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Prominent Expression of Xenobiotic Efflux Transporters in Mouse Extraembryonic Fetal Membranes Compared to Placenta

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Abstract

Fetal exposure to xenobiotics can be restricted by transporters at the interface between maternal and fetal circulation. Previous work identified transporters in the placenta, however, less is known about the presence of these transporters in the fetal membranes (i.e., yolk sac and amniotic membranes). The purpose of this study was to quantify mRNA and protein expression of xenobiotic transporters in mouse placenta and fetal membranes during mid- to late-gestation. Concepti (placenta and fetal membranes, gestation day 11) or placenta and fetal membranes (gestation days 14 and 17) were collected from pregnant mice and analyzed for expression of multidrug resistance-associated proteins (Mrps), multidrug resistance proteins (Mdr), multidrug and toxin extrusion proteins (Mate), breast cancer resistance protein (Bcrp), and organic anion transporting polypeptides (Oatps). Maternal liver and kidneys were also collected at day 14 for mRNA and immunohistochemical analysis. mRNA expression of Mrp, Mdr, Bcrp, Mate-1, Oatp isoforms was detected at day 11. The uptake carriers Oatp2a1, 3a1, 4a1, and 5a1 showed placenta-predominant expression. At days 14 and 17, fetal membranes expressed higher mRNA levels of the efflux transporters Mrp2 (7-fold), Mrp4 (5-fold), Mrp5 (3-fold), Mrp6 (12-fold), Bcrp (2-fold), and Mate-1 (7-fold) compared to placenta. Western blot of Mrp2, Mrp4, Mrp6, and Bcrp confirmed higher expression in fetal membranes. Immunostaining revealed apical (Mrp2 and Bcrp) and basolateral (Mrp4, 5, and 6) cellular localization in epithelial cells of the yolk sac. In conclusion, xenobiotic transporters in the fetal membranes may provide an additional route to protect the fetus against endogenous chemicals and xenobiotics.

INTRODUCTION

Bidirectional exchange of chemicals into and away from the fetus is critical for normal *in utero* development. Nutrients and endogenous chemicals are transferred from the maternal circulation to the fetus, thereby providing building blocks for organogenesis. Likewise, metabolic by-products produced by the fetus are transferred to the mother. Chemical and nutrient transport between fetus and mother occurs through the placenta and fetal membranes. In rodents, the fetal membranes are comprised of an inverted yolk sac and amniotic membrane that extend from the placenta and enclose the fetus. In addition to providing nutrition, immunologic defense, and gas exchange, the placenta and fetal membranes likely represent physical barriers that prevent fetal exposure to potentially harmful xenobiotics by limiting passage, enhancing removal, or both.

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Membrane transport proteins import and efflux chemicals into and from cells, respectively. Consequently, transporters play key roles in the clearance of drugs and endobiotics from the body as well as protection of sanctuary organs such as the testes and brain. Active transport of substrates into and from tissues depends on the orientation and localization of transporters in the plasma membrane of polarized cells. In the liver and kidney, members of the organic anion transporting polypeptide (Oatp) family participate in the uptake of chemicals into epithelial cells. In contrast, efflux transporters remove chemicals and their metabolites from hepatocytes and renal tubule cells. Families of efflux transporters include the multidrug resistance-associated proteins (Mrp), multidrug resistance proteins/p-glycoprotein (Mdr, Pgp), multidrug and toxin extrusion proteins (Mate), and the breast cancer resistance protein (Bcrp). Localization of these transporters to either the apical or basolateral membranes of hepatocytes and renal tubule cells determines the direction of chemical exchange. Although transporter isoforms tend to be expressed at the same side of polarized epithelia within different tissues, there are instances in which this does not occur. For example, Mrp4 protein is expressed on the basolateral membrane of hepatocytes, but on the apical face of proximal tubule cells (van Aubel et al., 2002; Aleksunes et al., 2006). Because of such discrepancies, it is important to identify the localization of transporters within various tissues.

Due to the critical role of the placenta in fetal development and protection, researchers have characterized mRNA and protein levels of transporters in rodent and human placenta. The uptake transporters, OATP2B1 (OATP-B), OATP3A1 (OATP-D), and OATP4A1 (OATP-E) are expressed in human placenta (St-Pierre et al., 2002; Sato et al., 2003; Ugele et al., 2003). Similarly, rodent Oatp1a1, 1a5, 1b2, 2a1, 2b1, and 4a1 homologs have been detected in rat placenta obtained during mid- to late gestation (Leazer and Klaassen, 2003; St-Pierre et al., 2004).

