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ARTICLE

Role of Elevated SFLT-1 on the Regulation of Placental Transporters in Women With Pre-Eclampsia

Dea Kojovic[1], Natalie V. Workewych[2] and Micheline Piquette-Miller[1,2,*]

Pre-eclampsia (PE) is an obstetric complication associated with elevated levels of fms-like tyrosine kinase 1 (sFlt-1) and dysregulated trophoblast differentiation. However, limited information exists on the expression and regulation of placental drug transporters in PE. Transporter mRNA and protein expression were analyzed in human placentas diagnosed with PE ($n = 34$) and gestational age-matched controls ($n = 24$), whereas placental BeWo cells were treated with angiogenic factors *in vitro*. Significant downregulation of breast cancer resistance protein (BCRP) and several other transporters were seen in placentas complicated by PE compared with controls, whereas mRNA levels of sFlt-1 were induced by 2.5-fold in PE placentas ($P < 0.01$). Treatment of BeWo cells with sFlt-1 resulted in an 85–90% downregulation of BCRP, which was attenuated by vascular endothelial growth factor. Our findings suggest that placental function is compromised during PE due to altered expression of clinically important transporters. Furthermore, our *in vitro* results show that sFlt-1 is involved in the regulation of BCRP.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Studies examining the impact of maternal diseases on the expression of transporters in human placenta have been inconsistent and mostly hindered by small sample sizes. However, dysregulation of these transporters in placenta and other tissues is common throughout the literature.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ The study explored effects of pre-eclampsia (PE) and associated angiogenic factors on the expression and regulation of placental transporters in human tissues.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ PE significantly modulated placental transporters in humans, and a novel role for sFlt-1 in the regulation of BCRP was identified *in vitro*.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ Therapeutic regimens may be optimized to minimize fetal exposure based on the transporter expression we observed seen in this population of pregnant women, ultimately improving maternal and fetal safety.

Pre-eclampsia (PE) is a severe pregnancy-specific disorder that is characterized by high maternal blood pressure, proteinuria, and edema. The incidence of PE is estimated to be between 3% and 10% of pregnancies worldwide and is responsible for 15% of premature births. Furthermore, some of the most severe cases of intrauterine growth restriction (IUGR) have been associated with PE, further hindering fetal outcomes. In the last several years, there has been mounting evidence supporting the involvement of a soluble vascular endothelial growth factor (VEGF) receptor (fms-like tyrosine kinase 1; sFlt-1) in the pathogenesis of PE, starting as early as the first trimester when the placental arteries begin to invade the uterine wall and placental development begins.[1] Indeed, studies have shown that women who have PE have elevated serum levels of sFlt-1 compared with their non-pre-eclamptic counterparts.[2] The endogenous ligands for sFlt-1 are the vascular endothelial growth factor (VEGF) and the placental growth factor (PLGF), which have also been shown to be dysregulated within this patient population, and are essential in maintaining proper endothelial function and proliferation of the placenta.[3] Although the only current known cure for PE is delivery of the fetus, therapeutic treatment is necessary to control maternal symptoms and maintain pregnancy into the third trimester.

Currently, more than half of pregnant women take four or more drugs during pregnancy and this is likely higher in women with PE. Optimal therapeutic management of PE is required to reduce negative maternal and fetal outcomes, and interventions involve antihypertensive therapy with labetalol, methyldopa, hydralazine, and/or nifedipine and corticosteroid administration to accelerate fetal lung maturity. Treatment of severe cases also includes magnesium sulfate to prevent seizures.[4] Furthermore, the use of statins to prevent PE is currently under clinical investigation.[5] As

[1]Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada; [2]Department of Pharmacology and Toxicology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada. *Correspondence: Micheline Piquette-Miller (m.piquette.miller@utoronto.ca)
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many of these drugs are substrates of transporters found in the placenta, understanding the potential disease-mediated changes in their expression is urgently needed to prevent potential teratogenicity and/or adverse effects to the developing fetus.

Serving as an interface between mother and developing fetus, the placenta expresses numerous ATP-Binding Cassette (ABC) efflux transporters within cytotrophoblast, and syncytiotrophoblast cells, which comprise the placental membranes. These transporters play a crucial role in limiting fetal toxicities by preventing entry, or by facilitating the active efflux of endogenous waste products and xenobiotics out of the fetal compartment. The breast cancer resistance protein (BCRP; *ABCG2*) is of particular importance as it is one of the most highly expressed transporters in the placenta,[6] and transports a large number of diverse chemicals, thus playing a protective role in limiting the passage of potentially harmful agents and teratogens. Substrates include, but are not limited to, antihypertensives, antivirals, and antibiotics; some of which are given for the treatment of PE (i.e., labetalol and nifedipine).[7] Other key members of the ABC transporter family that play a protective role include P-glycoprotein (P-gp; *ABCB1*) and the multidrug resistance associated proteins (MRPs; *ABCC*s).

The placenta also plays an important role in the uptake of endogenous compounds and nutrients that are essential for maintaining healthy pregnancy. Several solute carrier (SLC) uptake transporters play a critical role in cellular uptake of nutrients, steroids, and xenobiotics and are highly expressed in placental membranes. The major groups include the organic anion transporting polypeptides (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs), and the equilibrative nucleoside transporters.

