ABC transporter expression in human placenta as a function of pregnancy condition

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d) Abbreviations: PTB, preterm birth; PTL, preterm labor; PTLI, preterm labor with inflammation; PTLNI, preterm labor without inflammation; PTSPE, preterm no labor with indications for spontaneous preeclampsia; TNL, term no labor; TL, term labor; DEG, differentially expressed gene; FDR, false discovery rate; NS, no significant difference; GA, gestational age; wk, weeks; IHC, immunohistochemistry; MDR, multidrug resistance protein; BCRP, breast cancer resistance protein; MRP, multidrug resistance-associated protein
ABSTRACT

Fetal drug exposure is determined by the type and concentration of placental transporters and their regulation is central to the development of new treatments and delivery strategies for pregnant women and their fetuses. We tested the expression of several clinically important transporters in the human placenta associated with various pregnancy conditions (i.e. labor, preeclampsia, and preterm labor-inflammation).

Placentas were obtained from 5 groups of women at the time of primary cesarean section: (i) term no labor (TNL); (ii) term labor (TL); (iii) preterm no labor (delivered for severe preeclampsia, PTSPE); (iv) preterm labor without inflammation (PTLNI); (v) preterm labor with inflammation (PTLI). Samples were analyzed by Western blot and immunohistochemistry to identify changes in protein expression. Relative mRNA expression was determined by quantitative real-time PCR. A functional genomic approach was used to identify placental gene expression and elucidate molecular events that underlie the given condition. Placental expression of ABC transporters from laboring women and women with preeclampsia was unaltered. MDR1 and BCRP protein and mRNA expression increased in placentas of women with preterm labor-inflammation. Molecular pathways of genes up-regulated in PTLI samples included cytokine-cytokine receptor interactions and inflammatory response when compared to the PTLNI group. The mRNA expression of MDR1 and BCRP were correlated to that of interleukin-8, which also increased significantly in PTLI. These data suggest that the transfer of drugs across the placenta may be altered in preterm pregnancy conditions associated with inflammation through changes in MDR1 and BCRP.
INTRODUCTION

Drug treatment options during pregnancy and lactation are limited because few products have been tested for safety and efficacy in these two patient groups. The placenta is a partially protective barrier that limits fetal exposure to xenobiotics, which is attributed in part to the expression of transporter proteins on placental apical and basal membrane surfaces. Among the most abundant of the apically expressed xenobiotic transporters on the maternal side of the placenta are multidrug resistance protein (MDR) 1 (P-glycoprotein; ABCB1), multidrug resistance-associated protein 2 (MRP2/ABCC2), and breast cancer resistance protein (BCRP/ABCG2), which handle the efflux of xenobiotics and metabolites out of the fetoplacental compartment (Jonker et al., 2000). The localization of MDR3 (ABCB4) and MRP1 (ABCC1) are less clear, but studies suggest that these transporters may be positioned on the basolateral membrane of the placenta where they transport substrates from mother to fetus (Nagashige et al., 2003; Evseenko et al., 2006). Additional transporters, including BCRP and MRP1, line the fetal capillaries, providing yet another barrier against xenobiotic entry (St-Pierre et al., 2000; Yeboah et al., 2006).

Expression of these clinically important transporters is dependent on gestational age. However, drug transporter expression and regulation in placenta of women with pregnancy pathology requires further definition. Preterm labor is the leading cause of perinatal morbidity and mortality. Preeclampsia and inflammation -which is often secondary to uterine infection- are well-recognized causes of preterm birth and, when diagnosed, frequently result in clinically indicated premature delivery. Treatment for the prevention of preterm birth has thus far been unsuccessful and the rate of premature...
birth has increased over the years. We hypothesize that pregnancy conditions associated with preterm birth such as spontaneous preterm labor, preeclampsia, and preterm labor-inflammation, alter the expression of drug transporters in human placenta. We applied immunohistochemistry, western blot, and quantitative real time PCR to determine the localization, and protein and mRNA expression of transporters in a series of human placentas obtained from clinically diagnosed pregnancy conditions. In addition, we applied functional genomic profiling, an effective approach toward obtaining mechanistic understanding of underlying disease through changes in gene expression (Mason et al., 2006), to gain insight into the processes associated with abnormal labor. We postulate that these processes may mediate the observed changes in transporter expression.