A number of efflux transporters, including MRP1–3, 5, BCRP, and MDR1/PGP, are present in syncytiotrophoblasts and to a lesser extent, endothelial cells of capillaries, within human placenta (St-Pierre et al., 2000; Nagashige et al., 2003; Pascolo et al., 2003; Kolwankar et al., 2005; Mathias et al., 2005). There are notable changes in expression of some placental efflux transporters during gestation. Bcrp mRNA and protein levels peak in rodent placenta during the middle of gestation and decline during later stages (Yasuda et al., 2005; Wang et al., 2006; Kalabis et al., 2007). A recent publication demonstrated MRP1, 2, 5, and BCRP protein expression in term human amnion membranes obtained following caesarean section (Aye et al., 2007). Bcrp and Mrp1 proteins have also been detected on the apical membrane of syncytiotrophoblasts and endodermal epithelia of rodent yolk sac (St-Pierre et al., 2004; Wang et al., 2006; Kalabis et al., 2007). Pgp protein is localized to the luminal surface of trophoblasts (facing maternal circulation) (Novotna et al., 2004; Kalabis et al., 2005). Little is known about the expression of Mate-1 and Mrp4–6 in placenta and fetal membranes. Furthermore, the relative expression of transporters in the fetal membranes compared to placenta has been largely undetermined.

Therefore, the purpose of this study was to investigate the expression of uptake and efflux transporters in mouse placenta and fetal membranes during gestation (days 11, 14, and 17). The average gestation period in mice is 19–21 days. Implantation occurs during early gestation (days 0–5) followed by gastrulation (days 5–10) and organogenesis (days 10–14). Late gestation (days 14–19) includes late organogenesis, fetal growth and development. Identification and characterization of transporter proteins in placenta and fetal membranes will enable researchers to determine the types of substrates to which a fetus is likely to be exposed during different periods of gestation.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were mated overnight (beginning at 5 p.m.) and males were removed the following morning at 7 a.m. This was designated as gestation day 0 (average gestation period of 19 days). Average litter size for C57BL/6 mice is 7 in our facility. For RNA and protein analysis, the whole conceptus was carefully separated and removed from the uterine tissue at gestation days 11, 14, and 17 (n = 3–5 dams/time point). On day 11, the fetus was removed from the conceptus, however, the placenta and fetal membranes could not be accurately separated. On days 14 and 17, the placenta and fetal membranes were carefully dissected and separated. Maternal livers and kidneys were obtained along with the placenta and fetal membranes and a portion of each tissue was placed in 4% paraformaldehyde for 4 h at 4°C, then 18% sucrose in 10 mM PBS (pH 7.4) for 18–20 h at 4°C, and embedded in Tissue-Tek Cryo-OCT compound (Fisher Scientific, St. Louis, MO). Remaining liver, kidney, placenta, and fetal membranes were frozen in liquid nitrogen. Animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals. Studies were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

RNA Isolation

Fetal membranes from 3 concepti were pooled prior to RNA isolation and were designated a single sample (9 fetal membranes yielded 3 samples). Total RNA was isolated using RNABee reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. The concentration of total RNA was quantified spectrophotometrically at 260 nm.

Branched DNA Signal Amplification (bDNA) Assay

The mRNA expression of mouse Oatp1a5, 2a1, 2b1, 3a1, 4a1, 5a1, Mrp1–6, Mdr1a and 1b, Mate-1, and Bcrp were quantified using the bDNA signal amplification assay (QuantiGene, High Volume bDNA Signal Amplification Kit 1.0; Panomics, Fremont, CA). Multiple oligonucleotide probe sets (containing capture, label, and blocker probes) specific to mouse mRNA transcripts were designed using ProbeDesigner software v1.0 (Bayer Corp., Diagnostics Div.). Specific mouse probe sets were described previously (Cheng et al., 2005; Maher et al., 2005; Tanaka et al., 2005; Cheng and Klaassen, 2006).

