

## Special Section on Transporters in Drug Disposition and Pharmacokinetic Prediction

# Interindividual Regulation of the Breast Cancer Resistance Protein/*ABCG2* Transporter in Term Human Placentas<sup>[S]</sup>

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Received October 28, 2017; accepted January 25, 2018

### ABSTRACT

The breast cancer resistance protein (BCRP/*ABCG2*) is a maternally-facing efflux transporter that regulates the placental disposition of chemicals. Transcription factors and gene variants are important regulatory factors that influence transporter expression. In this study, we sought to identify the genetic and transcriptional mechanisms underlying the interindividual expression of BCRP mRNA and protein across 137 term placentas from uncomplicated pregnancies. Placental expression of BCRP and regulatory transcription factor mRNAs was measured using multiplex-branched DNA analysis. BCRP expression and *ABCG2* genotypes were determined using Western blot and Fluidigm Biomark genetic analysis, respectively. Placentas were obtained from a racially and ethnically diverse population, including Caucasian (33%), African American (14%), Asian (14%), Hispanic (15%), and mixed (16%) backgrounds, as well as unknown origins (7%). Between placentas, BCRP mRNA and

protein varied up to 47-fold and 14-fold, respectively. In particular, BCRP mRNA correlated significantly with known transcription factor mRNAs, including nuclear factor erythroid 2-related factor 2 and aryl hydrocarbon receptor. Somewhat surprisingly, single-nucleotide polymorphisms (SNPs) in the *ABCG2* noncoding regions were not associated with variation in placental BCRP mRNA or protein. Instead, the coding region polymorphism (C421A/Q141K) corresponded with 40%–50% lower BCRP protein in 421C/A and 421A/A placentas compared with wild types (421C/C). Although BCRP protein and mRNA expression weakly correlated ( $r = 0.25$ ,  $P = 0.040$ ), this relationship was absent in individuals expressing the C421A variant allele. Study results contribute to our understanding of the interindividual regulation of BCRP expression in term placentas and may help to identify infants at risk for increased fetal exposure to chemicals due to low expression of this efflux protein.

### Introduction

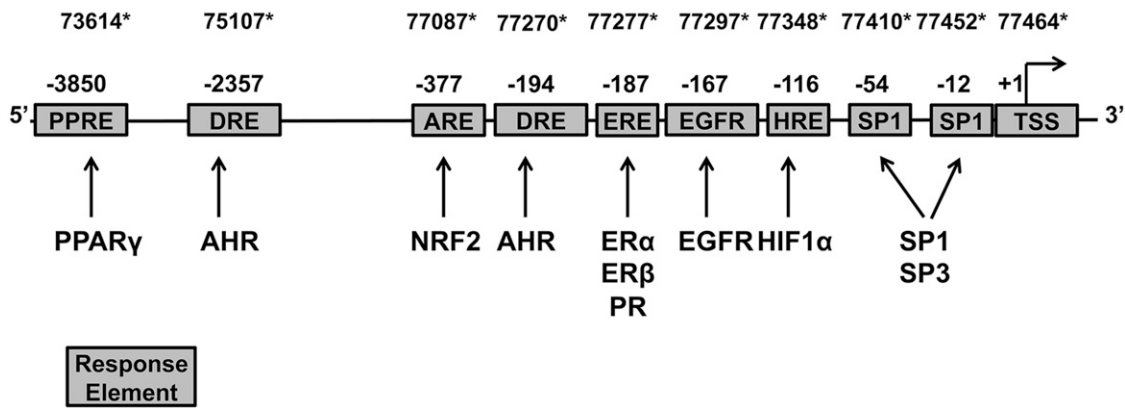
In the placenta, the breast cancer resistance protein (BCRP/*ABCG2*) efflux transporter plays an important role in limiting fetal exposure to chemicals,

This work was supported by National Institutes of Health National Institute of Environmental Health Sciences [Grants ES020522, ES005022, and ES007148]. A portion of tissue samples was provided by the National Institutes of Health Placental Bank in University of California, San Francisco, supported by National Institutes of Health Eunice Kennedy Shriver National Institute of Child Health and Human Development [Grant HD055764]. K.M.B. was supported by predoctoral fellowships from the American Foundation for Pharmaceutical Education and Pharmaceutical Research and Manufacturers of America. J.E.M. was supported by predoctoral fellowships from the American Foundation for Pharmaceutical Education and the National Institutes of Health Eunice Kennedy Shriver National Institute of Child Health and Development [Grant F31HD082965].  
<https://doi.org/10.1124/dmd.117.079228>.

[S] This article has supplemental material available at [dmd.aspetjournals.org](http://dmd.aspetjournals.org).

**ABBREVIATIONS:** AHR, aryl hydrocarbon receptor; BCRP, breast cancer resistance protein; ER, estrogen receptor; HIF1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; NRF2, nuclear factor erythroid 2-related factor 2; PBS/T, phosphate-buffered saline with 0.5% Tween 20; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; PR, progesterone receptor; SNP, single-nucleotide polymorphism; 5'UTR, 5' untranslated region.

ABCG2 Promoter Region



**Fig. 1.** Map of transcription factor and nuclear receptor response elements in the promoter region of the *ABCG2* gene. Location of response elements numbered with respect to the transcriptional start site (TSS, +1) and the first nucleotide of the gene (+1; AC084732).

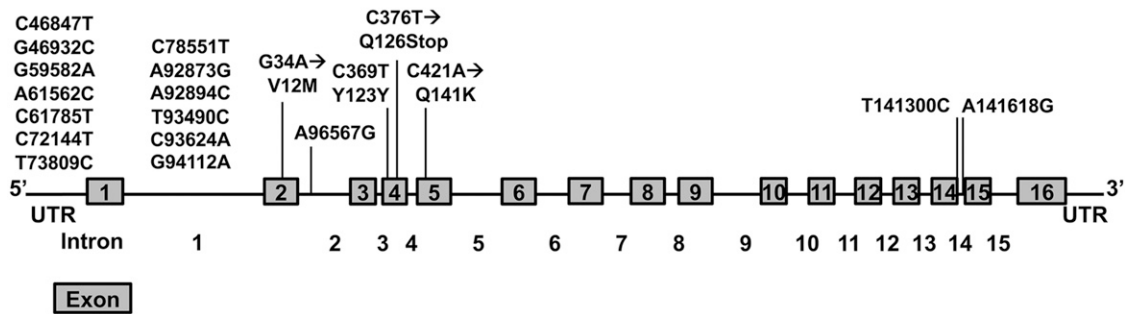
hydrocarbon receptor (AHR) (Tompkins et al., 2010), estrogen receptor  $\alpha$  (ER $\alpha$ /ESR1) and  $\beta$  (ER $\beta$ /ESR2) (Ee et al., 2004), hypoxia-inducible factor  $\alpha$  (HIF1 $\alpha$ ) (Krishnamurthy et al., 2004), nuclear factor erythroid 2-related factor 2 (NRF2) (Singh et al., 2010), peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (Sztamari et al., 2006), progesterone receptor (PR) (Wang et al., 2008), SP1 transcription factor (SP1) (Bailey-Dell et al., 2001), and SP3 transcription factor (SP3) (Yang et al., 2013). Despite the identification of functional regulatory elements, the relationship between these transcription factors and BCRP in term human placentas has largely been unexplored.

A number of SNPs have been identified in the *ABCG2* gene (chromosome 4q22, *ABCG2*; Fig. 2) (Zamber et al., 2003; de Jong et al., 2004; Kobayashi et al., 2005; Poonkuzhali et al., 2008). The most well-characterized genetic variants are localized to the coding region of the *ABCG2* gene and result in amino acid changes in the subsequent BCRP protein (i.e., G34A $\rightarrow$ V12M and C421A $\rightarrow$ Q141K) (Zamber et al., 2003; de Jong et al., 2004; Kobayashi et al., 2005). The C421A variant is often observed in Asian (allele frequency: 0.35) and Caucasian (allele frequency: 0.10) populations (Zamber et al., 2003; Kobayashi et al., 2005). Importantly, the C421A variant has been associated with the altered pharmacokinetics and pharmacodynamics of drugs in patients that express one or two variant alleles, most likely due to reduced BCRP function in the intestine, liver, and/or kidneys. For example, lung cancer

patients with one variant allele (421C/A) have a 3.7-fold greater risk of developing severe diarrhea, a toxic side effect of the BCRP substrate and chemotherapeutic drug gefitinib (Cusatis et al., 2006). Furthermore, healthy volunteers that were homozygous for the variant (421A/A) exhibited significantly greater area-under-the-curve values and maximal concentrations for the hypolipidemic drug rosuvastatin following a 20 mg oral dose as compared with those with no (421C/C) or one (421C/A) mutant allele (Keskitalo et al., 2009). Due to these data and additional reports, the International Transporter Consortium recommended that the C421A polymorphism be considered in clinical drug development (Giacomini et al., 2013).