Over the last decade, there has been mounting evidence indicating disease-mediated changes in placental transporters, which can ultimately alter transplacental passage of substrates and compromise important physiological functions. Studies in rodent models of bacterial and viral infection have repeatedly shown downregulation of important ABC transporters in placenta resulting in altered fetal exposure to their substrates, along with increases in pro-inflammatory cytokines.[8,9] Similar trends have also been observed in placenta obtained from women with chorioamnionitis.[10] Additionally, *in vitro* work using primary term trophoblasts demonstrated that treatment of cells with the pro-inflammatory cytokines, TNF-α or IL-1β, significantly decreases the expression of *ABCB1* and *ABCG2.*[11] Interestingly, women with PE have higher levels of circulating pro-inflammatory cytokines and imbalances of pro-inflammatory vs. anti-inflammatory cytokines are seen.[12]

Although PE is highly prevalent, and a leading cause of preterm birth and neonatal death, little is known about the effects of PE on the regulation of placental transporters. Furthermore, as these women are frequently on medication throughout pregnancy, changes in the expression of placental transporters may affect fetal exposure to their substrates. Therefore, our aim was to identify changes in placental transporter expression in human tissues of women with PE and to explore the role of sFlt-1 as well as pro-inflammatory cytokines in the regulation of these transporters *in vitro* in cultured human placental BeWo cells.

MATERIALS AND METHODS

Human placental sample acquisition

Human placental tissue samples from pregnancies meeting our inclusion/exclusion criteria were obtained from the Research Center for Women's and Infant's Health (RCWIH) BioBank program at Mount Sinai Hospital (MSH), Toronto, Canada, in accordance with the policies of the MSH Research Ethics Board (Protocol Reference #28225) and following the tenets of the Declaration of Helsinki. Sample acquisition and processing are detailed on the RCWIH Biobank website, http://biobank.lunenfeld.ca, as previously described. In order to determine the appropriate sample size for our study, a power analysis was conducted for a desired power of 0.80, with a 40% change between groups, and sample size of 25–30 in each group was determined. Placental samples were collected from women who were clinically diagnosed for PE (n = 34). Control placental samples (n = 25) were collected from healthy pregnancies matched for gestational age. Clinical data that were extracted from patient charts that were collected by the RCWIH BioBank program of MSH were made available to this study. Extensive review of clinical data was completed to ensure that all patients met our inclusion/exclusion criteria, which can be found in **Table S2**.

Cell culture

The BeWo continuous human choriocarcinoma-derived placental cell line was purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium/F12, supplemented with 10% fetal bovine serum, 0.1% nonessential amino acid, 0.1% penicillin/streptomycin, and cultured at 37°C under an atmosphere containing 5% CO_2. Cells were seeded into 12-well plates (50,000 cells/mL) and cultured for 48 hours prior to forskolin exposure (150 µm) for 72 hours to induce syncytialization. Cells were subsequently treated with sFlt-1 (1 and 10 ng/mL), VEGF (0.1 and 1 ng/mL) and PLGF (0.5 and 5 ng/mL) for 72 hours. Cytotoxicity was evaluated using a methylthiazol tetrazolium (MTT) assay. Following treatment, 5 mg/mL MTT solution was added in each well and incubated for 3 hours at 37°C. Absorbance was measured at 570 nm using a spectrophotometer (SPECTRAmax M2; SpectraMax, Sunnyvale, CA). Culture media and supplements were obtained from Gibco (ThermoFisher, Mississauga, Ontario, Canada) and growth factors were obtained from R&D Systems (Oakville, ON) and PeproTech (Montreal, Quebec, Canada).

[^{3}H] Topotecan uptake assay

Uptake assays were modified from a previously published protocol.[13,14] Cells for uptake assays were grown for 48 hours after seeding, treated with 150 µm forskolin, and grown for an additional 72 hours before treatment with 10 ng/mL SFLT1 for 96 hours before uptake assays were performed. On the day of the uptake, cells were incubated with N1 standard uptake buffer for 1 hour, followed by a 30-minute incubation with 10 µM ko143 inhibitor dissolved in DMSO in N1 buffer or

an equivalent volume of DMSO in N1 buffer for control wells. Cells were then incubated with 1 μM [^{3}H] topotecan and a 1:3,000 ratio of 14C mannitol to incubation buffer volume. After 1 hour, cells were washed twice with ice cold phosphate-buffered saline, lysed for 1 hour with 0.01 N NaOH, collected, and combined with scintillation fluid for radioactive counting. Cell lysates were also used in a standard bicinchoninic acid assay for protein quantification. All incubations were performed at 37 degrees. Experiments were performed in triplicate on three or more different experimental days. Data are expressed as normalized to mannitol uptake and protein concentrations from lysed cells determined by standard bicinchoninic acid assay.