Western Blotting

Placenta and fetal membranes were homogenized separately in sucrose-Tris buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) using a Potter-Elvehjem homogenizer. Protein concentrations were determined using Pierce BCA protein assay reagents according to the manufacturer's recommendations (Pierce Biotechnology, Rockford, IL). Homogenates (40 µg protein/lane) were loaded and separated on 8% SDS-polyacrylamide gels. Proteins were transferred overnight at 4°C to polyvinylidene difluoride membranes. Membranes were blocked for 1 hr in blocking buffer (2% non-fat dry milk with 0.5% Tween 20). All primary and secondary antibodies were diluted in blocking buffer. Primary antibody dilutions are as follows: Mrp1 (MRPr1, 1:2000), Mrp2 (M₂III-5, 1:600), Mrp3 (M₃II-2, 1:1000), Mrp4 (M₄I-10, 1:1000), Mrp5 (M₅I-10, 1:1000), Mrp6 (M₆II-68, 1:1000), Bcrp (BXP-53, 1:5000), Pgp (C219, 1:1000), and β-actin (ab8227, 1:2000). Mrp and Bcrp antibodies were provided by George Scheffer, VU Medical Center, Amsterdam, the Netherlands. The β-actin antibody was purchased from Abcam (Cambridge, MA). The C219 antibody was purchased from Signet Laboratories (Dedham, MA). Blots were subsequently incubated with a species-appropriate horseradish peroxidase-conjugated secondary antibody for 1 h (Sigma-Aldrich, St. Louis, MO). Protein-antibody complexes were detected using an ECL chemiluminescent kit (Pierce

Biotechnology, Rockford, IL) and exposed to medical X-ray film (Denville Scientific, Metuchen, NJ). The intensity of protein bands was quantified using Quantity One Software (Bio-Rad Laboratories, Hercules, CA).

Immunofluorescence Analysis

Staining for Mrp2, Mrp4, Mrp5, Mrp6, and Bcrp was performed as described previously (Aleksunes et al., 2006). Of note, the M₂III-5 antibody does not detect mouse Mrp2 protein by immunohistochemical staining. In turn, the Mrp2 antibody from Bruno Stieger (University Hospital Zurich, Zurich, Switzerland) was used for immunohistochemical analysis. Cryosections (5 μ m) were thaw-mounted onto Superfrost glass slides (Fisher Scientific) and stored at -80°C with dessicant until use. Briefly, cryosections were blocked with 5% goat serum in phosphate-buffered saline containing 0.1% Triton X (PBS-Tx) for 1 h and then incubated with primary antibody diluted 1:100 in 5% goat serum/PBS-Tx for 2 h at room temperature. Sections were subsequently washed in PBS-Tx, and incubated for 1 h with goat anti-rat Alexa 488 IgG for detection of Mrp4, Mrp5, Mrp6, and Bcrp, whereas goat anti-rabbit Alexa 488 IgG was used for detection of Mrp2 (Invitrogen Corporation, Carlsbad, CA). Secondary antibodies were diluted 1:200 in 5% goat serum/PBS-Tx. Rhodamine-phalloidin (1:200, Invitrogen Corp.) was added to secondary antibody incubations for detection of actin cytoskeleton. For double staining, anti-rabbit Alexa 594 F(ab')₂ fragment was used for detection of Mrp2 (Invitrogen Corporation, Carlsbad, CA). The sections were air dried and mounted in Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Corp.). Images were captured on an Olympus BX41 fluorescent microscope with a DP70 camera and DP Controller software. Each fluorescent channel was acquired sequentially and then merged to create the final image. Images were cropped and brightness and contrast were adjusted the same for each slide in Adobe Photoshop CS2 (San Jose, CA). Day 14 fetal membranes were used for immunofluorescent staining because protein expression of efflux transporters as determined by western blot was highest at this time point. All sections were both stained and imaged under uniform conditions for each antibody. Negative controls without antibody were included in the analysis (data not shown).

Statistical analysis

Quantitative results were expressed as mean $\pm$ standard error. For bDNA analysis, data are presented as relative light units (RLU) per 4 μ g total RNA. Immunoblot data are presented as trace density (O.D. x mm). Statistical analysis was not performed for tissue distribution analysis (Figs. 1 and 2). Quantitative results in Figs. 3-5 were analyzed using a one-way analysis of variance followed by Tukey's multiple range test ($p < 0.05$) using GraphPad Prism (v4) software (GraphPad Software, Inc, San Diego, CA). Two-way analysis was subsequently used to confirm the influence of tissue (placenta, fetal membranes) and time (gestational days 14 and 17) on transporter expression. Day 11 placenta/fetal membranes were excluded from statistical analysis because the two tissues could not be accurately dissected.

RESULTS

Tissue expression of uptake transporters

Placenta, fetal membranes, maternal liver, and kidney at gestation day 14 were analyzed for mRNA expression of six Oatp carriers in order to determine tissue distribution and relative expression between maternal and embryonic tissues (Fig. 1). mRNA levels of Oatp1a5 were similar among all 4 tissues, with a tendency of higher expression in maternal kidney. Expression of Oatp2a1, 4a1, and 5a1 mRNA was seen predominantly in mouse placenta, with lower levels in the other tissues analyzed. Oatp3a1 was also detected in mouse placenta, however its expression was half that in maternal kidneys. In contrast, Oatp2b1 mRNA was very low in placenta and fetal membranes, with markedly higher levels in maternal liver and

kidney. Expression of Oatp1a1, 1a4, and 1b2 mRNA in placenta and fetal membranes was less than 1% of adult mouse liver (data not shown).