Genetic variants that occur in the noncoding region of the *ABCG2* gene have begun to emerge as regulators of BCRP expression and are being evaluated for their clinical relevance. SNPs in the 5' untranslated region and intronic regions of the *ABCG2* gene have been associated with variation in BCRP mRNA expression in intestinal, liver, and lymphoblast samples (Poonkuzhali et al., 2008). Importantly, the effect of noncoding variants on BCRP expression and function in the placenta warrants investigation. Therefore, the purpose of this study was to identify factors, including infant sex, ethnicity/race, transcription factor relationships, and genetic variants, that contribute to the interindividual expression of BCRP mRNA and protein expression in term placentas from uncomplicated pregnancies.

ABCG2 Gene SNPs



**Fig. 2.** Map of SNPs in the noncoding and coding regions of the *ABCG2* gene. Noncoding SNPs are numbered according to the first nucleotide of the gene (+1; AC084732), and coding SNPs are numbered according to the translational start site (+1) and exclude nucleotides in noncoding regions. When applicable, *ABCG2* SNPs that result in amino acid changes in the BCRP protein are denoted with an arrow ( $\rightarrow$ ).

TABLE 1  
Placenta donor demographic information ( $N = 137$ )

Parameter	Clinical Characteristics of Pregnancies and Placentas Obtained
Gestational age (wk)	$38.8 \pm 1.1^a$
Maternal age (yr)	$31.2 \pm 4.9^a$
Placental weight (g) <sup>b</sup>	$660.0 \pm 154.5^a$
Birth weight (g)	$3355.3 \pm 426.3^a$
Infant sex <sup>c</sup>	Female = 65, Male = 69

<sup>a</sup>Mean  $\pm$  S.D.

<sup>b</sup>Wet, untrimmed weight.

<sup>c</sup>Sex was unknown for three infants.

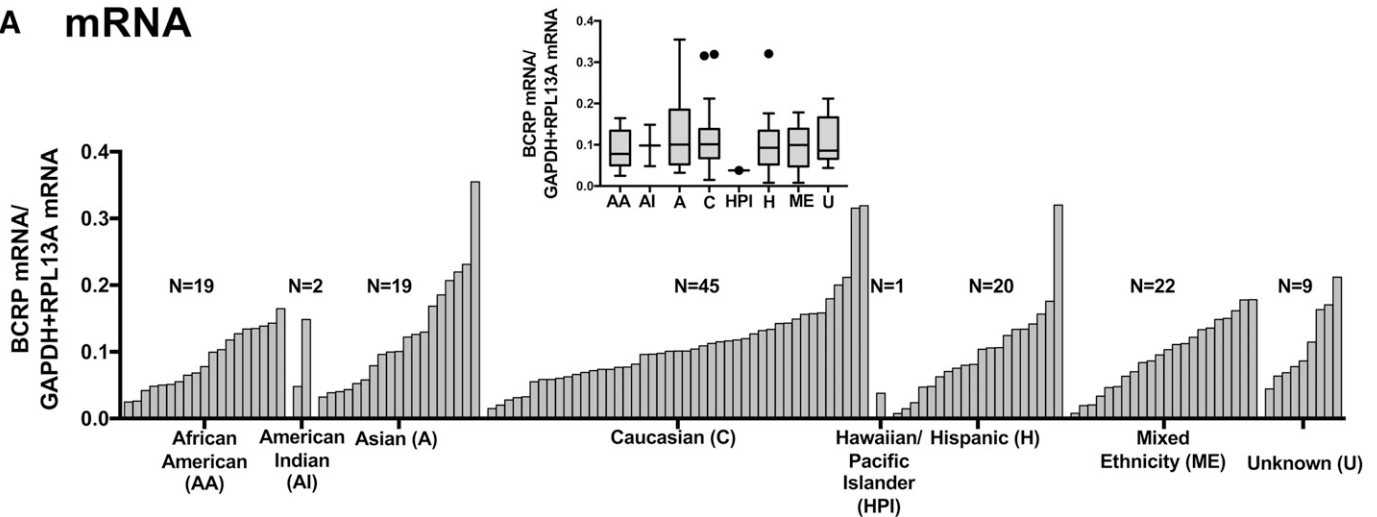
### Materials and Methods

**Placenta Donor Selection.** One hundred and thirty-seven placentas were obtained by written informed consent from women with uncomplicated pregnancies across two institutions (Robert Wood Johnson University Hospital, New Brunswick, NJ,  $N = 108$ ; University of California, San Francisco, CA,  $N = 29$ ). Participant inclusion criteria for this study were healthy women, ages 18–40,

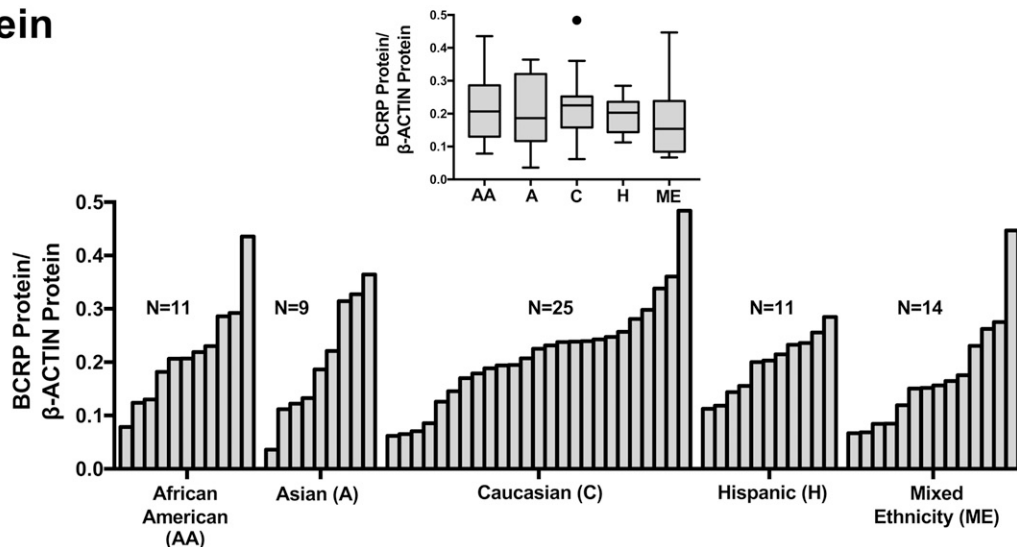
term gestation, scheduled Cesarean sections, and vaginal deliveries (University of California only). Exclusion criteria were pregnancy-induced medical conditions (i.e., pregnancy-induced hypertension, pre-eclampsia, gestational diabetes), chronic medical disorders (i.e., hypertension, diabetes, autoimmune diseases), maternal infection, clinical chorioamnionitis, medication use (except for prenatal vitamins), maternal alcohol or drug use, and known fetal chromosomal abnormalities. Placenta donor demographic information is listed in Table 1. Ethnicity and race were self-reported. Where possible, infant ethnicity and race were obtained from both maternal and paternal reporting. In the absence of paternal information, the ethnicity and race of the mother were used. This study was approved by the Institutional Review Boards of Robert Wood Johnson Medical School (Protocol 0220100258), Rutgers University (Protocols E12-024 and #E14-325), and the University of California (Protocol 10-00505).

