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Authors

Eclov, Rachel J

Kim, Mee J

Smith, Robin

et al.

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Rare Variants in the *ABCG2* Promoter Modulate In Vivo Activity

Rachel J. Eclov, Mee J. Kim, Robin Smith, Nadav Ahituv, and Deanna L. Kroetz

Department of Bioengineering and Therapeutic Sciences (R.J.E., M.J.K., R.S., N.A., D.L.K.) and Institute for Human Genetics (N.A., M.J.K., R.S., D.L.K.), University of California San Francisco, San Francisco, California

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ABSTRACT

ABCG2 encodes the breast cancer resistance protein (BCRP), an efflux membrane transporter important in the detoxification of xenobiotics. In the present study, the basal activity of the *ABCG2* promoter in liver, kidney, intestine, and breast cell lines was examined using luciferase reporter assays. The promoter activities of reference and variant *ABCG2* sequences were compared in human hepatocellular carcinoma cell (HepG2), human embryonic kidney cell (HEK293T), human colorectal carcinoma cell (HCT116), and human breast adenocarcinoma cell (MCF-7) lines. The *ABCG2* promoter activity was strongest in the kidney and intestine cell lines. Four variants in the basal *ABCG2* promoter (rs76656413, rs66664036, rs139256004, and rs59370292) decreased the promoter activity by 25%–50% in at least three of the four cell lines. The activity of these four variants was also examined in vivo using the hydrodynamic tail vein assay, and two single nucleotide polymorphisms (rs76656413 and rs59370292) significantly decreased in vivo liver promoter activity by 50%–80%. Electrophoretic mobility shift assays confirmed a reduction in nuclear protein binding to the rs59370292 variant probe, whereas the rs76656413 probe had a shift in transcription factor binding specificity. Although both rs59370292 and rs76656413 are rare variants in all populations, they could contribute to patient-level variation in ABCG2 expression in the kidney, liver, and intestine.

Introduction

The breast cancer resistance protein (BCRP) ABCG2 is an efflux membrane transporter and part of the ATP-binding cassette (ABC) transporter family. It transports a variety of dietary toxins, endogenous nutrients, and pharmaceutical compounds (Ni et al., 2010). BCRP is expressed in the side population of hematologic stem cells, endothelium of veins and capillaries (including in the brain), intestinal and colon epithelium, placental syncytiotrophoblasts, ducts and lobules of the breast, the bile canalicular membrane of hepatocytes, and to a lesser extent in renal cortical tubules (Robey et al., 2009). BCRP is essential for detoxification processes, the transport of nutrients into milk, and the protection of vital organs and tissues like the brain, fetus, prostate, and eye (Leslie et al., 2005). Interindividual expression of ABCG2 mRNA is highly variable, with reports of 500-fold differences among human livers without detectable copy number variation (Poonkuzhali et al., 2008), 1000-fold differences in leukemic blast cells (Ross et al., 2000) and 1.8-fold to 78-fold differences in human intestine (Zamber et al., 2003; Urquhart et al., 2008). Additionally, high ABCG2 expression has been linked to decreased disease-free survival in cancer (Mao and Unadkat, 2015). Understanding the mechanisms that regulate the expression of ABCG2 can help to predict cancer outcomes, drug response, and toxicity. These mechanisms could become clinical targets of epigenetic inhibitors to downregulate transporter expression and enhance the efficacy of pharmacotherapy (Chen et al., 2016).

BCRP is transcribed by *ABCG2*, which spans over 66 kilobases on the anti-strand of chromosome 4q22 (Allikmets et al., 1998). The basal *ABCG2* promoter is a TATA-less promoter identified as the 312 base pairs upstream of the transcription start site (TSS) (Bailey-Dell et al., 2001), whereas the 5′ promoter regulatory region has been described as >100 kilobases (Poonkuzhali et al., 2008). The basal promoter

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ABBREVIATIONS: ABC, ATP-binding cassette; ANOVA, analysis of variance; ApoE, apolipoprotein E; ARE, antioxidant response element; BCRP, breast cancer resistance protein; bp, base pair; ChIP-seq, chromatin immunoprecipitation coupled with sequencing; CpG, cytosine-phosphoguanine; EMSA, electrophoretic mobility shift assay; ENCODE, encyclopedia of DNA elements; FBS, fetal bovine serum; MAF, minor allele frequency; Max, myc-associated factor X; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; STAT5, signal transducer and activator of transcription 5; TSS, transcription start site; UCSF, University of California San Francisco; USF-1, upstream stimulatory factor-1; VDR, vitamin D receptor.