Tissue expression of efflux transporters

Analysis of Mrp1–6, Mdr1a and 1b, Bcrp, and Mate-1 mRNA expression in placenta, fetal membranes, maternal liver, and kidney at gestation day 14 revealed relatively higher expression of efflux transporters (Mrp2, Mrp4, Mrp5, Mrp6, Bcrp, and Mate-1) in fetal membranes compared to placenta (Fig. 2). These transporters were also highly expressed in maternal liver (Mrp2, Mrp6, Mate-1) and kidney (Mrp2, Mrp4, Mrp6, Bcrp, Mate-1). Hepatic and renal Mrp5 mRNA levels were similar to expression in mouse placenta. Mrp1, Mdr1a, and Mdr1b mRNA were higher in the placenta compared to liver, kidney (except Mrp1), and fetal membranes. Very low levels of Mrp3 were detected in mouse placenta and fetal membranes compared to liver and kidney.

Gestational expression of uptake transporters in placenta and fetal membranes

Because levels of a number of Oatp transporters were higher in mouse placenta compared to fetal membranes, a time-course study was designed to quantify expression of Oatps from gestation day 11 to 17 in these two tissue compartments (Fig. 3). Because placenta and fetal membranes could not be accurately separated at day 11, mRNA values at this time point reflect their combined expression. Oatp2a1, 2b1, 3a1, 4a1, 5a1, and to a lesser degree, Oatp1a5 mRNA were detected in mouse placenta/fetal membranes at gestational day 11. Similar to Fig. 1, gene expression of Oatp2a1, 3a1, 4a1, and 5a1 was higher in placenta compared to fetal membranes at days 14 and 17. Levels of Oatp1a5 and 2b1 mRNA were similar between fetal membranes and placenta over the time course. Expression of Oatp2a1 mRNA in placenta increased 1.6-fold from day 14 to 17, whereas the level of Oatp5a1 decreased 46%. Two-way ANOVA revealed significant effects of tissue-type (i.e., placenta) on the expression of Oatp3a1 and 4a1 and significant effects of tissue-type and gestational day on the expression of Oatp2a1 and 5a1.

Relative placenta/membrane expression demonstrates the following rank order at gestational day 14: Oatp4a1 (24-fold), 5a1 (21-fold), 3a1 (6-fold), 2a1 (3-fold), 2b1 (2-fold), 1a5 (2-fold). A similar rank order was observed at gestational day 17: Oatp4a1 (23-fold), 3a1 (8-fold), 5a1 (6-fold), 1a5 (3-fold), 2a1 (3-fold), 2b1 (80% of fetal membranes).

Gestational expression of efflux transporters in placenta and fetal membranes

Mrp1–6, Mdr1a, 1b, Mate-1, and Bcrp mRNA were detected in placenta and fetal membranes at gestational day 11. Similar to Fig. 2, the gestational time-course study revealed higher expression of Mrp2, Mrp4 (day 14 only), Mrp5, and Mrp6 mRNA in fetal membranes compared to placenta at days 14 and 17 (Fig. 4). Interestingly, Mrp4 expression in fetal membranes decreased from gestation day 14 to 17. Mrp1 mRNA was lower in fetal membranes with expression at 16 and 28% of placental levels at days 14 and 17, respectively. Two-way ANOVA revealed significant effects of tissue-type (i.e., fetal membranes) on the expression of Mrp1, 2, 5, and 6 and significant effects of tissue-type and gestational day on the expression of Mrp4.

At day 14, expression of Mate-1 and Bcrp mRNA was 7- and 2-fold higher in fetal membranes compared to placenta, respectively (Fig. 4). By day 17, Bcrp expression in fetal membranes decreased, whereas Mate-1 levels remained unchanged. Mdr1a mRNA was lower in fetal membranes compared to placenta at day 17. In contrast to Mdr1a, levels of Mdr1b were similar in placenta and fetal membranes at days 14 and 17. Two-way ANOVA revealed significant effects of tissue-type (i.e., fetal membranes/placenta) on the expression of Mdr1a and Mate-1 and significant effects of tissue-type and gestational day on the expression of Bcrp.