**Sample Collection.** Placentas were collected within 10 minutes of delivery for processing within 1 hour. Visible abnormalities and the location of the umbilical cord were assessed, and only normal placentas with central or eccentric cord insertions were used. A small piece of umbilical cord close to the placenta was placed in a PAXgene Tissue Container (Qiagen, Germantown, MD) in the PAXgene Tissue Fix for 4 hours at 4°C, moved to PAXgene Tissue Stabilizer, and stored at  $-80^\circ\text{C}$  for *ABCG2* SNP genotyping. To sample placental tissue, the

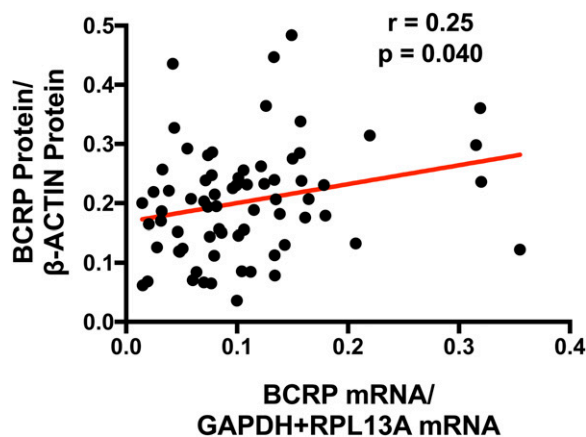
## A mRNA



## B Protein



**Fig. 3.** Evaluation of placental BCRP expression by infant ethnicity/race. (A) mRNA expression of BCRP was assessed in 137 term placentas using a multiplex-branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two reference genes, glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein L13A. (B) Protein expression of BCRP was assessed in 70 term placentas by Western blot analysis.  $\beta$ -Actin was used as a loading control. Data are presented as box and whisker plots using the Tukey method with medians marked by a horizontal line. The box contains 50% of the data, whereas the length of the whiskers from the edge of the box is equal to the lesser of either 1.5 times the interquartile range or the distance to the minimum/maximum value. Dots represent individual expression values that fall more than 1.5 times the interquartile range beyond the 25th and 75th quartiles. The bars represent individual placental BCRP expression.



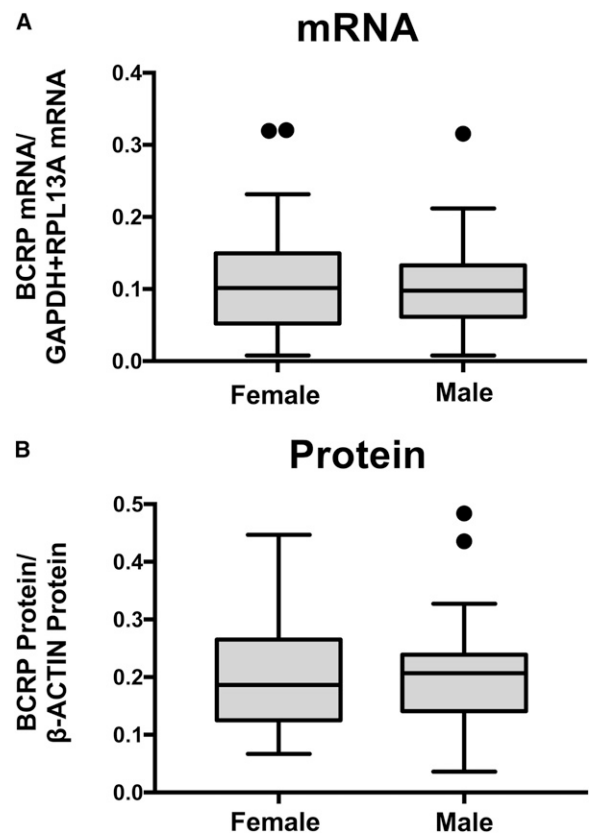
**Fig. 4.** Correlation between BCRP mRNA and protein expression. mRNA expression of BCRP was assessed in 70 term placentas using a multiplex-branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two reference genes, glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein L13A. Protein expression of BCRP was assessed in the same term placentas by Western blot analysis.  $\beta$ -Actin was used as a loading control. Correlation between BCRP mRNA and protein expression was determined using the Pearson linear method ( $r = 0.25$ ,  $P = 0.040$ ).

overlying membranes, maternal decidua, and chorionic plate were removed. From the maternal side of the placenta, two pieces of chorionic tissue were collected along the long axis approximately 1 cm distal to the cord insertion site, as described (Memon et al., 2014). It was previously shown that BCRP expression does not change across the placental disc (Memon et al., 2014). The dimensions of each sample were approximately  $2 \times 1 \times 0.5$  cm. After rinsing three times with sterile phosphate-buffered saline to remove maternal blood, each sample was further dissected into two subsegments of equal size for RNA and protein analysis. Tissues were placed in PAXgene Tissue Containers and processed, as described above. Samples were stored at  $-80^{\circ}\text{C}$  until further analysis. For assessment of protein expression, samples were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**Single-Nucleotide Polymorphism Genotyping.** Umbilical cord samples were homogenized, and total DNA was isolated with the PAXgene Tissue DNA Kit (Qiagen). A DropSense96 UV/Vis droplet reader was used to quantify total DNA and confirm the integrity of the DNA (Trinean, Gentbrugge, Belgium). The Fluidigm BioMark Genetic Analysis system was used to genotype 20 *ABCG2* gene SNPs (Fig. 2) in the Bionomics Research and Technology Center at Rutgers University. Primer sequences used for *ABCG2* SNP analysis are provided in Supplemental Tables 1–4.

**RNA Isolation and Multiplex-Branched DNA Assay.** PAXgene-stabilized placental tissues were homogenized using a TissueLyser (2 minutes, 50 Hz; Qiagen) in TR1 buffer provided in the PAXgene Tissue RNA Kit (Qiagen) plus 1%  $\beta$ -mercaptoethanol. Total RNA was isolated with the PAXgene Tissue RNA Kit (Qiagen), according to the manufacturer's instruction. Concentration of total RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and integrity was confirmed using a 2100 Bioanalyzer instrument (Agilent, Santa Clara, CA). A multiplex-branched DNA signal amplification assay (QuantiGene Assay; Affymetrix, Santa Clara, CA) and a Bio-Plex MAGPIX multiplex reader (Bio-Rad, Hercules, CA) were used to quantify mRNA expression of BCRP, AHR, ER $\alpha$ , ER $\beta$ , HIF1 $\alpha$ , NRF2, PPAR $\gamma$ , PR, SP1, and SP3. The number of target RNA molecules detected in each sample was proportional to the assay readout, median fluorescence intensity. Data were normalized to the geometric mean of the median fluorescence intensity for two reference genes that were previously determined to have low correlation with placental BCRP mRNA expression, glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein L13A (Memon et al., 2014). Probe information is provided in Supplemental Table 5.

**Western Blot.** Samples were processed for Western blot analysis, as previously described (Memon et al., 2014). Briefly, frozen placenta samples were homogenized in a sucrose (250 mM)–Tris base (10 mM) buffer (pH 7.5) with protease inhibitors (1%; Sigma-Aldrich, St. Louis, MO). Following centrifugation (100,000g) for 1 hour at  $4^{\circ}\text{C}$ , pellets containing crude membrane



**Fig. 5.** Evaluation of placental BCRP expression by infant sex. (A) mRNA expression of BCRP was assessed in 137 term placentas using a multiplex-branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two reference genes, glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein L13A. (B) Protein expression of BCRP was assessed in 70 term placentas by Western blot analysis.  $\beta$ -Actin was used as a loading control. Data are presented as box and whisker plots using the Tukey method with medians marked by a horizontal line. The box contains 50% of the data, whereas the length of the whiskers from the edge of the box is equal to the lesser of either 1.5 times the interquartile range or the distance to the minimum/maximum value. Dots represent individual expression values that fall more than 1.5 times the interquartile range beyond the 25th and 75th quartiles.

fractions were resuspended in sucrose–Tris base buffer with protease inhibitors. Protein concentrations were measured by a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed by adding 10  $\mu\text{g}$  total protein to polyacrylamide 4%–12% Bis–Tris gels (Life Technologies, Carlsbad, CA) that were resolved by electrophoresis. Gels were carefully trimmed, as previously described (Kiyatkin and Aksamitiene, 2009), and the same size proteins were transferred to polyvinylidene fluoride membranes in overnight transfer apparatuses onto the same membrane (Bio-Rad Criterion Blotter). This approach allowed protein samples from all placentas to be directly compared on the same transblotted membrane. This procedure was performed twice for each of the two sampling sites within the placentas. Membranes including all the samples were blocked in 5% nonfat dairy milk in phosphate-buffered saline with 0.5% Tween 20 (PBS/T) for 1 hour. Primary antibodies (BCRP, BXP-53, 1:5000, Enzo Life Scientific, Farmingdale, NY;  $\beta$ -actin, Ab8227, 1:2000, Abcam, Cambridge, MA) were diluted in 2% nonfat milk in 0.5% PBS/T and incubated with the membranes overnight at  $4^{\circ}\text{C}$ . After washing with PBS/T, horseradish peroxidase–conjugated secondary antibodies were added to the blots for 1 hour. Supersignal West Dura Extended Duration Substrate (Pierce Biotechnology) was used for chemiluminescent detection of proteins using a Fluorchem Imager (ProteinSimple, Sanata Clara, CA). Band densities were semiquantitated using the AlphaView Software (ProteinSimple). BCRP band density for each sample was normalized to its respective  $\beta$ -actin (loading control) band density.