Quantitative real-time polymerase chain reaction analysis

Levels of mRNA were determined using quantitative real-time polymerase chain reaction, as previously described.[15] Briefly, RNA was extracted from 50–100 mg of placental tissue or BeWo cells using Trizol (Invitrogen, Carlsbad, CA), according to manufacturer instructions, and concentrations were quantified using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA was treated with DNase (Invitrogen) and reverse transcribed using a high capacity cDNA reverse transcribed kit (Applied Bio Systems, Waltham, MA). The quantitative real-time polymerase chain reaction was performed using power SYBR green (ABI HT-7900). Primer sequences are listed in **Table S1**.

Western blotting

Crude membrane fractions were isolated from 300 mg of human placental tissue, as previously described.[10] BeWo cell homogenates were lysed and collected in RIPA buffer (Cell Signalling, 9806) followed by sonication. Following incubation at 4C for 1 hour, cell lysates were centrifuged (16,000 *g*) for 10 minutes at 4C and the resulting supernatant was collected. Protein concentrations for whole cell lysates and crude membrane fractions were measured using the Bradford assay. Isolated protein samples (30 μg) in Laemmli sample loading buffer were heated at 37C for 20 minutes, separated via 10% SDS-PAGE, and transferred to Polyvinylidene difluoride membranes (Bio-Rad Laboratories, Ontario, Canada) and incubated overnight at 4°C with a primary antibody in 2% nonfat milk (TRIS-buffered saline-Tween-20 (TBST)). The primary antibodies included and loading control (β-actiN) information can be found in **Table S3**. Membranes were then washed with TBST and incubated with a horseradish peroxidase-labeled secondary antibody in 2% nonfat milk -TBST (**Table S3**). Optical densities analysis was performed using a FluorChem Xplor imager (Alpha Innotech, San Leandro, CA) and quantified using Alpha Ease FC imaging software. Representative blots are provided in **Figure S2**.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (San Diego, CA). Human data were log-transformed to conform to normality. For comparison between two groups, we used a nonparametric unpaired *t*-test with Welch's correction. Significance was defined as $P < 0.05$. The distribution of human transporter, angiogenic markers, and cytokine mRNA expression is shown using a box and whisker plot as a percentage of the mean indicated y "+" with error bars representing minimum to maximum values. All other data results are expressed as the mean ± SEM.

RESULTS

Patient clinical characteristics

Relevant clinical characteristics of patients in the control and PE group are shown in **Table 1**. We collected 24 placentas from the control group, and 34 placentas from pregnancies complicated by PE. Placental control samples were collected from normal pregnancies and were not associated with pathological abnormalities upon histological examination. There were no statistically significant differences in maternal weight, body mass index, or gestational age between the two groups. As excepted, maternal blood pressure was significantly higher in the PE group compared with non-PE term matched controls ($P < 0.001$). Furthermore, placental weight as well as neonatal birthweight were significantly decreased in the PE group compared with non-PE term matched controls ($P < 0.001$). Fifty percent of the PE placentas collected were complicated by IUGR.

Impact of PE on mRNA expression of placental transporters

The expression of numerous clinically relevant ABC and SLC transporters were examined in placentas from women with PE and compared with non-PE term matched controls (**Figure 1a,b**). No significant

Table 1 Clinical data

Characteristic	Non – PE control (*n* = 24)	PE (*n* = 34)
Age	33.1	32
Ethnicity		
White	14 (58)	15 (44)
Black	4 (16)	9 (26.5)
Asian	2 (8)	8 (23.5)
Other	4 (16)	2 (6)
BMI	24.29 ± 4.5	25.8 ± 4.7
Mode of delivery		
Vaginal	12 (50)	2 (6)
C-Section	12 (50)	32 (94)
Gestational age	32.8 ± 2.1	31 ± 2
Placental weight	475.2 ± 148	286 ± 100*
Neonatal birth weight	2100 ± 658	1133 ± 385*
Blood pressure		
Systolic	120 ± 13	166 ± 16*
Diastolic	74 ± 14	103 ± 9*

BMI, body mass index; PE, pre-eclampsia.
Statistical analysis results are presented as mean ± SD. All other results are presented as frequency (% of total).
*Significantly different from controls ($P < 0.001$).