Relative membrane/placenta expression demonstrates the following rank order at gestational day 14: Mrp6 (12-fold), Mrp2 (7-fold), Mate-1 (7-fold), Mrp4 (5-fold), Mrp5 (3-fold), Bcrp (2-fold), Mrp3 (no difference), Mdr1a (50% of placenta), Mdr1b (33% of placenta), Mrp1 (28% of placenta). A similar rank order was observed at gestational day 17: Mrp2 (8-fold), Mate-1 (6-fold), Mrp6 (5-fold), Mrp5 (3-fold), Mrp4 (2-fold), Bcrp (2-fold), Mrp3 (no difference), Mdr1b (no difference), Mdr1a (41% of placenta), Mrp1 (16% of placenta).

Protein expression of efflux transporters in placenta and fetal membranes

Western blot analysis was performed on placenta and fetal membrane homogenates at days 14 and 17 (Fig. 5). Analysis was limited to Mrp1–6, Bcrp, and Pgp. Protein analysis of other transporters whose mRNA expression had been assessed by bDNA was not performed because there are no specific antibodies commercially available to detect these proteins in mice (e.g., Oatps and Mate-1). Individual blots are shown in Fig. 5A. Expression of Mrp1 protein increased 26-fold in mouse placenta from day 14 to 17 of gestation. Little Mrp1 protein was detected in fetal membranes. In contrast to Mrp1, other efflux transporters, including Mrp2, Mrp4, Mrp6, and Bcrp, demonstrated fetal membrane-predominant expression patterns. Mrp2 protein expression in fetal membranes was 5- and 2-fold higher than in the placenta at days 14 and 17 of gestation, respectively. Similarly, Mrp4 protein was 136-fold higher in fetal membranes than placenta at day 14 of gestation, however, Mrp4 levels were markedly reduced by day 17. Mrp5 was detected in placenta and membranes at day 14, with reduced expression in both tissues by day 17. Mrp6 protein was not detected in placenta, but was in fetal membranes, and decreased from day 14 to 17. Bcrp protein was 6- and 3-fold higher in fetal membranes than placenta at days 14 and 17, although the relative difference was not statistically significant at day 17. Placenta and fetal membranes had similar expression of Pgp, with reduced levels seen at day 17. Of note, the Pgp antibody (C219) does not discriminate the various Mdr isoforms (Vos et al., 1998). Mrp3 protein could not be detected in mouse placenta or fetal membranes (data not shown). It should be noted that placental expression of β -actin (loading control) is 2-fold higher than fetal membranes at both time points.

Immunofluorescent detection of efflux transporters in fetal membranes

Indirect immunofluorescent staining was performed on cryosections from gestational day 14 fetal membranes to determine patterns of cellular distribution and localization of transporters. Because hepatic and renal expression of efflux transporters is known, maternal liver and kidneys were also stained in order to confirm antibody staining patterns. Immunofluorescent staining of Mrp4, Mrp5, and Mrp6 (*green*) demonstrated staining of epithelial cells (endoderm-derived) in the visceral yolk sac at gestation day 14 (Fig. 6A, 6F, and 6K, respectively). Little staining was observed in the amnion membrane (data not shown). Higher magnification shows basolateral localization of these 3 efflux transporters (Fig. 6B, 6G, 6L). In order to differentiate Mrp4–6 staining with the entire plasma membrane, tissue sections were also stained with rhodamine-phalloidin which detects actin (*red*) (Fig. 6C, 6H and 6M). Comparison of Mrp4–6 and actin images demonstrates little transporter staining at the apical surface of the yolk sac.

As previously reported, Mrp4 protein expression in maternal kidney (Fig. 6E) was localized to the apical brush-border membrane (Leggas et al., 2004). Little hepatic Mrp4 protein staining was observed (Fig. 6D). Similarly, Mrp5 was not detected in maternal liver and kidney sections (Fig. 6I and J). Staining for Mrp6 protein revealed basolateral localization in maternal hepatocytes (Fig. 6N) and proximal tubules (Fig. 6O).

Bcrp and Mrp2 proteins (*green*) are expressed at gestation day 14 and exhibit apical cellular localization in epithelial cells (endoderm-derived) of the visceral yolk sac (Fig. 7A and 7F, respectively). Staining of the actin cytoskeleton (*red*) in Figs 7C and 7H confirms that Bcrp

and Mrp2 staining is not localized to the lateral or basal membranes (Fig. 7B and 7G). Little staining was observed in the amniotic membrane (data not shown). Similar to the fetal membranes, Bcrp and Mrp2 proteins are localized to apical membranes of maternal hepatocytes (canalicular membrane, Fig. 7D and 7I, respectively) and proximal tubule cells (brush-border membrane, Fig. 7E and 7J, respectively).