The results from these studies provide evidence for altered expression of MDR1 and BCRP during inflammation-associated spontaneous preterm labor. They also support the involvement of cytokine-mediated events as a means to explain the observed increase in MDR1 and BCRP expression. Overall, our data suggest that up-regulation of MDR1 and BCRP could alter drug transfer across the placenta. These results will help predict human fetal drug toxicity and drug delivery, and offer new insights into the regulation of placental drug transporters and the impact of various pregnancy conditions upon them.
METHODS

Study Design

Placenta were obtained under Institutional Review Board approval and after written consent from 5 groups of women at primary cesarean section performed at Yale University: (i) term no labor (TNL); (ii) term labor (TL); (iii) preterm no labor delivered for severe preeclampsia (PTSPE; mean gestational age (GA), 30.3 weeks; range, 25.6 – 33.0 weeks); (iv) preterm labor unassociated with inflammation (PTLNI; mean GA, 30.5 weeks; range, 25.3 – 36.6 weeks, histological chorioamnionitis, stage 0); (v) preterm labor with inflammation (PTLI; mean GA, 28.7 weeks; range, 28.0 – 33.3 weeks; histological chorioamnionitis, stage III). Labor was defined by the presence of regular uterine contractions accompanied by progressive cervical dilation. The diagnosis of intra-amniotic inflammation was based on an amniotic fluid mass restricted score of 3 or 4 plus >100 white blood cells/μL in the context of a positive amniotic fluid culture in a sample that was obtained by transabdominal amniocentesis (Buhimschi et al., 2005). These tests provide the most accurate tools currently available to maximize the likelihood of sample homogeneity. The mass restricted score provides qualitative information regarding the presence or absence of intra-amniotic inflammation. Briefly, the score ranges from 0-4, depending on the presence (assigned a value of 1) or absence (assigned a value of 0) of each of 4 protein biomarkers (Buhimschi et al., 2005). A score of 3-4 indicates inflammation, whereas a score of 0-2 excludes it. This biomarker pattern is predictive of preterm birth, histologic chorioamnionitis, and adverse neonatal outcome. A detailed description of the mass restricted method has been published previously (Buhimschi et al., 2005). Preeclampsia was defined according to
established criteria from the American College of Obstetricians and Gynecologists as a systolic blood pressure of 140 mmHg or a diastolic blood pressure of 90 mmHg and proteinuria of at least +1 on dipstick testing, each on two occasions 4–6 hours apart. In a 24-hr urine collection, proteinuria was defined as ≥300 mg of protein. Indications for cesarean delivery in the PTLNI group were related to spontaneous preterm labor. The indication for cesarean delivery in the TNL and TL group was related to breech presentation and an arrest of cervical dilation at ≥6 cm, respectively. Clinical data were retrieved from the medical records, and statistical analysis of patient demographics was performed using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls post hoc test for multiple comparisons.

**RNA Isolation and Microarray Preparation**

Total RNA isolation and gene profiling of placenta was performed in triplicate for term and preterm samples using the Affymetrix GeneChip Human Genome U133 Plus 2.0™ microarray (Affymetrix, Santa Clara, CA) as described previously (Mason et al., 2010).

**Microarray Data Processing and Statistical Analysis**

The quality of the microarray experiment was assessed as described by Chang and colleagues (Chang et al., 2007) using bioconductor packages for statistical analysis of microarray data. Multidimensional Scaling (MDS) analysis was performed with the signal estimates to assess sample variability. The quality assessment and MDS analyses identified and disqualified discordant sample chips. Signal data were obtained using the RMA algorithm. Differential gene expression between the individual pair-wise conditions was assessed by modified t-tests as described (Kedziorek et al., 2010). The
search for genes varying among the conditions was made by combining all the pair-wise comparisons above to construct an F-test, which is equivalent to a one-way ANOVA for each gene except that the residual mean squares have been moderated between genes (Smyth, 2004). The P-values of the tests provide a way to rank genes in terms of the evidence for differential gene expression in order to obtain the most likely differentially expressed genes (DEG) between and among conditions. P-values (P ≤ 0.05) and a 1.5-fold threshold were used as a cutoff for gene inclusion in our analysis.

Microarray Data Analysis

DAVID (Huang da et al., 2009), an ontology-based webtool, was utilized to evaluate statistical measures of knowledge-based groups of genes from publications and public resources. The biological functions of the genes in the placental groups were examined in DAVID on the basis of information from the Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and gene descriptions from various public databases. We distinguished genes that were up-regulated and down-regulated (differently expressed genes) and used DAVID to determine Gene Ontology categories which were overrepresented (enriched) with differentially expressed genes. The false discovery rate (FDR) filter identified categories (biological processes, pathways, or molecular functions) that were changed by random chance. The FDR was set at 10% and GO categories with FDR <10% were considered significantly enriched.