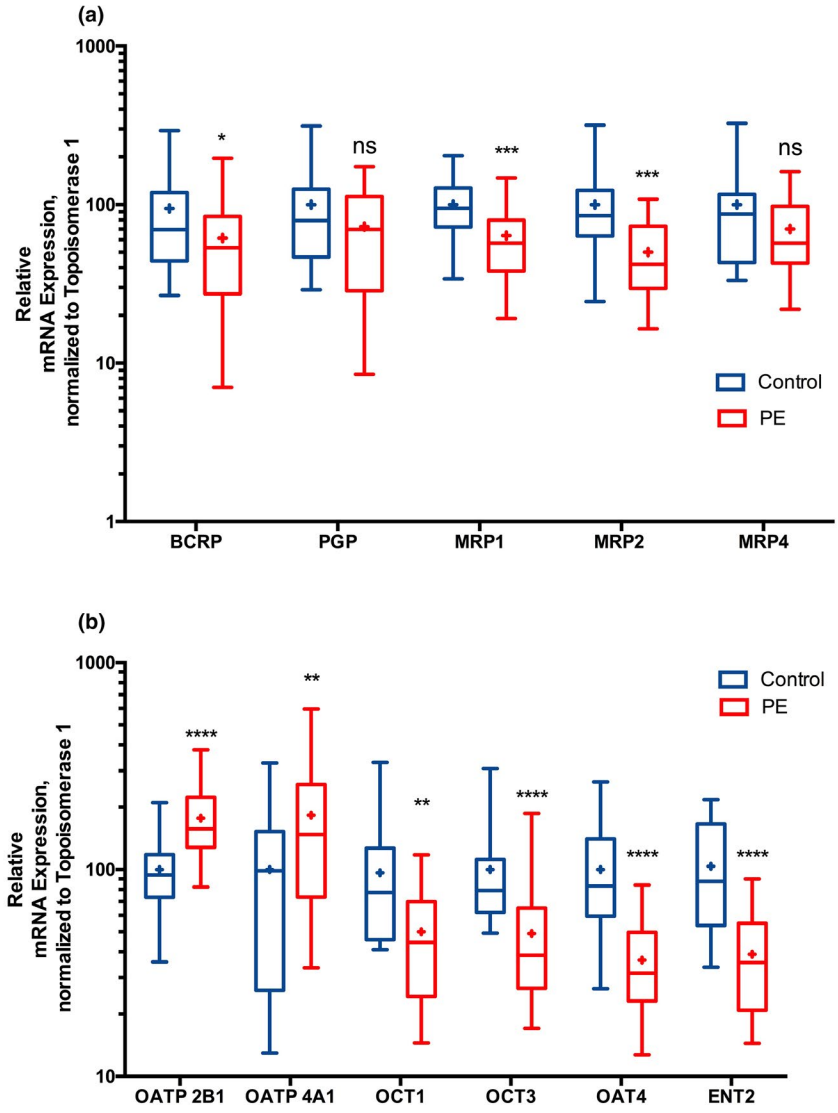

Figure 1 Impact of pre-eclampsia (PE) on the mRNA expression of (**a**) ABC transporters and (**b**) several solute carrier transporters in human placental tissues. The distribution of transporter mRNA expression in control (n = 25) vs. PE (n = 34) groups is shown using a box and whisker plot as a percentage of the mean indicated by "+." The dark line represents the median, the box represents the interquartile range, and the whiskers represent the minimum to maximum. Data are log-transformed to conform to normality where *P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.001. BCRP, breast cancer resistance protein; ENT, equilibrative nucleoside transporter; MRP, multidrug resistance associated protein; ns, not significant; OAT, organic anion transporters; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PGP, P-glycoprotein.

differences in transporter expression were seen between placentas collected from PE complicated by IUGR vs. PE not complicated by IUGR, therefore, these groups were combined. Levels of *BCRP* as well as MRP 1 and 2 were significantly downregulated by 40–60% in the PE group as compared with controls (P < 0.05–0.001). Levels of the SLC transporters, *OCT1*, *OCT3*, *OAT4*, and equilibrative nucleoside transporters *2* were significantly downregulated by 50–70% in placentas complicated by PE compared with controls, and *OATP2B1* and *OATP4A1* were significantly increased by 1.8-fold (P < 0.01–0.001). Although we saw trends toward a downregulation in P-gp gene expression, this change did not reach statistical significance (P = 0.051).

Impact of PE on protein expression of placental transporters

Overall, we saw a dysregulation in the protein levels of several ABC and SLC transporters in PE placentas (**Figure 2**). Consistent with mRNA data, we detected a significant 45% downregulation in the protein expression of BCRP in PE placentas as compared with controls (P < 0.01; n = 12 per group). On the other hand, protein levels of OATP2B1 and OCT3 were significantly upregulated PE placentas compared with controls (P < 0.05 and P < 0.001, respectively; n = 8 per group). Although some trends were seen, we did not detect significant differences between PE and control for PGP (P = 0.09) and OATP4A1 (P = 0.60).

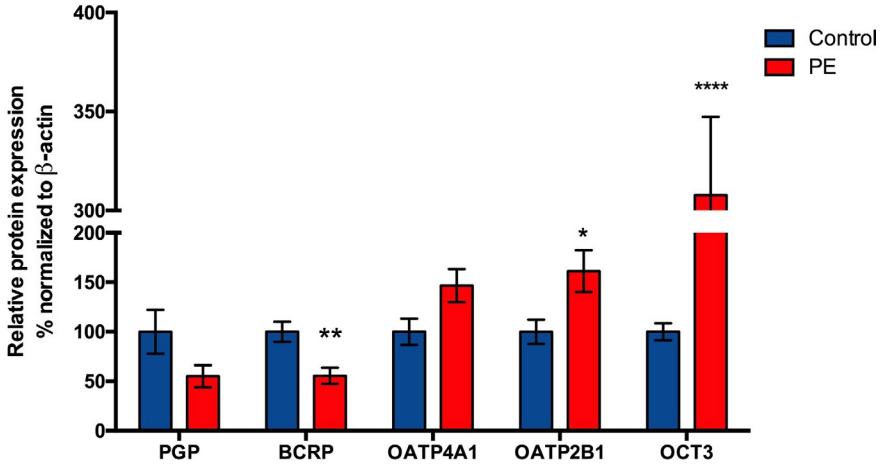

Figure 2 Impact of pre-eclampsia (PE) on the protein expression of clinically important ABC and several solute carrier transporters in human placental tissues. Data are presented as a percentage of the mean ± SEM (n = 8–12 per group. *P < 0.05, **P < 0.01, and ***P < 0.001. BCRP, breast cancer resistance protein; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PGP, P-glycoprotein.