Double immunostaining directed against Bcrp (*green*, Fig. 8A) and Mrp2 (*red*, Fig. 8B) confirmed that both efflux transporters are localized to the apical surface of the yolk sac (Fig. 8C). This is in contrast to double staining of Mrp2 (*red*, Fig. 8E) and Mrp4 (*green*, Fig. 8D), in which the two proteins demonstrate distinct localization to the apical and basolateral membranes, respectively (Fig. 8F). Double staining of Mrp2 with either Mrp5 or Mrp6 did not reveal overlap in localization (data not shown).

DISCUSSION

The present study characterizes the expression of uptake and efflux transporters in mouse placenta and fetal membranes during gestation. The relative mRNA expression of transporters between placenta, fetal membranes, and maternal liver and kidney were isoform-dependent. In general, Oatp carriers (Oatp 2a1, 3a1, 4a1, and 5a1) were expressed to a higher extent in placenta compared to fetal membranes. In contrast to most Oatps, there was a trend towards higher mRNA and protein expression of efflux transporters (Mrp2, Mrp4, Mrp5, Mrp6, Mate-1, and Bcrp) in mouse fetal membranes compared to placenta.

The novel finding of efflux transporters in fetal membranes warranted further immunofluorescent analysis in order to determine their cellular localization. Distinct staining patterns demonstrated both apical/maternal-facing (Mrp2 and Bcrp) and basolateral/fetal-facing (Mrp4, Mrp5, Mrp6) localization in the plasma membrane of the visceral yolk sac epithelium. Comparison to maternal tissues revealed similar localization for Mrp2, Mrp6, and Bcrp in yolk sac to that in hepatocytes and proximal tubule cells. A summary of transporter expression and localization in mouse placenta and fetal membranes is provided in Figure 9.

Data generated in the current study suggest placental transporter expression in mice parallels patterns published for humans. Mrp1, Mrp5, Pgp, and Bcrp proteins were detected in mouse placenta similar to that noted previously in human placenta (Nagashige et al., 2003; Mathias et al., 2005; Meyer Zu Schwabedissen et al., 2005a). Human and mouse placenta express the same uptake transporter isoforms including OATP2B1/2b1, 3A1/3a1, and 4A1/4a1 (St-Pierre et al., 2002; Sato et al., 2003; Ugele et al., 2003). In addition to similarities in placental expression, MRP1/Mrp1, MRP2, MRP5, and BCRP/Bcrp proteins have been detected in human amnion and rodent yolk sac (St-Pierre et al., 2004; Aye et al., 2007; Kalabis et al., 2007).

Whereas rodent and human placenta are both hemochorial, the physical structure of the placental unit differs between the two species (Carter, 2007). Invasion of trophoblasts into uterine arteries is more extensive in human placenta, with a single layer of syncytial trophoblast as the site of exchange. In contrast, rodent placenta is comprised of three trophoblast layers. In addition, human yolk sac floats free in exocoelom during the first trimester whereas rodents exhibit an inverted visceral yolk sac that functions until term. Despite these structural differences, the rodent remains a useful model for assessing pharmacokinetics within the embryo due to the general similarities in transporter expression noted above. Use of knockout mice is gaining popularity for determining the role of a particular transporter in the accumulation of xenobiotics and teratogens within the fetus. The antibiotic nitrofurantoin accumulates within fetuses of Bcrp-null mice, resulting in a 4-fold increase in the fetal-to-maternal plasma area under the curve ratio, compared to wild-type mice (Zhang et al., 2007).

Mdr1a/1b double null mice exhibit increased fetal-to-maternal plasma levels of digoxin, saquinavir, and paclitaxel, confirming a role for Pgp in limiting transplacental passage of drugs (Smit et al., 1999). In addition, CF-1 mice with a natural mutation in Pgp have increased accumulation of the L-652,280 isomer of the pesticide, avermectin, in the fetal compartment, resulting in an elevated incidence of cleft palate malformations (Lankas et al., 1998). Knowledge generated in the present study will enable the design of additional *in vivo* disposition and toxicology studies for transporters (i.e., Mrp2 and Mrp6) not previously investigated in the placenta and fetal membranes.