Quantitative Real Time PCR

Primer sequences for amplifications were chosen based on previously published cDNA sequences (Table 1). For normalization of the mRNA data, the endogenous
reference gene 18s rRNA was used. All primer sets were tested to ensure efficiency of amplification over a wide range of template concentrations. SYBR green (BioRad, Hercules, CA) was used for amplicon detection. A melt curve was used after amplification to ensure all samples exhibited a single amplicon. Each sample was assayed in triplicate. The average Cₜ value (cycle threshold for target or endogenous reference gene amplification) was estimated using the software associated with the i-cycler real-time PCR detection system (BioRad, Hercules, CA). Relative changes in mRNA expression of the target genes were analyzed using delta-delta Cₜ method (2⁻ΔΔCₜ) (Livak and Schmittgen, 2001). In this method, the average ΔCₜ was calculated by subtracting the average Cₜ value of the endogenous reference gene (18s rRNA) from the average Cₜ of the target gene for the condition and control placental groups. Fold changes in mRNA expression of target genes in placenta from the condition groups (TL, PTSPE, PTLNI, PTLI) were expressed relative to that of the TNL placental control group.

To validate (biological and statistical) the microarray results, we performed qRT-PCR on select genes that were differentially expressed and/or significantly different in comparisons of either PTLI versus PTLNI or PTLI versus TL.

**Western blot analysis**

Human placentas were processed according to methods previously described (Novotna et al., 2004). Briefly, placentas were homogenized in buffer containing 250mM sucrose, 10mM Tris, 5mM EDTA and complete protease inhibitor cocktail (Roche, Indianapolis, IN) supplemented with PMSF (1mM), pH 7.4. Crude membrane fractions were obtained through differential centrifugation. The homogenate was initially
centrifuged at 10,000 x g for 10 min at 4 °C. The resulting supernatant was then centrifuged at 36,000 x g for 70 min at 4 °C. Protein concentration was determined using Bradford (Bio-rad, Hercules, CA) assays according to the manufacturer’s protocol. Membrane protein (50-75 µg) was subject to SDS-PAGE on precast Tris-HCl gels (Bio-rad, Hercules, CA). The separated proteins were transferred to PVDF membranes and blocked using 5% non-fat dry milk. Placental transporters were detected by incubating the membranes overnight at 4 °C with a 1:500 dilution of the monoclonal primary antibody for MDR1 (F4; Sigma, St. Louis, MO) and BCRP (BXP21; Chemicon, Temecula, CA), a 1:500 dilution for MDR3 (clone P3 II-26; Chemicon, Temecula, CA), a 1:400 dilution for MRP1 (MRPr1; Alexis Biochemicals, San Diego, CA) and a 1:50 dilution for MRP2 (M2III-6; Chemicon, Temecula, CA). The membranes were immunoblotted using peroxidase-conjugated secondary antibody and detected using the ECL detection systems (Amersham Biosciences, Piscataway, NJ). Equivalence of protein loading was confirmed by secondary immunoblotting with anti-β-actin antibody.

Immunohistochemical (IHC) Analysis

Immunohistochemical detection of MDR1 and BCRP was performed on frozen sections of placenta from each of the 5 groups of women (n=3 / group). Placental sections were blocked and incubated overnight at 4 °C with the MDR1 and BCRP monoclonal antibodies and dilutions used for western blots. Biotin-labeled secondary antibodies were visualized using peroxidase-conjugated streptavidin (Vectastain ABC kit, Vector Laboratories, USA) with diaminobenzidine (DAB; Sigma, St. Louis, MO) as the substrate. Slides were then counterstained with hematoxylin followed by dehydration in a graded series of ethanol dilutions, cleared by xylene substitute, and
mounted with DPX mountant (Sigma, St. Louis, MO). Control incubations were without primary antibody.

**Statistical Analysis**

Statistical analysis was done with Graphpad Prism version 4.0 (Graphpad Software, Inc., San Diego, CA). Quantitative real-time PCR results were reported as fold change in mRNA expression of target genes (mean ± SEM) for each placental group relative to the mRNA expression found in the TNL control placental group. Mean fold changes in mRNA expression in all the groups were compared by one-way analysis of variance (ANOVA) followed by post-hoc Student-Newman-Keuls multiple comparison test. Pearson coefficient analysis was used to determine the correlation between the fold changes in mRNA expression of the target genes. Statistical significance was set at p < 0.05.