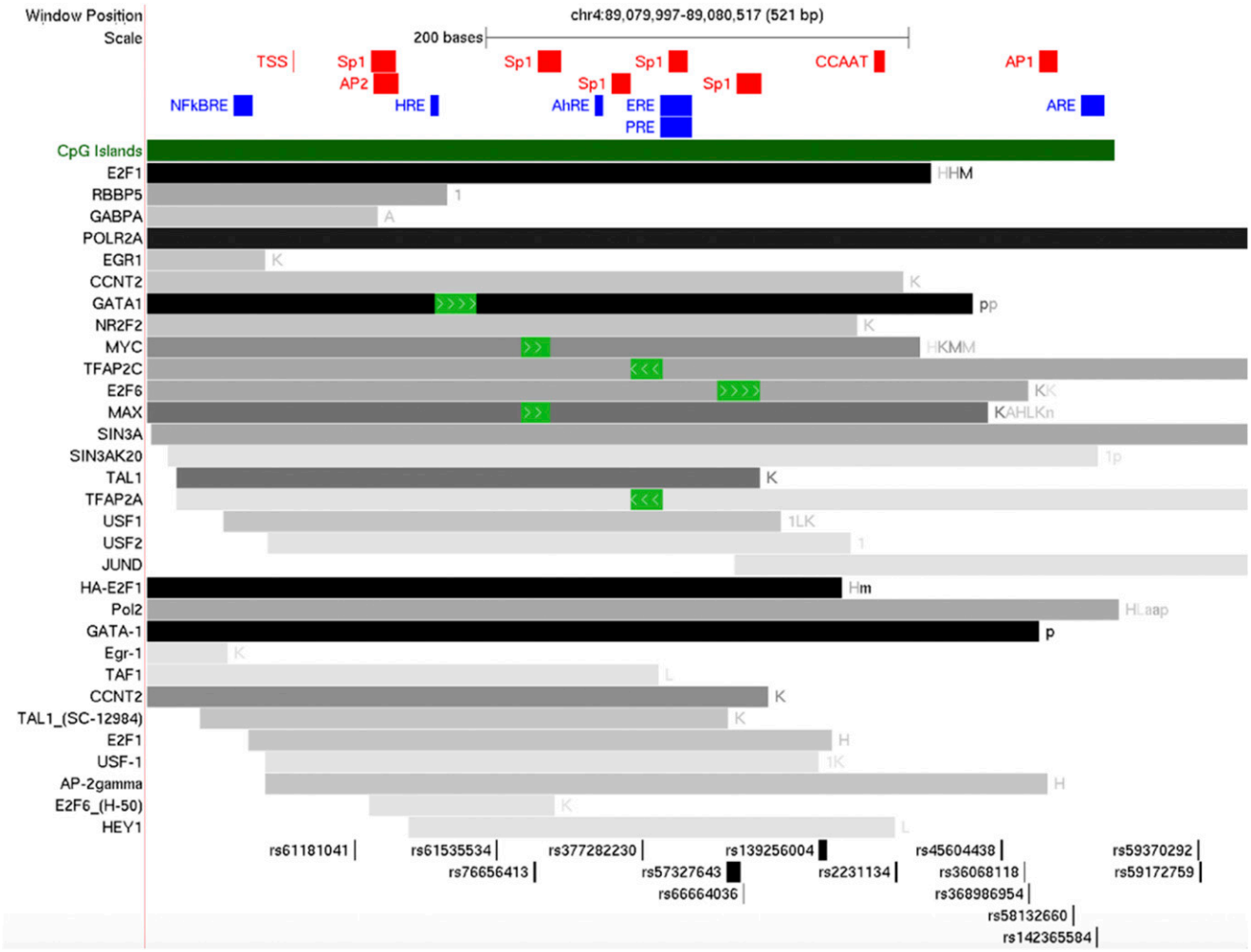

Fig. 1. Schematic of the *ABCG2* promoter region. The genomic region chr4:89079997-89080517 (hg19) with red boxes indicating location of basal transcription factors and the TSS, and blue boxes indicating locations for nuclear factor-κB response element (NFkBRE), hypoxia response element (HRE), aryl hydrocarbon response element (AhRE), estrogen response element (ERE), progesterone response element (PRE), and ARE. The promoter is also covered by a CpG island, which is indicated by a dark green bar. The binding of transcription factors determined by ChIP-seq from ENCODE data is indicated by the horizontal bars, with the length and shading of the bar indicating the breadth and strength of the peak. Within the bar, a light green box indicates the site of a canonical motif for the corresponding factor. Cell lines for peaks are indicated by letters, as follows: K, K562; H, HeLa-S3; L, HepG2; A/a, A549; p, PBDE; M/m, MCF-7; 1, H1-hourESC; n, NB4. Finally, the location and rs number for SNPs reported in dbSNP 138 are indicated.

includes a CCAAT box and numerous binding sites for specificity protein 1 and activator proteins 1 and 2 (Fig. 1) (Bailey-Dell et al., 2001). The proximal promoter of *ABCG2* has a functional aryl hydrocarbon receptor response element (Tan et al., 2010; To et al., 2011) that overlaps with progesterone elements (Wang et al., 2008b) and estrogen response elements (Ee et al., 2004) (Fig. 1). It also has a nuclear factor-κB response element that works in concert with estrogen to increase ABCG2 expression (Pradhan et al., 2010), a hypoxia-inducible factor 1α response element (Krishnamurthy et al., 2004), and an antioxidant response element (ARE) (Singh et al., 2010) (Fig. 1). A large cytosine-phosphoguanine (CpG) island covers most of the *ABCG2* proximal promoter (Fig. 1) (Tan et al., 2010). To date, there are no systematic evaluations of the effect of genetic variation on the activity of the *ABCG2* promoter.