The fetus is particularly sensitive to xenobiotic toxicity during early gestational periods. Limited availability of tissue in pregnant mice makes it difficult to quantify transporter expression during the first half of gestation (days 1 to 10). A number of studies using human preterm placenta suggest that transporter expression declines with gestation. Levels of MRP2, MRP5, PGP, and BCRP are higher in placenta obtained at preterm compared to term (Mathias et al., 2005; Meyer Zu Schwabedissen et al., 2005a; 2005b; 2006; Sun et al., 2006). Of note, one conflicting report demonstrates increased BCRP protein in term placenta compared to preterm (6–13 weeks) (Yeboah et al., 2006). In the current study, we were able to quantify expression of transporters during mid- to late gestation (days 14 to 17). mRNA expression of Bcrp and Mdr1b tended to decrease by day 17, consistent with previous reports (Kalabis et al., 2005; 2007). Expression of Mrp4 mRNA in fetal membranes was also reduced at day 17. Mrp2, Mrp5, and Mrp6 mRNA were expressed similarly in fetal membranes at days 14 and 17, however, Mrp5 and Mrp6 protein levels decreased at day 17. Although gestational patterns of efflux transporters are isoform-specific, there are general trends of reduced expression near term.

In addition to pharmaceuticals, a number of endogenous chemicals are substrates for Oatp and Mrp transporters. The placenta is a major source of essential steroid hormones necessary for fetal development and to maintain pregnancy. Sulfate conjugates of the steroids dehydroepiandrosterone and estrone, are substrates for OATP2B1-mediated transport (Hagenbuch and Meier, 2004). Similarly, OATP4A1 transports the bile acid, taurocholate. The uptake transporters OATP2A1, 3A1, and 4A1 are also able to transport prostaglandins. It has more recently been demonstrated that some OATPs can function bidirectionally (Mahagita et al., 2007). Additional research should investigate the functional activity of OATPs in the placental passage of endogenous chemicals.

MRP4 and MRP5 efflux proteins also transport endobiotics. MRP4 transports cyclic nucleotides (i.e., cAMP and cGMP), prostanoids (PGE₁, PGE₂, TXB₂), steroids, bile acids, folate, and urate (Chen et al., 2001; 2002; Rius et al., 2005; 2006). MRP5 can similarly transport cyclic nucleotides and folate (Jedlitschky et al., 2000; Wielinga et al., 2003). Maternal-fetal exchange of these chemicals may be important in fetal development and parturition.

In conclusion, uptake and efflux transporters are expressed in mouse placenta and fetal membranes. Detection of Mrp proteins in mouse yolk sac suggests an additional route of substrate transfer between mother and fetus in addition to the placenta. The exact function of these transporters during gestation is uncertain, however, possible roles include exchange of endobiotics and metabolic by-products, paracrine signaling, and fetal protection.

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Nonstandard Abbreviations

bdNA, branched DNA; rodent: Bcrp, human: BCRP breast cancer resistance protein; DAPI, 4',6-diamidino-2-phenylindole; rodent: Mate, human: MATE multidrug and toxin extrusion proteins; rodent: Mdr, human: MDR multidrug resistance proteins; rodent: Mrp, human: MRP multidrug resistance-associated proteins; rodent: Oatp, human: OATP organic anion transporting polypeptides; PBS-Tx, phosphate-buffered saline with 0.1% Triton X; rodent: Pgp, human: PGP p-glycoprotein; RLU, relative light units.

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Figure 1. Tissue expression of uptake Oatp transporters

Total RNA was isolated from placenta, fetal membranes, maternal liver and kidney on day 14 of gestation. RNA was analyzed by bDNA assay for mRNA expression of Oatp1a5, 2a1, 2b1, 3a1, 4a1, and 5a1. The previous nomenclature for Oatp isoforms is presented in parentheses. The data are presented as mean relative light units (RLU) per 4 µg total RNA ± SE (n = 3).

Figure 2. Tissue expression of efflux Mrp, Mdr, Bcrp, and Mate transporters

Total RNA was isolated from placenta, fetal membranes, maternal liver, and kidney on day 14 of gestation. RNA was analyzed by bDNA assay for expression of Mrp1–6, Mdr1a, 1b, Bcrp, and Mate-1. The data are presented as mean relative light units (RLU) per 4 µg total RNA ± SE (n = 3).

Figure 3. Gestational expression of uptake Oatp transporters in placenta and fetal membranes
Total RNA was isolated from concepti (day 11) and placenta and fetal membranes (days 14 and 17). RNA was analyzed by bDNA assay for expression of Oatp1a5, 2a1, 2b1, 3a1, 4a1, and 5a1. The previous nomenclature for Oatp isoforms is presented in parentheses. The data are presented as mean relative light units (RLU) per 4 µg total RNA $\pm$ SE (n = 3–5). Asterisks (*) represent statistical differences (p<0.05) between placenta and fetal membranes. Daggers (†) represent statistical differences (p<0.05) compared to Day 14.