**RESULTS**

*Clinical characteristics of placental samples*

There were no pathological changes in placentas from TNL, TL, and PTLNI groups based histological evaluation. The samples from PTLI group were associated with histological stage III chorioamnionitis (full-thickness inflammation of both chorion and amnion). This was complemented by inflammation of the amnion (range, grades 1-3; mode, grade 3), inflammation of the chorion-decidua (range, grades 3-4; mode, grade 3) and funistis (range, grade 1-4; mode, grade 3). Histological grading was based on four grade system devised by Salafia and colleagues (Salafia et al., 1989). Pathological
abnormalities associated with placentas from PTSPE consisted of placental infarcts less than 3 cm, fibrin deposition, decidual vascular thrombosis, decidual hemorrhage, necrosis, and hyperplastic arteriosclerosis. Further, there were no statistically significant differences in gestational age or fetal birth weight among those women who delivered term (no labor versus labor) or preterm (preeclampsia, inflammation, labor, and no labor) or in maternal age (data not shown). Thus, the observed differences in the ABC transporters among the placental groups are likely the result of the pregnancy condition rather than any variation in maternal age, or variation in gestational age within term or preterm groups (i.e. PTLNI vs PTLI).

Expression and localization of placental drug transporters

Protein levels of MDR1, MDR3, MRP1, MRP2, and BCRP were determined from immunoblot analyses. There was greater expression of MDR1 and BCRP observed in placenta of women who were preterm (Figure 1A and B) compared to term. Furthermore, their protein expression appeared highest in the placenta of women with inflammation (PTLI) compared to women in preterm labor unassociated with inflammation (PTLNI; Figure 1A and B). Other laboratories have shown that MDR1 expression is not dependent on the region of the sample, nor cesarean versus vaginal delivery (Camus et al., 2006; Sun et al., 2006). MRP1, MRP2, and MDR3 were present in all samples but their expression was variable and did not appear to be dramatically affected by pregnancy condition (Figure 1C, D, and E).

The proper cellular localization is essential for transporters to perform their transport function. IHC analysis verified that the observed changes were due to MDR1
and BCRP (Figure 2) protein expression at the membrane of the syncytiotrophoblast cells. BCRP was also localized to fetal blood vessel endothelial cells (Fig. 2).

In many instances, the regulation of these transporters occurs at transcription. Given the range of gestational ages in each placental group (condition), we increased the number of placental samples \( n = 6-10 \), following Western blot analysis and immunohistochemistry, used for semi-quantitative real-time PCR. We found significant increases in MDR1 and BCRP gene expression in the preterm placental samples associated with inflammation (PTLI) (Figure 3), which corresponds with their observed protein levels in this condition. There were no changes in MRP1 and MRP2 gene expression among the given conditions. MDR3 mRNA levels were significantly increased in the PTLI group and there were higher levels in PTSPE compared to TL and TNL.

Functional characteristics of genes overexpressed in preterm labor inflammation (PTLI)

a) PTLI compared to preterm labor no inflammation (PTLNI)

We identified 127 genes overexpressed \( \geq 1.5; p \leq 0.05 \) in PTLI compared to PTLNI. The enrichment of these genes was categorized by GO (pathways, biological processes, and molecular functions). Pathway analysis using the KEGG database revealed only one significantly enriched pathway, cytokine-cytokine receptor interaction (FDR 7.7). Significantly up-regulated genes were further categorized by biological processes and molecular functions. We identified seven biological processes that were significantly enriched: 1) response to wounding (FDR 0.6); 2) inflammatory response (FDR 0.8); 3) regulation of cell motion (FDR 3.9); 4) regulation of cell proliferation (FDR 5.7); 5) defense response (FDR 6.4); 6) positive regulation of signal transduction (FDR
7.7); and 7) positive regulation of cell motion (FDR 8.0). There were three molecular functions significantly enriched: 1) growth factor binding (FDR 0.1); cytokine binding (1.3); and 3) cytokine receptor activity (FDR 8.4).

b) PTLI compared to term labor (TL)

One hundred and thirty seven genes were overexpressed (≥ 1.5; p ≤ 0.05) in PTLI when compared to TL. We found only the focal adhesion pathway (FDR 9.9) to be significantly enriched. There were nine biological processes enriched: 1) female pregnancy (FDR 0.4); 2) tube development (FDR 3.3); 3) ossification (3.8); 4) positive regulation of kinase activity (FDR 4.3); 5) bone development (FDR 5.1); 6) positive regulation of transferase activity (FDR 5.2); 7) wound healing (FDR 7.08); 8) regulation of locomotion (FDR 7.3); 9) anion transport (FDR 9.4) and two molecular functions enriched: 1) growth factor binding (FDR 1.95); and 2) actin binding (FDR 5.7).