Impact of PE on mRNA expression of placental angiogenic factors and cytokines

As compared with controls, we observed a significant 2.2-fold increase in *sFlt-1* mRNA expression in PE placentas compared with controls (P < 0.005), whereas VEGF and PLGF were significantly downregulated in PE (**Figure 3a**). We also examined the mRNA profile of pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, and IL-8 in our control and PE cohorts (**Figure 3b**). Although there was much greater variability within the PE group, there were no significant differences between the two groups.

Effect of angiogenic factors on BCRP (*ABCG2*) expression and activity

The effect of sFlt-1, VEGF, and PLGF on regulation of BCRP expression was assessed *in vitro* in cultured human placental BeWo cells (**Figure 4**). Treatment with sFlt-1 resulted in an 80–90% downregulation of BCRP transcript levels compared with vehicle-treated control cells after 72 hours (P < 0.05). Treatment with VEGF on the other hand did not alter BCRP mRNA expression. Interestingly, combination treatment with sFlt-1 and VEGF (1 ng/mL) showed a threefold to fourfold upregulation of BCRP mRNA compared with controls (P < 0.05). Interestingly, treatment with PLGF resulted in a similar threefold to fourfold upregulation of BCRP compared with controls (P < 0.05). Although there was a trend toward BCRP downregulation at the protein level after 72 and 96 hours of sFlt-1 treatment, this did not reach statistical significance (**Figure 4b**). An accumulation assay for [^{3}H] topotecan was used to assess BCRP activity in BeWo cells after administration of sFlt-1 for 96 hours. The BCRP-specific inhibitor KO143 was used as a positive control, resulting in a twofold increase in the retention of [^{3}H] topotecan (**Figure 4c**). Consistent with the downregulation of BCRP mRNA and protein, the intracellular retention of [^{3}H] topotecan, was significantly increased by 1.5-fold with pretreatment with sFlt-1. The cell viability was not impacted by treatments as measured by the MTT assay (data not shown).

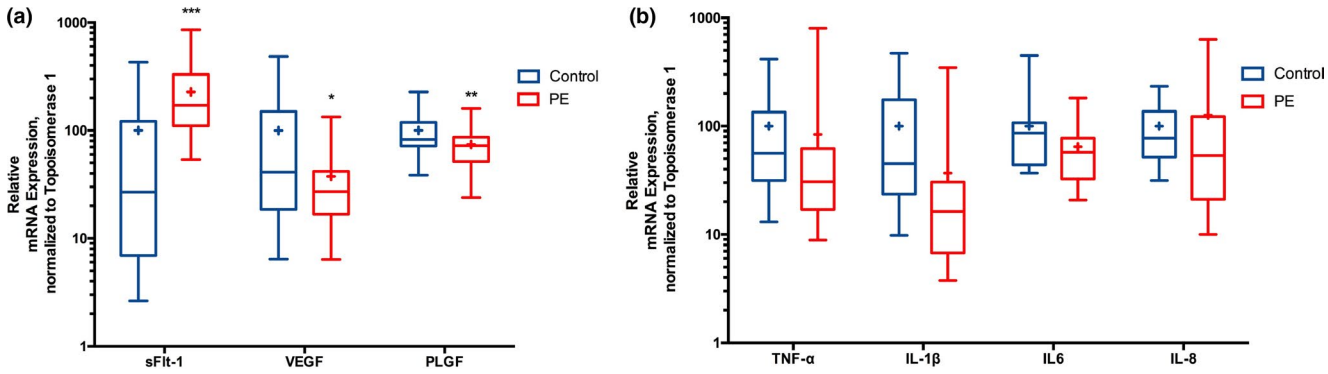

Figure 3 Impact of pre-eclampsia (PE) on (**a**) placental angiogenic factors (fms-like tyrosine kinase 1 (sFlt-1), vascular endothelial growth factor (VEGF), and placental growth factor (PLGF)) and (**b**) placental cytokines. The distribution of mRNA levels in control (n = 25) vs. PE (n = 34) groups is shown using a box and whisker plot as a percentage of the mean indicated by "+.". The dark line represents the median, the box represents the interquartile range, and the whiskers represent the minimum to maximum. Data are log-transformed to conform to normality where *P < 0.05, **P < 0.01, and ***P < 0.005.