Figure 4. Gestational expression of efflux Mrp, Mdr, Bcrp, and Mate transporters in placenta and fetal membranes

Total RNA was isolated from concepti (day 11) and placenta and fetal membranes (days 14 and 17). RNA was analyzed by bDNA assay for expression of Mrp1–6, Mdr1a, Mdr1b, Mate-1, and Bcrp. The data are presented as mean relative light units (RLU) per 4 µg total RNA $\pm$ SE (n = 3–5). Asterisks (*) represent statistical differences (p < 0.05) between placenta and fetal membranes. Daggers (†) represent statistical differences (p < 0.05) compared to Day 14.

Figure 5. Protein expression of efflux transporters in placenta and fetal membranes

Western immunoblots were performed using homogenates (40 μ g protein/well) from placenta and fetal membranes at 14 and 17 days of gestation. The data are presented as individual blots (A) and as mean trace density (O.D. x mm) $\pm$ S.E (n = 3) (B) for Mrp1, Mrp2, Mrp4, Mrp5, Mrp6, Bcrp, and Pgp. Blots were probed for β -actin staining to confirm equal protein loading. Due to differences in relative expression of β -actin between placenta and fetal membranes, transporter western blot data was not normalized to β -actin. Asterisks (*) represent statistical differences (p<0.05) between placenta and fetal membranes. Daggers (†) represent statistical differences (p<0.05) compared to Day 14.

Figure 6. Immunofluorescent detection of Mrp4, Mrp5, and Mrp6 transporters in fetal membranes Indirect immunofluorescence against Mrp4, Mrp5, Mrp6 (*green*), and actin cytoskeleton (*red*) was conducted on day 14 fetal membrane cryosections. Images are shown at 40X (A, F, K) magnification. Cropped images were enlarged and provided as inserts (B, G, L). Actin staining is shown at high magnification (C, H, M). Sections were mounted in Prolong Gold containing DAPI for nuclear staining (*blue*). Maternal liver (D, I, N) and kidney (E, J, O) cryosections from gestational day 14 were stained for Mrp4, Mrp5, and Mrp6 proteins. Bar, 50 μ m.

Figure 7. Immunofluorescent detection of Bcrp and Mrp2 transporters in fetal membranes
Indirect immunofluorescence against Bcrp, Mrp2 (*green*), and actin cytoskeleton (*red*) was conducted on day 14 fetal membrane cryosections. Images are shown at 40X (A, F) magnification. Cropped images were enlarged and provided as inserts (B, G). Actin staining is shown at high magnification (C, H). Sections were mounted in Prolong Gold containing DAPI for nuclear staining (*blue*). Maternal liver (D, I) and kidney (E, J) cryosections from gestational day 14 were stained for Bcrp and Mrp2 proteins. Bar, 50 μ m.

Figure 8. Immunofluorescent co-localization analysis of Mrp2/Bcrp and Mrp2/Mrp4 proteins in fetal membranes

Double indirect immunofluorescence against Bcrp (*green*, A) and Mrp2 (*red*, B) proteins was performed on day 14 fetal membrane cryosections. Images A and B were merged to create image C demonstrating co-localization of Bcrp and Mrp2 at the apical surface. Double indirect immunofluorescence against Mrp4 (*green*, D) and Mrp2 (*red*, E) proteins was similarly performed on day 14 fetal membrane cryosections. Images D and E were merged to create image F demonstrating distinct localization of Mrp4 and Mrp2 to the basolateral and apical surfaces, respectively. 40X magnification; Bar, 50 µm.

Figure 9. Summary of xenobiotic transporter expression in mouse placenta and fetal membranes
 The mouse placenta expresses a number of uptake (*Oatp1a5*, *2a1*, *3a1*, *4a1*, *5a1*) and efflux (*Mrp1*, *Mrp5*, *Mdr/Pgp*, *Bcrp*) transporters. Kalabis *et al.* demonstrated localization of *Bcrp* to the apical surface of the syncytiotrophoblasts in mouse placenta (Kalabis *et al.*, 2007). Localization of *Oatp*, *Mrp* and *Mdr* transporters in mouse placenta is currently unknown. The amniotic membrane and inverted yolk sac enclose the fetus and are together known as the fetal membranes. Endoderm-derived epithelial cells of the yolk sac express transporters on the apical/maternal face (*Mrp2* and *Bcrp*) and the basolateral/fetal face (*Mrp4*, *5*, *6*) (see insert). *Mdr/Pgp*, *Mate-1*, and *Oatp1a5* transporters are also detected in the fetal membranes, although their cellular localization is unknown.