Functional characteristics of genes underexpressed in preterm labor inflammation (PTLI)

a) PTLI compared to preterm labor no inflammation (PTLNI)

We identified 216 genes that were underexpressed (≥ 1.5; p ≤ 0.05) in PTLI compared to PTLNI. There was only one significantly enriched pathway, ECM-receptor interaction (FDR 1.7). Ten biological processes were found to be significantly enriched: 1) unsaturated fatty acid metabolic process (FDR 0.6); 2) fatty acid metabolic process (FDR 1.1); 3) branching morphogenesis of a tube (FDR 1.8); 4) morphogenesis of a branching structure (FDR 3.2); 5) icosanoid metabolic process (FDR 3.9); 6) tube morphogenesis (FDR 6.8); 7) negative regulation of binding (FDR 8.7); 8) lipid
biosynthesis process (FDR 8.9); 9) positive regulation of cell adhesion (FDR 9.2); and 10) icosanoid biosynthesis process (FDR 9.4). There were six molecular functions significantly enriched: 1) lipid binding (FDR 0.4); 2) coenzyme binding (FDR 1.8); 3) cofactor binding (FDR 3.6); 4) actin binding (FDR 6.7); 5) peroxidase activity (FDR 8.1); and 6) oxidoreductase activity, acting on peroxide as acceptor (FDR 8.1).

b) PTLI compared to term labor (TL)

There were 140 genes underexpressed (≥ 1.5; p ≤ 0.05) in PTLI compared to TL. There were no pathways or biological processes that were significantly enriched (FDR < 10%). Lipid binding (FDR 1.8) was the only molecular function that was significantly enriched.

Biological validation of microarray gene expression

To verify the microarray results, highly differentially expressed genes including beta-1 adrenergic receptor (ADRB1), eosinophil major basic protein- also referred to as proteoglycan 2 (MBP or PRG2), stanniocalcin 1 (STC1), and hydroxysteroid (11-beta) dehydrogenase 2 (HSD11β2) were selected and analyzed by qRT-PCR. We confirmed changes in expression (direction and magnitude) of these genes between PTLI and PTLNI (Supplemental Table 2), and PTLI and TL (Supplemental Table 3). Overall, the direction of change in gene expression by qRT-PCR was consistent with the microarray analysis of these four genes. Additional genes encoding human chorionic gonadotropin beta polypeptide (β-hCG), retinoid x receptor alpha (RXRα), and GATA binding protein 2 (GATA2) were used to confirm statistical significance of microarray genes.

Changes in the mRNA expression of pro-inflammatory cytokines in various placental
Previous reports have indicated inverse correlations between MDR1 and pro-inflammatory cytokines. However, neither TNF-α nor IL-6 mRNA expression was altered, and fold changes in IL-8 mRNA expression was significantly increased (12.1-fold, p < 0.001) in PTLI compared to TNL (Figure 4). IL-8 mRNA expression in PTLI was greater than in other conditions including PTSPE where the fold change in IL-8 mRNA expression (6.3, p < 0.05) compared to TNL was greater than that of TL but not PTL with and without inflammation (Figure 4). The fold changes in mRNA expression of IL-8 were correlated with that of MDR1 (Pearson r = 0.50, p < 0.05, respectively) and BCRP (Pearson r = 0.65, p < 0.00) among the placental groups.

DISCUSSION

Expression patterns of placental ABC transporters vary with gestational age and medical condition during pregnancy. The general consensus is that MDR1 and BCRP expression decline (Gil et al., 2005; Mathias et al., 2005; Meyer zu Schwabedissen et al., 2006; Sun et al., 2006), whereas MRP2 and MDR3 levels increase with gestational age towards term (Patel et al., 2003; Meyer zu Schwabedissen et al., 2005). This may reflect a physiological adaptation to the changing requirements for fetal protection, especially in the preterm period. However, several discrepancies have been observed, particularly in humans. For example, Mathias and colleagues reported that BCRP expression in human placenta does not significantly change with gestational age (Mathias et al., 2005), whereas Yeboah and co-workers showed that placental BCRP protein levels increased toward term while mRNA expression remained unchanged.
(Yeboah et al., 2006). The placental samples used here encompass two very distinct gestational time points: a preterm pregnancy period (28-31 weeks) and a term pregnancy period (38-41 weeks. Because this is not a continuous time course analysis, we cannot infer gestational regulation of the inspected transporters. However, we do observe relatively higher protein expression of MDR1 and BCRP in placental samples from preterm women compared to term (Fig. 1A, B).