Genetic polymorphisms in the proximal promoter of transporter genes have been linked to variation in gene expression (Ha Choi et al., 2009; Hesselson et al., 2009; Li et al., 2009; Yee et al., 2009). Additionally, genetic variation in promoters for transporters and enzymes have been linked to adverse drug reactions (Innocenti et al., 2004; Wang et al., 2008a; Kenna et al., 2009; McLeod et al., 2010; Toffoli et al., 2010). Previous studies of the effect of regulatory variants on ABCG2 expression (Zamber et al., 2003; Poonkuzhali et al., 2008; Eclov et al., 2017a) have only considered regions outside the primary promoter. In the present study, the basal activity of the major *ABCG2* promoter (−499 to +21 bp relative to the TSS) was investigated in transiently transfected kidney, liver, intestine, and breast cell lines. The activity of 11 variant *ABCG2* promoters was characterized in these same cell lines to identify SNPs that alter *ABCG2* promoter activity. Rare variants were included in this study because they have recently been shown to contribute significantly to individual gene expression profiles (Li et al., 2014). Variants that caused significant in vitro reductions in *ABCG2* promoter activity were also tested in the mouse hydrodynamic tail vein assay for their effect on in vivo promoter activity. Electrophoretic mobility shift assays (EMSAs) were performed on SNPs with significantly altered in vivo activity to understand how sequence affects transcription factor binding.

Materials and Methods

Chemicals and Materials. The vectors pGL4.11b [*luc2*P], pGL4.74 [*hRluc*/TK], pGL4.13 [*luc2*/SV40], and the Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). The human embryonic kidney (HEK293T/17), human colorectal carcinoma (HCT116), human hepatocellular carcinoma (HepG2), and human breast adenocarcinoma (MCF-7) cell lines were