**Statistical Analysis.** Separate linear regression models were used to compare both relative BCRP mRNA expression and relative BCRP protein expression to

TABLE 2  
Univariate associations between individual transcription factors and BCRP mRNA expression

	Parameter Estimate	S.E. of Parameter Estimate	t-Statistic	Unadjusted P Value	Adjusted P Value
NRF2	0.85	0.11	7.97	6.1e-13	5.5e-12
AHR	0.29	0.04	7.05	8.6e-11	7.7e-10
SP3	0.40	0.06	6.16	7.9e-09	7.1e-08
SP1	1.14	0.24	4.67	7.2e-06	6.5e-05
HIF1 $\alpha$	0.64	0.23	2.76	6.7e-03	6.0e-02
ESR2	36.57	19.86	1.84	6.8e-02	6.1e-01
ESR1	10.76	8.10	1.33	1.9e-01	1.0e+00
PR	10.86	8.19	1.33	1.9e-01	1.0e+00
PPAR $\gamma$	1.20	0.94	1.27	2.1e-01	1.0e+00

race and ethnicity, infant sex, placenta weight, infant weight, transcription factor expression (BCRP mRNA expression only), and SNPs (using an additive model). For relative BCRP mRNA expression, these linear regression models also included a fixed effect of collection site to account for overall differences in expression between collection sites. Adjusted relative BCRP mRNA expression values were used for figures and were calculated from the linear regression model that included collection site. Because BCRP protein expression values were calculated only at one site, no adjustment was needed. Effects are reported as the parameter estimate (b) from the regression model, i.e., the relative change in BCRP mRNA or protein expression associated with a 1 U change in the predictor variable. The comparison between protein expression and BCRP mRNA expression used a Pearson correlation coefficient and only included samples from one site. To compare transcription factor expression to one another, a pairwise Pearson correlation analysis was employed. All statistical analyses were carried out using the R statistical package (Version 3.4.1). Statistical significance was set to  $P < 0.05$ .  $P$  values were adjusted using a Bonferroni correction to account for multiple comparisons.

## Results

### Interindividual Expression of BCRP in Term Human Placentas.

From the lowest- to the highest-expressing placenta, BCRP mRNA and protein expression varied up to 47-fold ( $N = 137$ ) and 14-fold ( $N = 70$ ), respectively (Fig. 3). There was no association between infant

ethnicity/race and either BCRP mRNA or protein (Fig. 3). Interestingly, BCRP protein expression demonstrated a weak, but significant, correlation with BCRP mRNA expression ( $r = 0.25$ ,  $P = 0.040$ ) (Fig. 4). There were no significant differences in BCRP mRNA or protein between term placentas from female and male infants (Fig. 5). Additionally, neither infant weight nor placental weight correlated with BCRP mRNA expression (Supplemental Fig. 1).

**Correlation of Transcription Factor Expression with BCRP Expression.** The mRNAs of nine transcription factors known to regulate BCRP expression (AHR, ER $\alpha$ , ER $\beta$ , HIF1 $\alpha$ , NRF2, PPAR $\gamma$ , PR, SP1, SP3) were quantified in human term placentas ( $N = 137$ ). Univariate regression analysis revealed that BCRP mRNA correlated most closely to NRF2 ( $b = 0.85$ ;  $P < 0.0001$ ) and AHR ( $b = 0.29$ ;  $P < 0.0001$ ) mRNAs (Table 2). Furthermore, the mRNA expression of multiple transcription factors correlated well with each other, notably NRF2 with SP1 ( $r = 0.74$ ;  $P < 0.0001$ ) and AHR with SP3 ( $r = 0.73$ ;  $P < 0.0001$ ) (Supplemental Fig. 2; Supplemental Table 6).

**Assessment of ABCG2 Genetic Variants as Contributors to Variation in Placental BCRP Expression.** Twenty SNPs in the noncoding and coding regions of the *ABCG2* gene were assessed in 137 human term placentas (Fig. 2). Although allele frequencies varied by SNP, noncoding region SNPs occurred more frequently than those in the

TABLE 3  
Allele frequencies of SNPs separated by ethnicity/race

dbSNP <sup>d</sup>	Gene Location	Gene Position <sup>a</sup>	Allele Frequency <sup>b</sup>						
			African American	Asian	Caucasian	Hispanic	Mixed	Unknown	Overall
rs2127862	5'UTR	C46847T	0.47	0.21	0.45	0.28	0.3	0.28	0.35
rs2127861	5'UTR	G46932C	0.11	0	0.13	0.1	0.07	0.17	0.097
rs13135956	5'UTR	G59582A	0.53	0.13	0.20	0.15	0.21	0.06	0.22
rs141093520	5'UTR	A61562C	0.026	0.16	0.078	0.075	0.071	0	0.074
rs55930652	5'UTR	C61785T	0.11	0.13	0.17	0.13	0.18	0.22	0.15
rs2725226	5'UTR	C72144T	0.19	0.53	0.41	0.6	0.5	0.61	0.45
rs3114020	5'UTR	T73809C	0.32	0.53	0.41	0.63	0.5	0.61	0.47
rs2622604	Intron 1	C78551T	0.11	0.16	0.13	0.10	0.14	0.22	0.13
rs3109823	Intron1	A92873G	0.61	0.26	0.22	0.15	0.36	0.22	0.29
rs3114018	Intron 1	A92894C	0.24	0.55	0.47	0.70	0.57	0.78	0.52
rs2725250	Intron 1	T93490C	0.34	0.26	0.35	0.28	0.33	0.17	0.31
rs2622620	Intron 1	C93624A	0.24	0.53	0.38	0.58	0.45	0.78	0.44
rs2046134	Intron 1	G94112A	0.42	0.083	0.033	0.05	0.13	0	0.11
rs2231137	Exon 2	G34A <sup>c</sup>	0	0	0.011	0.13	0.09	0	0.036
rs186749266	Intron 2	A96567G	0.056	0.18	0.10	0.2	0.14	0.056	0.13
rs2231139	Exon 4	C369T <sup>c</sup>	0.11	0	0	0.025	0	0	0.019
rs72552713	Exon 4	C376T <sup>c</sup>	0	0	0.023	0	0.024	0	0.011
rs2231142	Exon 5	C421A <sup>c</sup>	0.026	0.32	0.011	0.2	0.048	0.28	0.11
rs2725267	Intron 14	T141300C	0.64	0.11	0.11	0.43	0.43	0	0.28
rs2231164	Intron 14	A141618G	0.63	0.37	0.13	0.6	0.45	0.22	0.36

<sup>a</sup>AC084732 (first nucleotide of gene = 1).

<sup>b</sup>Allele frequency was calculated by dividing the number of variant alleles by the total number of alleles (number of individuals  $\times$  2) in a population.

<sup>c</sup>Starting at translational start site (+1) and excluding nucleotides in noncoding regions.

<sup>d</sup>dbSNP: NCBI SNP database number.

TABLE 4  
Univariate associations between SNPs and BCRP mRNA expression

SNP <sup>a,b</sup>	Regression Parameter Estimate for Additive Effect on BCRP mRNA Expression	S.E. of Parameter Estimate for Additive Effect on BCRP mRNA Expression	t-Statistic for SNP Effect on BCRP mRNA Expression	P Value for SNP Effect on BCRP mRNA Expression	Bonferroni-Adjusted P Value for SNP Effect on BCRP mRNA Expression
C46847T	-0.0082	0.0082	-1.00	0.318	1.000
G46932C	0.0014	0.0129	0.11	0.912	1.000
G59582A	0.0016	0.0094	0.17	0.864	1.000
A61562C	-0.0294	0.0155	-1.90	0.060	1.000
C61785T	0.0019	0.0104	0.18	0.857	1.000
C72144T	0.0040	0.0074	0.55	0.586	1.000
T73809C	0.0060	0.0074	0.82	0.415	1.000
C78551T	0.0066	0.0109	0.61	0.543	1.000
A92873G	0.0031	0.0079	0.39	0.695	1.000
A92894C	-0.0062	0.0074	-0.84	0.401	1.000
T93490C	0.0035	0.0081	0.43	0.665	1.000
C93624A	0.0091	0.0075	1.22	0.225	1.000
G94112A	-0.0147	0.0109	-1.36	0.177	1.000
A96567G	-0.0072	0.0127	-0.57	0.570	1.000
C421A <sup>c</sup>	0.0029	0.0109	0.26	0.794	1.000
T141300C	-0.0013	0.0085	-0.15	0.882	1.000
A141618G	-0.0009	0.0074	-0.12	0.903	1.000

<sup>a</sup>AC084732 (first nucleotide of gene = 1).