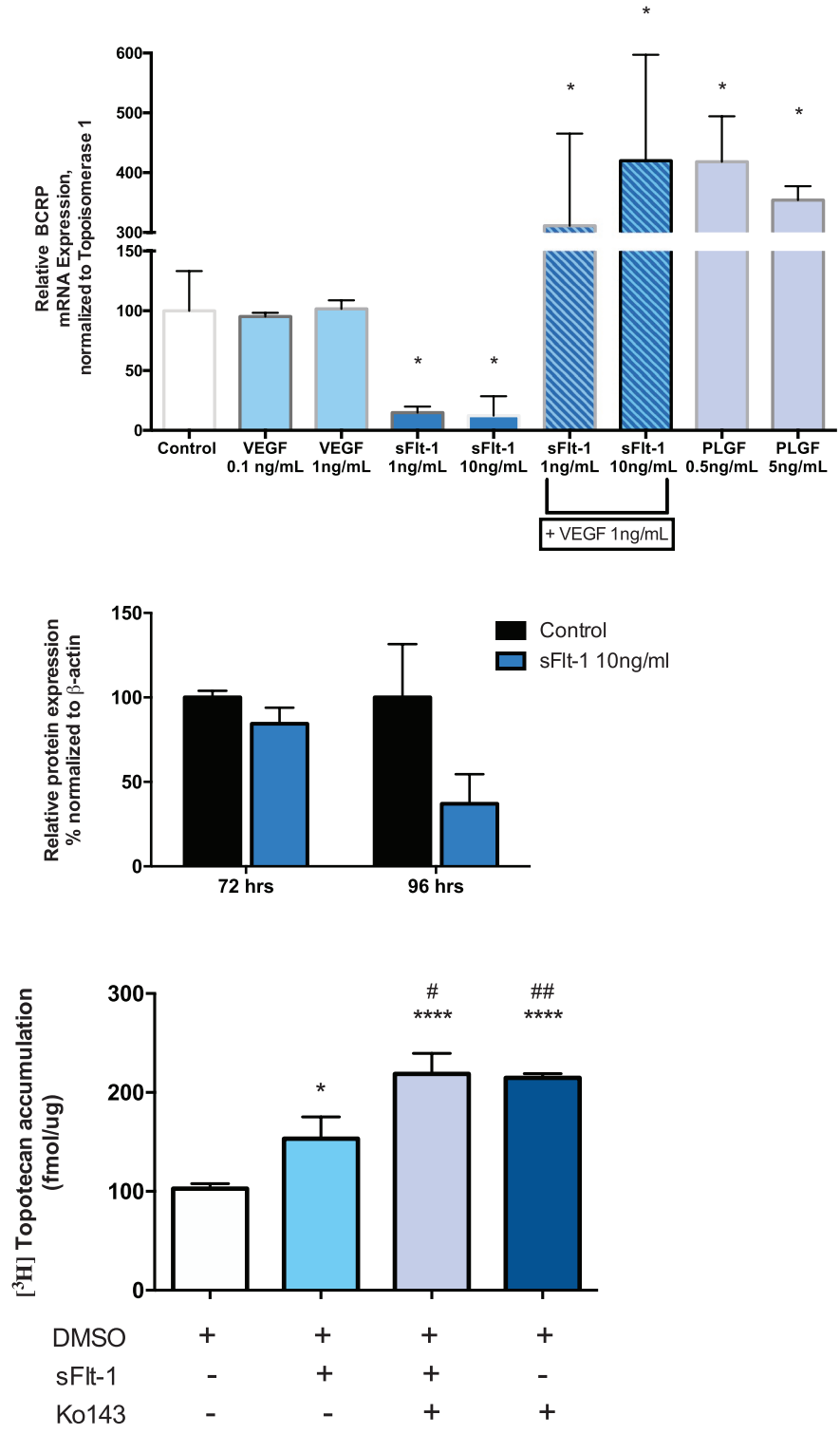

Figure 4 Effect of angiogenic factors on breast cancer resistance protein (BCRP; *ABCG2*) (**a**) mRNA expression in BeWo cells after administration of vascular endothelial growth factor (VEGF), fms-like tyrosine kinase 1 (sFlt-1; alone or in combination with VEGF), and placental growth factor (PLGF) for 72 hours and (**b**) protein expression of BCRP after treatment with sFlt-1 for 72 and 96 hours. Results are expressed as mean ± SEM relative to the vehicle control group (0.1 BSA) of at least three separate experiments done in triplicates. (**c**) Effect of sFlt-1 on BCRP activity in BeWo cells. The intracellular retention of [^{3}H] topotecan was measured after exposure to sFlt-1. Ko143 was used as a positive control inhibitor of BCRP function. There were $n = 3$ experiments, and four to five replicates/experiment. Data are presented as mean ± SEM. *Indicates significantly different from control. #Indicates significantly different from sFlt-1 treatment. $^{*}P < 0.05$, $^{\#\#}P < 0.01$, and ****, $^{\#\#\#\#}P < 0.001$.