purchased from the American Type Culture Collection (Manassas, VA). High-glucose Dulbecco's modified Eagle's medium, Opti-MEM, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Penicillin and streptomycin were purchased from the University of California San Francisco (UCSF) Cell Culture Facility (San Francisco, CA). Phusion High-Fidelity DNA Polymerase, NheI, HindIII, and DpnI, were purchased from New England BioLabs (Ipswich, MA). Fetal bovine serum (FBS) (Axenia BioLogix, Dixon, CA), GenElute HP Endotoxin-Free Maxiprep Kits (Sigma-Aldrich, St. Louis, MO), Improved Minimum Essential Medium without phenol red (Mediatech Inc., Manassas, VA), TransIT EE In Vivo Gene Delivery System (Mirus Bio, Madison, WI), CD1 mice (Charles River Laboratories, Wilmington, MA), PolyJet DNA In Vitro Transfection Reagent (SignaGen Laboratories, Rockville, MD), Odyssey EMSA Buffer Kit (LI-COR, Lincoln, NE), HepG2 Nuclear Extract (Abcam, Cambridge, MA), and PfuTurbo DNA Polymerase (Agilent Technologies, Santa Clara, CA) were purchased from the indicated manufacturers.

***ABCG2* Promoter Plasmid Construction.** A 524-bp region of the *ABCG2* promoter (chr4:89079995-89080518, hg19) was polymerase chain reaction (PCR) amplified using the forward primer 5′-TCAGGCTAGCAAGCATCCACTTTCTCAGA-3′ and reverse primer 5′-TTATAAGCTTCAGGCAGCGCTGACACGAA-3′. This region included the proximal promoter (−312 bp upstream of the TSS), adjacent transcription factor response elements, and the CpG island that extends to ~500 bp upstream of the TSS (Fig. 1) (Bailey-Dell et al., 2001). The sequences for restriction sites NheI and HindIII were added to the forward and reverse primers, respectively (underlined in the above sequences). The region was amplified from human placenta genomic DNA using PfuTurbo DNA polymerase following the manufacturer protocol. PCR conditions were 95°C for 2 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 1 minute at 68°C, then a final extension of 10 minutes at 72°C. The 542-bp PCR product was gel purified, enzyme digested, and ligated into the pGL4.11b vector. The reaction was purified and transformed into competent cells, and colonies containing the reverse promoter (forward transcription direction) orientation were isolated. DNA for pGL4.11b promoter plasmids and empty pGL4.11b, pGL4.74, and pGL4.13 vectors were isolated using the GenElute HP Endotoxin-Free Maxiprep Kit (Sigma-Aldrich) following the manufacturer protocol.

Genetic Analysis of *ABCG2* Promoter Region. SNPs in the *ABCG2* promoter region were retrieved for all available ethnic populations from publicly available databases, including 1000 Genomes (phase 1 release February 14, 2012) (Abecasis et al., 2010), dbSNP build 137, and HapMap release 28 (Frazer et al., 2007). These SNPs were combined with sequencing results from the *ABCG2* promoter region (−674 to +85 bp) of the SOPHIE cohort and reported in the Pharmacogenetics of Membrane Transporter Database (UCSF) (Hesselson et al., 2009; Kroetz et al., 2010). Haplotypes were determined by downloading genotype and information files from the 1000 Genomes browser (phase 1 release May 21, 2011) for all available ethnic groups combined and analyzed with Haploview version 4.2 (Barrett et al., 2005).

Site-Directed Mutagenesis. *ABCG2* promoter SNPs were introduced into the reference promoter plasmid using the Phusion High-Fidelity DNA Polymerase, following the manufacturer protocol. Reaction conditions for all site-directed mutagenesis primers (Supplemental Table 1) except rs139256004 are as follows: an initial cycle for 30 seconds at 98°C, followed by 20 cycles for 10 seconds at 98°C, the primer pair melting temperature for 30 seconds and 3 minutes at 72°C, then a final extension for 10 minutes at 72°C. The deletion SNP rs139256004 was introduced into the *ABCG2* promoter using a special protocol for deletion mutagenesis (Liu and Naismith, 2008). The PCR components were the same as described above with the following PCR conditions: an initial cycle at 95°C for 5 minutes; then 12 cycles at 95°C for 1 minute, 45°C for 1 minute, and 72°C for 9 minutes; with a final cycle for 1 minute at 36°C and 30 minutes at 72°C. Promoter SNP rs57327643 was also attempted via this protocol, but no colonies were isolated. The site-directed mutagenesis PCRs were digested with the DpnI enzyme, purified, and transformed into competent cells. Plasmids were isolated and sequenced to confirm the presence of the SNP. All DNA used for the in vitro and in vivo luciferase assays was endotoxin free.

