression of mOct1 and mMate1 was minimally affected by pregnancy. OCT3/Oct3 was the major isoform expressed in term or near-term human and mouse placentas. Oct3 expression in mouse placenta was gestational age dependent, and its expression is activated between early and middle pregnancy. In contrast, expression of hOCT3 was high, even in early stage human placenta, and hOCT3 protein only showed a moderate increase in second-trimester and term placentas. Together, our studies provide new insights into the effect of pregnancy on the expression of polyspecific OC transporters and support an important role of OCT3 in OC transport at the maternal-fetal interface.

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Authorship Contributions

Participated in research design: Lee, Hebert, Wang.

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Contributed new reagents or analytic tools: Easterling, Kelly, Unadkat.

Performed data analysis: Lee, Prasad, Wang.

Wrote or contributed to the writing of the manuscript: Lee, Hebert, Prasad, Wang.

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