DISCUSSION

In this study, we demonstrated that several clinically important ABC and SLC transporters are significantly modulated in the placentas of women with clinically confirmed cases of PE as compared with non-PE gestational-term matched controls. Consistent with the pathogenesis of PE, angiogenic factors sFlt-1, VEGF, and PLGF were significantly changed in PE placentas. Of note, *in vitro* results in BeWo cells demonstrated that these angiogenic factors can significantly alter BCRP expression. To our knowledge, this is the first study demonstrating, *in vitro*, that these growth factors play a role in modulating placental transporter expression. As transporters play a key role in the placental transfer of numerous endogenous and exogenous substances, obtaining insight into their regulation during prevalent obstetric complications, such as PE, is required to ensure proper fetal development and prevent unnecessary toxicities.

Overall, we found that mRNA levels of *BCRP*, *MRP 1*, and *2*, *OCT1*, and *3*, and *OAT4* were all significantly downregulated in the PE group compared with controls, whereas *OATP2B1* and *OATP4A1* were significantly upregulated. In general, transporter transcript and protein levels were indicative of one another, with significant decreases in BCRP and significant increases in OATP2B1 within the PE group. Furthermore, there were trends toward a downregulation of P-gp and an upregulation of OATP4A1. One exception was seen with OCT3, the most highly abundant SLC transporter in placenta,[16] which was significantly downregulated at the mRNA level while there was a threefold increase at the protein level in the PE group. Although there is a discrepancy between the mRNA and protein levels of OCT3, consistent changes in transcript and protein levels were seen for the other SLC transporters in the PE group. Studies have shown that SLC transporters are the target of various post-translational modifications[17] and, as such, OCT3 may be subject to additional regulatory mechanisms. As immuno-detectable levels of proteins are measured in membrane fractions rather than whole cell lysates, it is also possible that increased trafficking of OCT3 to the membrane occurs in response to reduced transcript levels.[18]

The ABC transporters, PGP, BCRP, and the MRPs along with the SLC transporters, OCT3, OATP2B1, and OATP4A1 are together responsible for the transport of numerous endogenous substrates, including steroid hormones, metabolites, and neurotransmitters, as well as exogenous substrates, including statins, glycosides, and antivirals, among others. These transporters are found at both the apical and basolateral membranes, supporting the bidirectional flow of substrates across the placenta. The increases seen in the SLC transporter group, which are predominantly found at the fetal facing surface for uptake, coupled with a decrease in efflux transporters P-gp and BCRP at the maternal facing side indicate that this population of pregnant women may have a greater risk of accumulating both endogenous and exogenous substrates within placental cells; which could potentially lead to placental and fetal toxicities. There is, in fact, significant substrate overlap between ABC and SLC transporters and numerous groups have suggested that the two families coordinate the vectoral transfer of many substrates across placental cells.[19–21] Furthermore, it is also possible that the upregulation of uptake transporters occur in response to the decreased expression of efflux transporters in order to provide additional fetal protection against the accumulation of toxins. To date, the implications of these findings remain unsolved as there are insufficient studies that have adequately measured placental transfer for these substrates within the fetal compartment.[22,23]

PE is a multifactorial disorder, associated with altered levels of angiogenic factors, cytokines, as well as hypoxia.[24–26] Elevated serum levels of several pro-inflammatory cytokines have been consistently reported in women with PE, including IL-2, 6, 8, 10, 15, 16, and 18.[27–29] However, reports on PE-associated changes in cytokine mRNA levels in placental tissues have been inconsistent.[30,31] Although numerous *in vivo* and *ex vivo* studies have demonstrated a link between an induction of pro-inflammatory cytokines and transporter dysregulation,[8–10,32] we did not see altered transcript levels of TNF-α, IL-1β, IL-6, or IL-8 in our PE group. This could suggest that cytokine induction is not involved in the transporter dysregulation observed in PE. On the other hand, as several women in our PE group received one or two doses of corticosteroids several days prior to delivery, this may have suppressed levels of pro-inflammatory cytokines at the time of sampling (i.e., birth) in our cohort. Thus, it is still possible that cytokines could play a role in the observed dysregulation of transporters. It is important to note that glucocorticoids were administered as single doses days before labor, and, thus, we do not expect this would significantly confound our results with respect to alterations in transporter levels.

Elevated serum levels of sFlt-1 are also seen in PE and this often occurs well before clinical symptoms arise.[33,34] Research has demonstrated that trophoblasts derived from PE placentas produce higher levels of sFlt-1 compared with normotensive controls.[35] Likewise, we also found a pronounced induction of sFlt-1 expression in PE placentas, whereas expression of VEGF and PLGF transcript were decreased. Although inductions of serum VEGF have been reported, changes in placental VEGF and PLGF transcript levels are inconsistent. Xu and Cheng[36] as well as our own data show significantly decreased transcript levels of VEGF in placentas of women with PE compared with controls, however, inductions have been observed by other groups.[37,38] Interestingly, we found significant associations among VEGF mRNA expression with transcript levels of MRP1, MRP4, OCT1, and OAT4 (**Figure S1**).