There were no apparent changes in expression levels of the ABC transporters in response to labor (term or preterm), which is consistent with prior reports where expression levels of BCRP in human reproductive tissues (fetal membranes and attached deciduas) (Yeboah et al., 2008) and MDR1 in human placenta (Sun et al., 2006) were not altered by labor at term. Our data further supports that MDR1 and BCRP expression does not change with preterm labor. Changes in MRP1, MRP2, and MDR3 protein expression were less apparent in crude membrane fractions of placental tissue. These preparations differ from isolated syncytiotrophoblasts in purity, and may explain potential differences with other results; specifically in the extent of BCRP and MRP1 protein expression, which are also localized to the fetal capillary endothelial cells. However, immunohistochemistry revealed that the cellular localization of MDR1 and BCRP was not altered in the placental groups. Further, mRNA expression appears to parallel that of protein expression. We suspect that the observed differences in protein expression are due to the specific pregnancy condition rather than variation in experimental design.

Preterm birth is the leading cause of perinatal morbidity and mortality. A large proportion of preterm births are associated with preeclampsia and inflammation- often
secondary to infection. It is increasingly clear that inflammation (outside of that associated with pregnancy) affects the expression of drug transporters (Petrovic et al., 2007). We found that both MDR1 and BCRP (protein and mRNA expression) are highest in placentas from women with inflammation (Figures 1A, B and 3A, B). Given their high WBC count, it is probable that inflammation (i.e. stage III chorioamnionitis) is a response to uterine infection. These data represent the first evidence of direct infection-mediated transporter regulation.

Our findings differ with prior literature noting transporter down-regulation during inflammation caused by inflammatory cytokines such as TNF-α, IL-6 and endotoxin (i.e. LPS) in rats (Sukhai et al., 2001; Chen et al., 2005; Wang et al., 2005) and human primary placental cells (Evseenko et al., 2007). We offer several possible explanations for these differences: the impact of LPS-induced inflammation on drug transporters has yet to be evaluated at different gestational stages, and previous reports have indicated that preterm placentas respond differently to LPS compared to those at term, specifically in their patterns of cytokine release (Holcberg et al., 2007). More importantly, common clinical infections of the reproductive compartments are associated with microorganisms that lack LPS, such as Ureaplasma sp., Mycoplasma hominis, and Group B Streptococcus. It is evident that different pathogens or pathogen components elicit diverse patterns of gene expression and cytokine release (Flad et al., 1993; Ueyama et al., 2005). For example, IL-8 was significantly elevated in amniotic fluid and umbilical cord blood in cases of intrauterine Ureaplasma infection, which was not observed with other pathogens (Witt et al., 2005). Taken together, stimulation of alternative cytokines or inflammatory mediators could have contrasting affects on ABC
transporters. Thus, observed differences in transporter regulation among various experimental models are not surprising. This is evident in cases of patients with inflammation from rheumatoid arthritis where an increase, rather than decrease, in MDR1 expression is observed (Llorente et al., 2000). It is clear that the impact of inflammation on drug transporters in the human placenta is still in a nascent stage. The development of models that more closely mimic that of the human pathological pregnancy condition will expound differences in transporter regulation; this includes the need to evaluate various inflammatory pathogens and or stimuli during pregnancy.

Hence, we adopted a functional genomic approach to identify potential mechanisms driving changes in gene expression during preterm labor associated with inflammation (PTLI). We hypothesized that underlying inflammatory events may account for the observed MDR1 and BCRP regulation. Because the PTLI group is defined, in part, by labor, it was logical to compare this placental group to those also associated with labor, specifically PTLNI and TL. In general, functional pathways and biological processes associated with pregnancy and development were found to be enriched (overrepresented) with genes overexpressed in PTLI compared to TL. Interestingly, these events appeared to be similar in comparisons of genes up-regulated in PTLNI compared to TL (Supplemental Data Section). When we compared PTLI to PTLNI we found that genes were up regulated in processes associated with inflammation and cellular regulation, in particular the cytokine-cytokine receptor interaction pathway as were molecular functions related to cytokine activity. These results provide biological relevance for the given PTLI condition and further suggest that proinflammatory cytokines may be involved in the pathways regulating MDR1 and BCRP. Thus, we
evaluated the correlation between expression of well-recognized proinflammatory cytokines, IL-6, IL-8, and TNF-α, and MDR1 and BCRP.