<sup>b</sup>SNPs G34A, C369T, and C376T were removed from analysis due to small sample size.

<sup>c</sup>Starting at translational start site (+1) and excluding nucleotides in the noncoding region.

exonic regions (Table 3). Most of the genetic variants located in the coding region of the *ABCG2* gene occurred at low frequencies (G41A, C369T, C376T; Table 3), such that statistical analysis in relation to BCRP mRNA or protein expression could not be performed.

Overall, individuals heterozygous for the A61562C variant in the 5' untranslated region (5'UTR) tended to exhibit reduced BCRP mRNA expression (Table 4), although this was not statistically significant. Interestingly, there were no individuals homozygous for the A61562C SNP. No other associations between SNPs and BCRP mRNA expression were observed.

In a representative population of samples from a single collection site ( $N = 70$ ), analysis of BCRP protein expression revealed a negative trend for two SNPs in noncoding regions, C72144T (5'UTR) and C93624A (intron 1) (Table 5). The C421A SNP located in exon 5 occurred at 11% of the overall sample population but was most frequent in Asian and Hispanic populations occurring at 32% and 20%, respectively. Although

the C421A variant was not associated with altered BCRP mRNA expression (Fig. 6; Table 4), at the protein level, the C421A genotype had a significant effect on BCRP protein expression. Importantly, individuals that were homozygous for the variant (421A/A) had 50% lower BCRP expression in placentas as compared with the wild-type genotype (421C/C) (Fig. 6; Table 5). Of note, heterozygous individuals (421C/A) similarly exhibited 40% lower BCRP protein expression. Finally, BCRP mRNA and protein expression correlated in 421C/C individuals ( $r = 0.29$ ,  $P = 0.032$ ). This relationship was not observed in placentas expressing one or two variant alleles (421C/A,  $r = 0.13$  and  $P = 0.75$ ; 421A/A,  $r = 0.16$  and  $P = 0.84$ ) (Fig. 6C).

## Discussion

This study characterized interindividual differences in the mRNA and protein expression of the BCRP transporter in term placentas from

TABLE 5  
Univariate associations between SNPs and BCRP protein expression

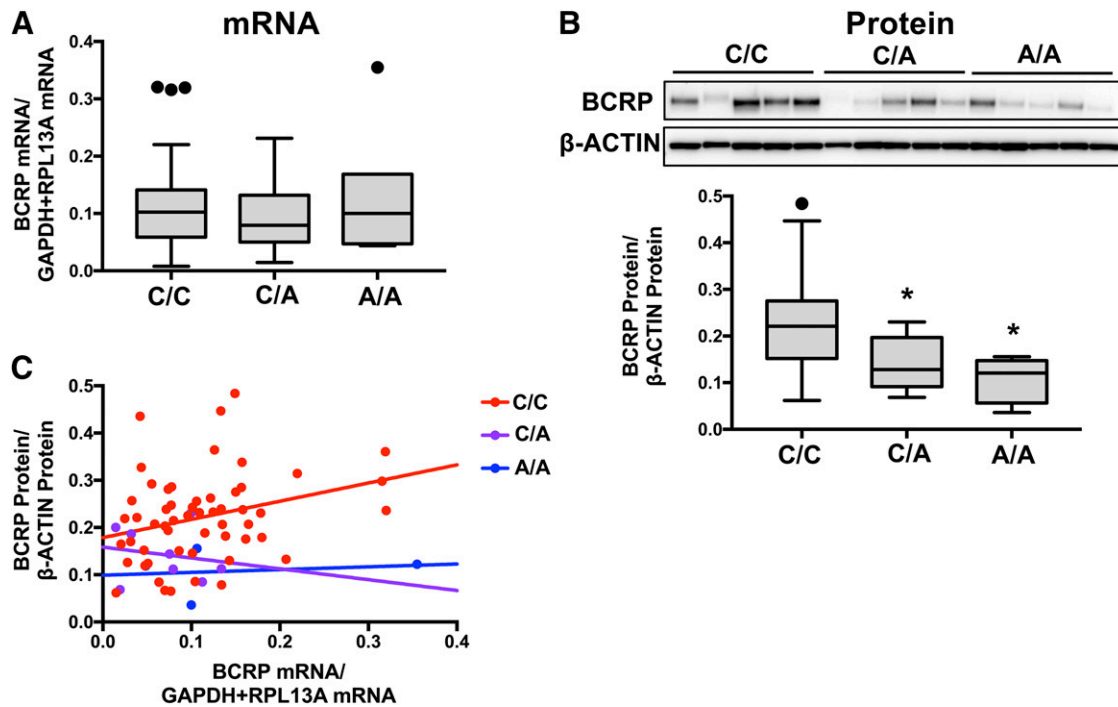
SNP <sup>a,b</sup>	Regression Parameter Estimate for Additive Effect on BCRP Protein Expression	S.E. of Parameter Estimate for Additive Effect on BCRP Protein Expression	t-Statistic for SNP Effect on BCRP Protein Expression	P Value for SNP Effect on BCRP Protein Expression	Bonferroni-Adjusted P Value for SNP Effect on BCRP Protein Expression
C46847T	0.0247	0.0169	1.46	0.149	1.000
G46932C	-0.0023	0.0256	-0.09	0.928	1.000
G59582A	0.0213	0.0178	1.20	0.235	1.000
A61562C	0.0097	0.0326	0.30	0.767	1.000
C61785T	0.0120	0.0215	0.56	0.578	1.000
C72144T	-0.0279	0.0148	-1.88	0.065	1.000
T73809C	-0.0210	0.0147	-1.42	0.159	1.000
C78551T	0.0227	0.0227	1.220	0.227	1.000
A92873G	0.0142	0.0158	0.90	0.373	1.000
A92894C	0.0207	0.0151	0.137	0.175	1.000
T93490C	0.0102	0.0180	0.57	0.574	1.000
C93624A	-0.0294	0.0154	-1.91	0.060	1.000
G94112A	0.0104	0.225	0.46	0.646	1.000
A96567G	0.0008	0.0274	0.03	0.976	1.000
C421A <sup>c</sup>	-0.0666	0.0195	-3.42	0.001	0.018
T141300C	0.0097	0.0184	0.53	0.598	1.000
A141618G	-0.0227	0.0147	-1.54	0.127	1.000

<sup>a</sup>AC084732 (first nucleotide of gene = 1).

<sup>b</sup>SNPs G34A, C369T, and C376T were removed from analysis due to small sample size.

<sup>c</sup>Starting at translational start site (+1) and excluding nucleotides in the noncoding region.





**Fig. 6.** Association of the C421A coding region SNP with placental BCRP expression. (A) mRNA expression of BCRP was assessed in 137 term placentas using a multiplex-branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two reference genes, glyceraldehyde-3-phosphate dehydrogenase and ribosomal protein L13A. (B) Protein expression of BCRP was assessed in 70 term placentas by Western blot analysis.  $\beta$ -Actin was used as a loading control. Data are presented as a representative Western blot and according to C421A genotype using box and whisker plots using the Tukey method with medians marked by a horizontal line. The box contains 50% of the data, whereas the length of the whiskers from the edge of the box is equal to the lesser of either 1.5 times the interquartile range or the distance to the minimum/maximum value. Dots represent individual expression values that fall more than 1.5 times the interquartile range beyond the 25th and 75th quartiles. Asterisks (\*) represent statistically significant differences ( $P < 0.05$ ) compared with wild-type control (C/C) using a Kruskal–Wallis nonparametric test with a Dunn's multiple testing correction. (C) Association of BCRP mRNA and protein expression by genotype was assessed by Pearson correlation.

ethnically and racially diverse pregnant women with uncomplicated pregnancies. There were no differences in BCRP mRNA or protein expression according to infant sex or ethnicity/race. Additionally, expression of BCRP mRNA weakly correlated with protein levels in a representative population of placentas ( $N = 70$ ). BCRP mRNA expression correlated to the greatest extent with the mRNA expression of two transcription factors, NRF2 and AHR. Overall, SNPs in the noncoding regions of the *ABCG2* gene were not associated with changes in BCRP mRNA or protein expression. However, there was up to a 50% reduction in BCRP protein in term placentas expressing a common SNP located in the fifth exon (C421A). Taken together, these data suggest that the C421A genetic variant has the greatest influence on placental BCRP protein expression.

We observed no significant differences in BCRP mRNA or protein expression based on infant sexes (Fig. 5). In contrast, other laboratories have demonstrated that BCRP is more highly expressed in the intestines and livers of adult females compared with males (Zamber et al., 2003; Prasad et al., 2013). Differences may be due to variation in the expression of BCRP regulatory factors across tissues or during different developmental ages.