Although we found that numerous ABC transporters were altered within the placentas of women with PE, we focused our attention on further exploring the regulation of BCRP due to the critical role it plays in placenta and fetal protection. BCRP is one of the highly expressed transporters at term and exhibits substrate redundancies with several other ABC and SLC transporters along with similar regulatory pathways. As sFlt-1 is consistently elevated in the serum and placentas of women with PE, and was induced within the placentas in our cohort of women, we examined whether it was playing a part in the regulation of BCRP. Clinically relevant concentrations were used as

found in high-risk pregnant women with PE.[22] Indeed, *in vitro* studies in human placental BeWo cells demonstrated that sFlt-1 significantly downregulated BCRP mRNA expression by 80–90%, with trends toward a decrease at the protein level. Furthermore, treatment with sFlt-1 resulted in increased intracellular accumulation of the BCRP substrate [^{3}H] topotecan in cells. This is the first report, to our knowledge, showing that sFlt-1 impacts BCRP expression in placental cells. It is plausible that this occurs through the sequestering of its endogenous ligands, VEGF, and/or PLGF, which may be responsible for downstream signaling and regulation of BCRP. Although PLGF was found to induce BCRP, VEGF itself did not alter levels of BCRP, therefore, it was unexpected that administration of sFlt-1 in conjunction with VEGF resulted in an induction of BCRP similar to that seen with PLGF. VEGF and PLGF are angiogenic factors involved in endothelial function and vascular proliferation, which function through two tyrosine kinase receptors, Flt-1 and Flk-1. VEGF has higher affinity for sFlt-1 (as well as its membrane bound form) than PLGF[39] and, thus, it is possible that sFlt-1 cotreatment sequesters VEGF allowing endogenous PLGF to bind to membrane receptors. In this manner, activation of PLGF signaling pathways may increase BCRP transcription. Further to this point, it is plausible that the observed decrease in PLGF expression in PE placenta along with elevated sFlt-1 could play a role in BCRP downregulation.

In addition to altered cytokines and growth factors, PE is frequently associated with placental hypoxia.[40] Interestingly, a downregulation of BCRP in response to low oxygen levels has been reported in BeWo cells.[41] Thus, the observed downregulation of BCRP could occur due to simultaneous and additive events rather than through a single pathway. Although hypoxia is more commonly associated with a reduction in VEGF expression,[42] it has been reported that low oxygen tension caused an increase in transcript levels of VEGF along with increased BCRP in human placental explants, which further supports involvement of angiogenic factors. Whereas our overall findings support the notion that sFlt-1, VEGF, and PLGF may be involved in placental BCRP regulation by hindering angiogenic endothelial signaling, it is clear that further studies are needed to elucidate the involvement of these factors in the regulation of BCRP.

PE is a pregnancy-specific disorder without a known cure aside from delivery of the fetus. As such, most pre-eclamptic pregnancies result in preterm labor to halt progression of the disease to eclampsia. As the progression of pregnancy modulates placental transporters, we correspondingly collected only preterm matched placental samples as controls. Consequently, one limitation of our study was unequal sample sizes between the two groups as it was harder to obtain preterm placentas from "healthy" pregnancies without any known obstetric complications. Although it is unusual for a healthy pregnancy to spontaneously end before term, clinical characteristics indicate that whereas the gestational ages between groups do not significantly differ, the placental birth weights and neonatal birth weights were twofold higher in the control group indicating a healthier progression of pregnancy in the control group of women.

In addition to defining regulatory pathways, it is important to consider the potential clinical consequences of reduced placental expression of BCRP in PE. As previously mentioned, BCRP transports a chemically diverse range of substrates; therefore, reduced expression can potentially alter fetal drug concentrations. Pregnant women continue prescribed treatments throughout gestation for various conditions, such as epilepsy, depression, and hypertension, as well as for new onset disorders, such as gestational diabetes, pre-eclampsia, or bacterial infections. Substrates for BCRP include antibiotics, antiretrovirals, calcium channel blockers, estrogen, porphyrins, and flavonoids.[43–45] Although many of these drugs are thought to be safe during pregnancy, few adequate or well-controlled studies have been carried out in pregnant women. Furthermore, apart from more obvious teratogenic effects, the potential long-term adverse effects of increased fetal accumulation are not known. For instance, whereas glucocorticoids are frequently administered to women at risk for preterm labor, *in utero* exposure, which is restricted by P-gp expression, can affect neonatal outcomes. These risks likely become worse in cases where P-gp expression is compromised, as this would lead to increased fetal exposure. It is also noteworthy that many potentially toxic or teratogenic drugs are substrates for BCRP and P-gp.

In summary, our results demonstrate that PE alters the expression of clinically important drug efflux and uptake transporters in human placenta. The transporters found in placenta are essential in providing nutrients and hormones and in maintaining a homeostatic environment for the developing fetus. Furthermore disease-mediated changes in these transporters can significantly alter transplacental passage of therapeutic agents used during pregnancy. Understanding the impact of PE and other highly prevalent obstetric conditions on the regulation of placental transporters is important in predicting fetal drug exposure and optimizing therapeutic regimens for management of these conditions during pregnancy. As prescription drug use during pregnancy is dramatically increasing worldwide, understanding the factors that affect regulation of placental transporters provides additional insight to optimize maternal and fetal safety.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

Supplementary Data.

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