IL-8 is a potent chemotactic and activates neutrophils, potentiating host defense mechanism against inflammation. It is thought to be constitutively produced by the human placenta (Shimoya et al., 1992) independent of preterm vs. term delivery (Keelan et al., 1999). IL-8 is increased in placental tissue during chorioamnionitis (Lockwood et al., 2006) as well as in amniotic fluid and cord blood from women with intrauterine infection. We found a significant fold increase in IL-8 mRNA expression in placenta of inflammation-associated pregnancy (PTLI), while there were no differences in placentas from preterm vs. term pregnancy as demonstrated in comparisons between PTLNI versus TL and TNL (Fig. 4). These results are consistent with the literature. Fold changes in IL-8 mRNA expression were correlated with that of MDR1 and BCRP. Based on the aforementioned association between IL-8 and inflammation-infection, these data support altered expression of MDR1 and BCRP in placentas of women with preterm labor-inflammation. Interestingly, there were no changes in mRNA expression of other pro-inflammatory cytokines, TNF-α and IL-6. However, changes in these cytokines may be more apparent in the amniotic fluid or the maternal or fetal serum.

We observed elevated placental mRNA expression of IL-8 in women diagnosed with preterm preeclampsia (PTSPE) compared to term (TNL and TL). These results are consistent with reports of increased IL-8 production in trophoblasts cells (Bowen et al., 2005) and elevated IL-8 levels in maternal and umbilical cord serum as well as amniotic fluid of preeclamptic women (Nakabayashi et al., 1998; Laskowska et al., 2007). In contrast, Wang and co-workers found a decrease in placental IL-8 production in
preeclampsia (Wang et al., 1999). Additional experiments may be required to determine the association of preeclampsia and cytokine-specific production.

In these studies, we did not detect significant changes in protein or mRNA expression of the multidrug associated proteins, MRP1 and MRP2. LPS and pro-inflammatory cytokines have been shown to down-regulate MRP2 expression in the liver of rodents (Teng and Piquette-Miller, 2008), however, there is currently no data to support inflammatory-induced changes in MRP2 and MRP1 expression in humans and in placental tissue. Although it has generally been considered a liver-specific transporter, MDR3 expression in human term and preterm placenta has been described (Patel et al., 2003); however, its physiologic function in syncytiotrophoblasts remains speculative. We observed that MDR3 protein levels were not altered to the same extent as its mRNA expression. Others have also indicated discrepancies in MDR3 protein and mRNA expression in trophoblasts, which may be attributed, in part, to translational regulation (Evseenko et al., 2006).

In the present study, we found that MDR1 and BCRP are significantly regulated in human placenta. Prior studies have observed that MDR1 and BCRP are co-regulated in various tissue barriers to enhance tissue protection from xenobiotics. For example, de Vries and colleagues showed that these two transporters act in concert to limit the penetration of topotecan at the blood-brain barrier (de Vries et al., 2007). Like the blood-brain barrier, the placenta protects against harmful toxic substances and restricts the entry of therapeutic agents. Therefore, changes in placental expression of these transporters could have a profound impact on drug efficacy or toxicity. We further demonstrated that both MDR1 and BCRP expression increase in association with
underlying inflammation. Upregulation of MDR1 and BCRP in placenta during preterm inflammation and/or labor could significantly impair therapeutic intervention. For example, MDR1 and BCRP transport a variety of drugs including drugs necessary for fetal therapy. BCRP/Bcrp1 significantly limits the fetal level of nitrofurantoin, an antibiotic commonly used to treat urinary tract infections during pregnancy (Zhang et al., 2007) while MDR1/Mdr1a/b transports antibiotics such as azithromycin, erythromycin, clarithromycin, levofloxacin, and rifampin (Thuerauf and Fromm, 2006). These are agents currently used to prevent materno-fetal infections. MDR1 may also limit the transplacental transfer of Protease Inhibitors (PI) such as nelfinavir, ritonavir, saquinavir, lopinavir which are used in HIV-infected women to prevent transmission to the fetus. Currently, perinatal drug therapy in an inflamed and/or infected maternal-fetal milieu is secondary to clinical premature fetal delivery. Future studies will need to demonstrate that the placental MDR1 and BCRP expression during preterm inflammatory conditions directly correlates with drug exposure and outcome. There are a variety of placental transporters that localize to the maternal interface of the placenta, the fetal membrane surface, or both. Additional studies should focus on other important placental transporter proteins and their regulation under the various pregnancy conditions.
Authorship Contributions