Of the transcription factors assessed, AHR and NRF2 mRNAs exhibited the greatest associations with BCRP mRNA (Table 2). This is similar to the relationships reported between mouse placental *Ahr* and *Bcrp* mRNAs ( $r = 0.78$ ,  $P < 0.01$ ) compared with other screened transcription factor mRNAs (*Hif1 $\alpha$* , *Er $\alpha$* , *Er $\beta$* , *Pr*) (Wang et al., 2006). However, in human placenta cell culture, two studies have reported conflicting findings regarding the regulation of BCRP by the AHR signaling pathway. The prototypical AHR activator 3-methylcholanthrene (24 hour) significantly enhanced the mRNA expression of AHR target gene *CYP1A1* without changing BCRP

levels in human term placental trophoblasts (Stejskalova et al., 2011). By comparison, the AHR antagonist CH223191 prevented a maximal 10-fold upregulation of BCRP mRNA by opioid receptor agonists methadone, buprenorphine, and norbuprenorphine in placenta choriocarcinoma cells (BeWo and JEG3) and primary human villous trophoblasts (Neradugomma et al., 2017). Based on these findings, additional mechanistic investigations are needed to demonstrate a causal relationship between AHR and BCRP expression in term placentas.

The relationship between the NRF2 transcription factor and BCRP has not been explored in the placenta. Typically, NRF2 localizes to antioxidant response elements in the promoter region of genes involved in detoxification pathways, including NAD(P)H quinone oxidoreductase 1. In rat brain capillaries, Bcrp protein and function were significantly increased following exposure to the prototypical NRF2 activator sulforaphane (Wang et al., 2014). Moreover, Singh et al. (2010) reported NRF2 directly interacts with an antioxidant response element in the promoter of the *ABCG2* gene and observed a downregulation of BCRP mRNA in human lung cancer cells in response to the genetic knockdown of NRF2 with a targeted short hairpin RNA. In human choriocarcinoma JEG3 cells, NRF2 is expressed and, upon treatment with acetaminophen (48 hour), resulted in an approximate 40% increase in NAD(P)H quinone oxidoreductase 1 gene expression; however, BCRP mRNA expression was not assessed (Blazquez et al., 2014). A detailed investigation of the relationship between human placental NRF2 and BCRP may provide mechanistic information regarding the fetoprotective pathways of the placenta during healthy conditions and in the presence of oxidative and cellular stress.

Although BCRP mRNA expression correlated with AHR and NRF2 mRNA expression in term placentas, it is unclear whether this relationship

is established early in pregnancy. There are conflicting data regarding the expression of BCRP across gestation as three independent research groups reported a reduction (mRNA and protein) (Meyer zu Schwabedissen et al., 2006), no change (protein and mRNA) (Mathias et al., 2005), and an increase (only protein) (Yeboah et al., 2006) in BCRP expression with advancing gestational age. Future studies should assess the mRNA and protein expression of BCRP, AHR, and NRF2 of first-, second-, and third-trimester placentas to better characterize the relationship between BCRP and the two transcription factors during placental development.

In a prior study, noncoding SNPs in the *ABCG2* gene correlated with high (A61562C, G94112A) or low (G46932C, C61785T, C78551T) BCRP mRNA expression in liver, intestine, and lymphoblasts (Poonkuzhali et al., 2008). The proposed underlying mechanisms for differential BCRP mRNA expression included changes in the binding of specific transcription factors (promoter region, 5'UTR) and/or disruption of gene splicing (introns) (Boccia et al., 1996; Wang and Sadee, 2016). Since the initial investigation by Poonkuzhali et al. (2008), three of the noncoding *ABCG2* SNPs have been linked to altered pharmacodynamics of BCRP substrates. In the first intron, the C78551T and A92873G variants have been associated with the development of severe myelosuppression as a side effect of the anticancer drug irinotecan (Cha et al., 2009). Also, in the first intronic region, the A92894C variant has been associated with altered pharmacokinetic parameters in patients treated with the epilepsy drug, lamotrigine; significantly higher blood concentrations were observed in subjects heterozygous or homozygous for the A92894C variant (Zhou et al., 2015).

This is the first report investigating the association of placental BCRP expression with genetic variants in the noncoding region of the *ABCG2* gene. Only one SNP in the 5'UTR (A61562C) tended to be associated with lower BCRP expression. Despite prior associations between non-coding variants and BCRP expression in liver, intestine, and lymphoblasts (Poonkuzhali et al., 2008), there may be differences in gene regulation across tissues. Tissue-specific effects have been reported for intronic SNPs occurring in the *CYP3A4* gene. Human livers with the intronic variant CYP3A4\*22 exhibited lower CYP3A4 mRNA expression, which was not observed in the intestines (Wang and Sadee, 2016). Segregation of mRNA expression data by ethnicity and genotype may uncover additional associations between *ABCG2* SNP and BCRP mRNA expression; however, our sample size limited the ability to conduct this analysis. We also examined SNPs occurring in the coding region of the *ABCG2* gene. Individuals that were homozygous for the coding region variant (C421A) had 50% less BCRP protein than the wild-type controls, with no differences in BCRP mRNA. This is in agreement with a study performed in 2005, which examined BCRP expression in 100 placentas from healthy Japanese women (Kobayashi et al., 2005).

We observed a weak but statistically significant correlation between BCRP mRNA and protein (Fig. 4), suggesting that, in some cases, mRNA levels may be a surrogate measure for understanding BCRP protein expression in term human placentas. However, the correlation coefficient ( $r = 0.25$ ) revealed that variation in BCRP protein is not solely dependent on mRNA expression and that other regulatory mechanisms, including microRNAs (Saito et al., 2013) and post-transcriptional modifications (Imai et al., 2005), may be important. Two other studies found no correlation between the BCRP gene and protein expression in other normal human tissues, including intestine ( $N = 42$ ) and liver ( $N = 65$ ) (Zamber et al., 2003; Prasad et al., 2013), further emphasizing the importance of understanding tissue-specific differences in the regulation of BCRP expression.

In summary, these data demonstrate the interindividual expression of placental BCRP in term placentas from uncomplicated pregnancies and reveal associations of BCRP with genetic variants as well as transcription factors. These findings add to our understanding of the regulation of

placental BCRP expression in normal term pregnancies and provide a foundation for identifying individuals that may be at risk for reduced BCRP expression and function.

## Authorship Contributions

*Participated in research design:* Bircsak, Aleksunes.

*Conducted experiments:* Bircsak, Moscovitz, Wen.

*Contributed new reagents or analytic tools:* Archer, Yuen, Mohammed, Memon, Vetrano, Weinberger.

*Performed data analysis:* Saba.

*Wrote or contributed to the writing of the manuscript:* Bircsak, Aleksunes.

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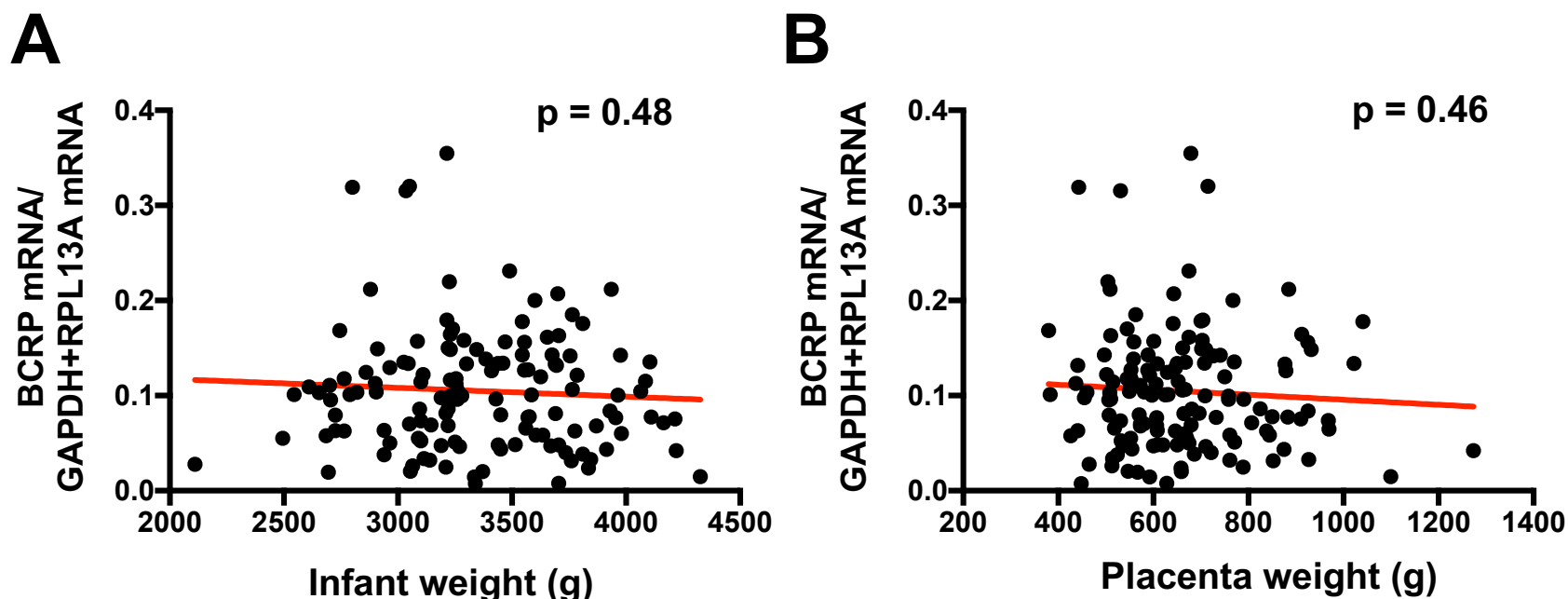
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## Interindividual Regulation of the BCRP/ABCG2 Transporter in Term Human Placentas

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Anna M Vetrano, Lauren M Aleksunes

*Drug Metabolism and Disposition*



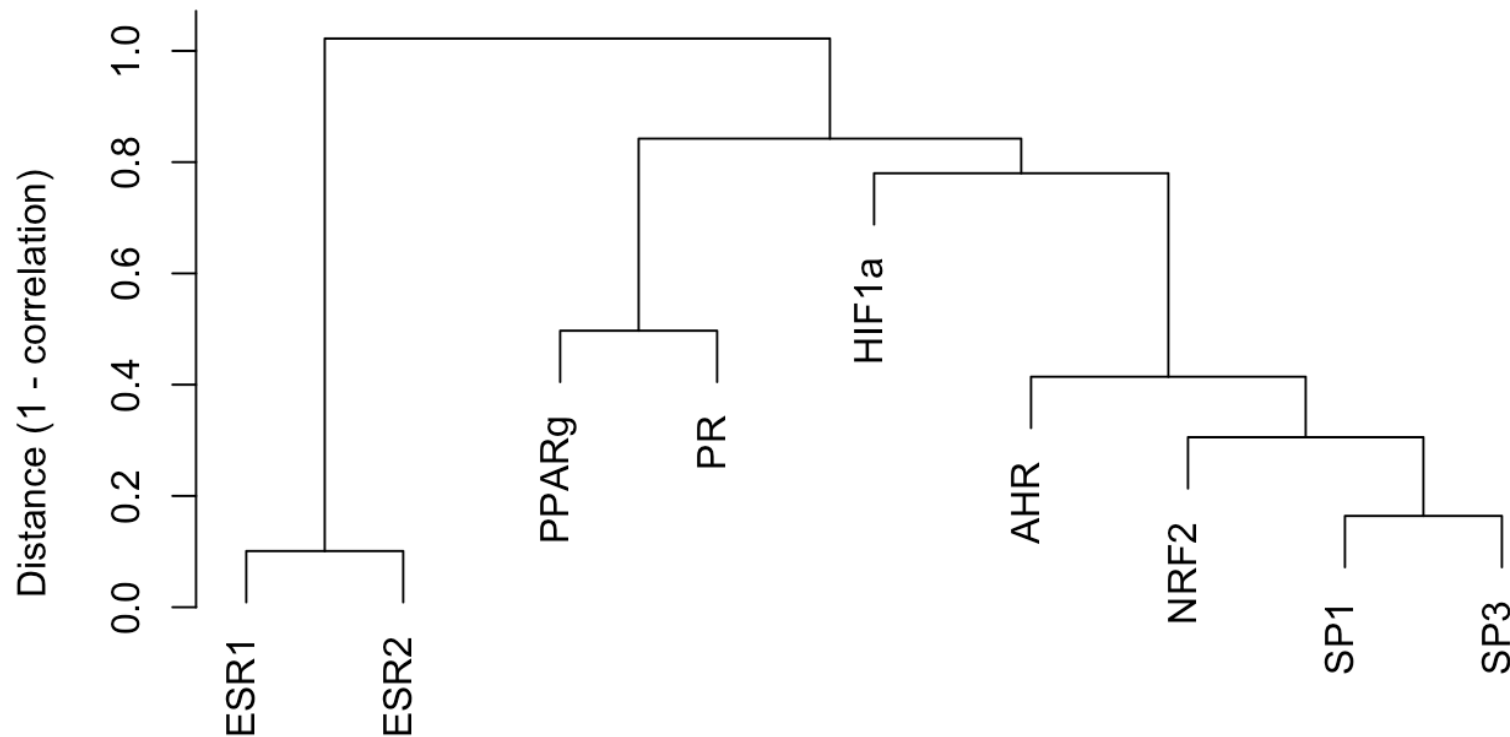
### Supplemental Figure 1. Evaluation of placenta BCRP mRNA expression by infant and placenta weight.

Messenger RNA expression of BCRP was assessed in 137 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. Linear regression analysis was used to determine the relationship between BCRP mRNA and (A) infant weight or (B) placenta weight after adjustment for collection site.

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### *Drug Metabolism and Disposition*



**Supplemental Figure 2. Relationship among mRNA expression levels of transcription factors.** Messenger RNA expression of nine transcription factors (AHR, ER $\alpha$ , ER $\beta$ , HIF1 $\alpha$ , NRF2, PPAR $\gamma$ , PR, SP1, SP3) was assessed in 137 term placentas using a multiplex branched DNA signal amplification assay. The geometric mean of two reference genes, GAPDH and RPL13A were used to normalize mRNA expression values. Pairwise correlation analysis was used to evaluate the relationship between placenta transcription factor mRNAs.

## **Supplemental Tables**

### **Interindividual Regulation of the BCRP/*ABCG2* Transporter in Term Human Placentas**

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*Drug Metabolism and Disposition*

**Supplemental Table 1.** *ABCG2* SNP allele specific primer 1 (forward) sequences used for genotyping.

<i>ABCG2</i> SNP ID	Nucleotide Change	Allele Specific Primer <u>1</u> (Forward)
rs2127862	C46847T	TTGCCCAGGCTAGAGTACG
rs2127861	G46932C	GCATGGTGGCACATGCTTC
rs13135956	G59582A	CAGAAGTAGGAGGCCAAGGT
rs141093520	A61562C	AAAAACACCAAAAGCAATTGCAACA
rs55930652	C61785T	GCCACATAGTATTACAGACATGCG
rs2725226	C72144T	CGGGTTCAAGCGATTCTACTG
rs3114020	T73809C	TGATTTACCACTGCCTCCCAG
rs2622604	C78551T	AAACATCTCTTCCTTACTACTCTGGC
rs3109823	A92873G	ACCTCACAAAAGTGTCTTTAAAATTTCCA
rs3114018	A92894C	TCTTAAGAGTATCAAATAACTTATTGCTTGAATTTA
rs2725250	T93490C	GTGCACACCCATGGTTTCG
rs2622620	C93624A	G TTCACACCTGTAGTCCCAGA
rs2046134	G94112A	ACTGATTCTGTCTAAGTACCCATTTTGT
rs2231137	G34A*	CCAGTAATGTCGAAGTTTTTATCCCAA
rs186749266	A96567G	CCTGTGAGGTTCACTGTAGGTAAA
rs2231139	C369T*	ACCCACTAATACTTACTTGTACCACG
rs72552713	C376T*	CAGAAAATGCAAACCCACTAATACTTACTTA
rs2231142	C421A*	CCGAAGAGCTGCTGAGAACTT
rs2725267	T141300C	CAACTGGTTCTAATGAAAACAAACAAGAAC
rs2231164	A141618G	CAGGGCAAGGTAAACAGTTTTTAAATTTT

<sup>a</sup> AC084732 (1st nucleotide of gene = 1)