*Participated in research design:* Mason, Swaan, Weiner

*Conducted experiments:* Mason, Dong

*Contributed clinical samples:* Buhimschi, IA & Buhimschi, CS

*Performed data analysis:* Mason, Swaan

*Wrote or contributed to the writing of the manuscript:* Mason, Swaan, Weiner
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FOOTNOTES PAGE

a) Unnumbered footnote

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FIGURE LEGENDS

Figure 1. Immunoblot analysis of protein expression of MDR1, BCRP, MRP2, MRP1, and MDR3 in human placenta from women after primary c-section during term no labor (TNL), term labor (TL), preterm spontaneous preeclampsia (PTSPE), preterm labor without inflammation (PTLNI), and preterm labor with inflammation (PTLI).

Figure 2. Immunohistochemical localization of MDR1 and BCRP in human placenta. Results show MDR1 and BCRP localization to the membrane of the syncytiotrophoblast cells (arrows) in all tissue conditions (n=3 per tissue group). BCRP was also localized to the fetal blood vessel endothelial cells (arrowheads). The MDR1 and BCRP controls are indicative of immunostaining without primary antibody. Images are 40x magnification. Scale bars: (in Control), 120 µm.

Figure 3. Relative changes in the gene expression of ABC transporters was determined by real time PCR in human placenta (n = 6-10) from women of various pregnancy conditions. The mean fold change in ABC transporter genes, normalized to the endogenous reference gene, 18s rRNA, and relative to the expression of the TNL control was calculated in each sample by the $2^{-\Delta\Delta Ct}$ method. Differences between all possible pairs of group means were determined by one-way ANOVA followed by Student-Newman-Keuls multiple comparison post-hoc test. Data are represented as mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001, NS, no significant difference. There were no differences in the mean fold changes of MRP1 and MRP2. Relative mRNA expression in PTLI was significantly higher for MDR1 (p < 0.01), BCRP (p <
0.001), and MDR3 (p < 0.05), by 6.4-fold, 3.7-fold, and 12.7-fold, respectively, than that in the TNL placental group.

**Figure 4.** Relative changes in mRNA expression of proinflammatory cytokines. [A] mRNA levels of IL-6, TNF-α, and IL-8 were analyzed by quantitative real time PCR. The mean fold change in ABC transporter genes, normalized to the endogenous reference gene, 18s rRNA, and relative to the expression of the TNL control was calculated in each sample by the $2^{-\Delta\Delta Ct}$ method. Differences between all possible pairs of group means were determined by one-way ANOVA followed by Student-Newman-Keuls multiple comparison post-hoc test. Data shown are the mean ± SEM from 6-10 independent placenta from each of the 5 groups. *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, no significant difference. There were no differences in the mean fold changes of TNF-α and IL-6. Relative mRNA expression in PTSPE and PTLI was significantly higher for IL-8 by 6.3-fold (p < 0.05) and 12.1-fold (p < 0.001), respectively, than that in the TNL placental group. [B] Fold changes in mRNA expression of IL-8 transcripts were correlated with MDR1 and BCRP in the human placentas of varying pregnancy conditions. Correlation analysis was performed using Pearson correlation.
Figure 4

A. Mean Fold Change in mRNA Expression (relative to TNL)

- **TNF-α**
  - TL: [graph]
  - PTSPE: [graph]
  - PTLNI: [graph]
  - PTLI: [graph]

- **IL-6**
  - TL: [graph]
  - PTSPE: [graph]
  - PTLNI: [graph]
  - PTLI: [graph]

- **IL-8**
  - TL: [graph]
  - PTSPE: [graph]
  - PTLNI: [graph]
  - PTLI: [graph]

B. Pearson r:
- IL-8 (mRNA expression) vs. MDR1 (mRNA expression): 0.50; p < 0.05
- IL-8 (mRNA expression) vs. BCRP (mRNA expression): 0.66; p < 0.001