\*Excluding nucleotides from untranslated regions

**Supplemental Table 2.** *ABCG2* SNP allele specific primer 2 (forward) sequences used for genotyping.

<i>ABCG2</i> SNP ID	Nucleotide Change	Allele Specific Primer <u>2</u> (Forward)
rs2127862	C46847T	TTGCCCAGGCTAGAGTACA
rs2127861	G46932C	GCATGGTGGCACATGCTTG
rs13135956	G59582A	AGAAGTAGGAGGCCAAGGC
rs141093520	A61562C	AAAACACCAAAAGCAATTGCAACC
rs55930652	C61785T	GGCCACATAGTATTACAGACATGCA
rs2725226	C72144T	GCGGGTTCAAGCGATTCTACTA
rs3114020	T73809C	TGATTTACCACTGCCTCCCAA
rs2622604	C78551T	AAAAAACATCTCTTCCTTACTACTCTGGT
rs3109823	A92873G	ACCTCACAAAAGTGTCTTTAAAATTTCCG
rs3114018	A92894C	TCTTAAGAGTATCAAATAACTTATTGCTTGAATTTC
rs2725250	T93490C	GTGCACACCCATGGTTTCA
rs2622620	C93624A	GTTACACCTGTAGTCCCAGC
rs2046134	G94112A	CTGATTCTGTCTAAGTACCCATTTTGC
rs2231137	G34A*	CCAGTAATGTCTGAAGTTTTTATCCCAG
rs186749266	A96567G	CCTGTGAGGTTCACTGTAGGTAAG
rs2231139	C369T*	AAACCCACTAATACTTACTTGTACCACA
rs72552713	C376T*	AGAAAATGCAAACCCACTAATACTTACTTG
rs2231142	C421A*	CCGAAGAGCTGCTGAGAACTG
rs2725267	T141300C	ACAACCTGGTTCTAATGAAAACAAACAAGAAT
rs2231164	A141618G	CAGGGCAAGGTAAACAGTTTTTAAATTTTC

<sup>a</sup> AC084732 (1st nucleotide of gene = 1)

\*Excluding nucleotides from untranslated regions



**Supplemental Table 3.** *ABCG2* SNP locus specific primer (reverse) sequences used for genotyping.

<i>ABCG2</i> SNP ID	Nucleotide Change	Locus Specific Primer (Reverse)
rs2127862	C46847T	GGAGAATCACTTGAGCCAAGGAG
rs2127861	G46932C	GCCTCCTGAGTGGCTAGGAAT
rs13135956	G59582A	GACTGGTCTTGAGCTCCTGGA
rs141093520	A61562C	ACCATGCCCAGCTGATGATAGT
rs55930652	C61785T	CATGGAGAAACCCTGTCTGTCTCT
rs2725226	C72144T	GCATGGTGGCAAGTGCTTGT
rs3114020	T73809C	TGATGTTTCATATTTATAGGTCGGTTTATTTGCTAACT
rs2622604	C78551T	AGCTCTCTTATCATTGAATGTCAGCTAGT
rs3109823	A92873G	AGACTTCTTAAGAGTATCAAATAACTTATTGCTTGAATTT
rs3114018	A92894C	GTGGAAACCTCACAAAAGTGTCTTTAAAATTTC
rs2725250	T93490C	CAATCTCTGCCTCCTGGGCT
rs2622620	C93624A	CCTCGAATTCCTGCGTTCAAGT
rs2046134	G94112A	ACAGGCAACCTGAGAGAGAAGG
rs2231137	G34A*	GGGAAGCCATTGGTGTTCCT
rs186749266	A96567G	CAAGTATGTACATGAACTTGTA AAAAAGACAGCTT
rs2231139	C369T*	GCGACCTGCCAATTTCAAATG
rs72552713	C376T*	GCGACCTGCCAATTTCAAATG
rs2231142	C421A*	TGATGGGCACTCTGACGGT
rs2725267	T141300C	GTGCTCAATGAGTATTGTTAATTTACAGTGACA
rs2231164	A141618G	GGCATGATTGTGATAACTCTTTGGAAACT

<sup>a</sup> AC084732 (1st nucleotide of gene = 1)

\*Excluding nucleotides from untranslated regions

**Supplemental Table 4.** *ABCG2* SNP specific target amplification primer sequences used for genotyping.

<i>ABCG2</i> SNP ID	Nucleotide Change	Specific Target Amplification Primer
rs2127862	C46847T	ACGAAAATTAGAGCCACTAAATAAATTCAGC
rs2127861	G46932C	GCAAAATAGTAACACACCTTCTCCA
rs13135956	G59582A	GTGGGTACAGTGGCTTATACCTA
rs141093520	A61562C	GCATGAGCAAAGACTTCATGACAA
rs55930652	C61785T	TCCAAAGGACATGAACTCATTCTTT
rs2725226	C72144T	GCAACCTCTGCCTTGCG
rs3114020	T73809C	TTATTTGATTTACCACTGCCTCCC
rs2622604	C78551T	ACTCTGAAAGCACTGTTTTGTAAA
rs3109823	A92873G	TTGTTGCCTTTTATTTGTGGAAACC
rs3114018	A92894C	CAGCCACTGAGGAAAAGACTT
rs2725250	T93490C	GCCGAGTATGCTGGTGC
rs2622620	C93624A	TTCTGCATCTGAAGATTCAACCAGT
rs2046134	G94112A	CTGGCCAGTATTTCTTCAAATACTGA
rs2231137	G34A*	GATAAAAACCTCTCCAGATGTCTTCCA
rs186749266	A96567G	CCAGTTTCTTGGAAATAGCCAAAAC
rs2231139	C369T*	GAAACAGAGGAAACAGAAAATGCAA
rs72552713	C376T*	GCACTTACCCATATAGAAACAGAGG
rs2231142	C421A*	CATGATTCGTCATAGTTGTTGCAAG
rs2725267	T141300C	AACCAAGCTTCTAACATCCAAGAATT
rs2231164	A141618G	GCCTATAACACAAGTGGGAGC

<sup>a</sup> AC084732 (1st nucleotide of gene = 1)

\*Excluding nucleotides from untranslated regions

**Supplemental Table 5.** QuantiGene multiplex branched DNA assay probe information.

Accession Number	Gene	Protein	Sequence Length	Probe Set Region
NM_004827	<i>ABCG2</i>	Breast cancer resistance protein (BCRP)	4445	681-1252
NM_001621	<i>AHR</i>	Aryl hydrocarbon receptor (AhR)	6247	641-1361
NM_000125	<i>ESR1</i>	Estrogen receptor $\alpha$ (ER $\alpha$ )	6330	5671-6292
NM_001437	<i>ESR2</i>	Estrogen receptor $\beta$ (ER $\beta$ )	2169	1435-1831
NM_002046	<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	1401	2-407
NM_001530	<i>HIF1A</i>	Hypoxia-inducible factor 1 $\alpha$ (HIF1 $\alpha$ )	4082	1538-1909
NM_006164	<i>NFE2L2</i>	Nuclear factor erythroid 2-related factor 2 (NRF2)	2859	1731-2270
NM_005037	<i>PPARG</i>	Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ )	1818	567-1044
NM_000926	<i>PGR</i>	Progesterone receptor (PR)	13037	2609-3194
NM_012423	<i>RPL13A</i>	Ribosomal protein L13A (RPL13A)	1142	90-498
NM_138473	<i>SP1</i>	SP1 Transcription Factor (SP1)	7667	1054-1399
NM_003111	<i>SP3</i>	SP3 Transcription Factor (SP3)	6359	435-963

**Supplemental Table 6.** Pairwise correlations between transcription factors.

	<b>AHR</b>	<b>ESR1</b>	<b>ESR2</b>	<b>HIF1<math>\alpha</math></b>	<b>NRF2</b>	<b>PPAR<math>\gamma</math></b>	<b>PR</b>	<b>SP1</b>	<b>SP3</b>
<b>AHR</b>	1.00	0.03	0.02	0.22	0.67	0.26	0.21	0.59	0.73
<b>ESR1</b>	0.03	1.00	0.90	-0.02	0.13	0.06	0.19	0.26	0.17
<b>ESR2</b>	0.02	0.90	1.00	0.06	0.14	0.08	0.23	0.30	0.22
<b>HIF1<math>\alpha</math></b>	0.22	-0.02	0.06	1.00	0.34	0.16	0.23	0.44	0.43
<b>NRF2</b>	0.67	0.13	0.14	0.34	1.00	0.63	0.51	0.74	0.69
<b>PPAR<math>\gamma</math></b>	0.26	0.06	0.08	0.16	0.63	1.00	0.50	0.51	0.30
<b>PR</b>	0.21	0.19	0.23	0.23	0.51	0.50	1.00	0.43	0.28
<b>SP1</b>	0.59	0.26	0.30	0.44	0.74	0.51	0.43	1.00	0.84
<b>SP3</b>	0.73	0.17	0.22	0.43	0.69	0.30	0.28	0.84	1.00