Cell Culture, Transfections, and Luciferase Assays. HEK293T/17, HCT116, and HepG2 cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The MCF-7 cell line was grown in Improved Minimum Essential Medium without phenol red, supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. All cell lines were grown in a 5% CO_2 incubator at 37°C. Transient transfections were performed as previously described (Eclov et al., 2017b) and the firefly and *Renilla* luciferase activity of cell lysates were measured using the Dual-Luciferase Reporter Assay System (Promega) in a GloMax 96-Microplate Dual Injector Luminometer (Promega) following the manufacturer protocol. Each experiment included the empty pGL4.11b vector as the negative control and the pGL4.13 vector as the positive control. Promoter plasmid firefly activity was normalized to *Renilla* activity and then displayed relative to the normalized activity of empty pGL4.11b.

TABLE 1

ABCG2 promoter SNPs

Variant	Position[a]	ΔNT[b]	MAF (%)[c]				
			AFR	AMR	EAS	EUR	SAS
rs61181041	−84	C > T	NR	NR	0.01	NR	NR
rs61535534	−151	G > C	0.00	0.00	0.00	0.03	0.00
rs76656413	−169	C > T	0.00	0.00	1.00	0.00	0.00
rs57327643	−266	AGTGTTT >-	1.20	3.70	0.00	7.60	5.00
rs66664036	−267	-> G	NR	3.40	NR	NR	NR
rs139256004	−267	GTTA>-	2.80	0.10	0.00	0.00	0.00
rs2231134	−307	G > C	0.20	2.60	0.00	4.10	0.10
rs45604438	−340	G > T	6.80	0.60	0.00	0.20	0.00
rs58132660	−400	G > A	0.20	0.10	0.01	0.00	0.01
rs142365584	−424	C > G	0.00	0.10	0.00	0.00	0.0
rs59370292	−435	C > T	NR	NR	0.80	NR	NR
rs59172759	−483	T > A	5.50	0.30	0.00	0.20	3.60

AFR, African American; AMR, Mixed American; EAS, Eastern Asian; EUR, European; NR, not reported; NT, nucleotide; SAS, South Asian.

[a]SNP position is noted relative to the TSS.

[b]Nucleotide change of the reference allele to the variant allele on the anti-strand as obtained from the UCSF genome browser.

[c]MAF for AFR, AMR, EAS, EUR, and SAS reported in dbSNP release 37 by 1000 Genomes or PMT.

Hydrodynamic Tail Vein Assay. Positive in vitro variant promoter plasmids were screened for their effect on in vivo promoter activity through the hydrodynamic tail vein injection as previously described (Eclov et al., 2017b). Briefly, 10 μg of promoter plasmid or the *ApoE* (Simonet et al., 1993) positive control liver enhancer, along with 2 μg of pGL4.74 was injected into the tail vein of four to five male CD1 mice (Charles River Laboratories) weighing 21–25 g using the TransIT EE In Vivo Gene Delivery System (Mirus Bio) following the manufacturer protocol. After 24 hours, liver lysates harvested from the euthanized mice were measured for firefly and *Renilla* luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer protocol in a Synergy 2 (BioTek Instruments, Winooski, VT) microplate reader. The firefly activity in each sample was normalized to *Renilla* activity and expressed as fold activation relative to pGL4.11b. All mouse work was approved by the UCSF Institutional Animal Care and Use Committee.

Electrophoretic Mobility Shift Assay. EMSAs were performed using a 2.5 nM 5′ IRDye 700 (LI-COR)–labeled probe, incubated with 5 μg of HepG2 nuclear extract using the Odyssey EMSA Buffer Kit (LI-COR) as previously described (Eclov et al., 2017a). Competition assays were performed by adding a 40-fold molar excess of unlabeled reference oligonucleotide. DNA/protein complexes were separated from free probe by gel electrophoresis and imaged using the LI-COR system (Odyssey). Transcription factors that bound over the ABCG2 promoter and its SNPs were obtained from ChIP-seq data from the Encyclopedia of DNA Elements (ENCODE) database (ENCODE Project Consortium, 2011).

Statistical Analysis. Normalized promoter activity is expressed relative to pGL4.11b. Basal forward and reverse promoter activity in the HEK293T and HepG2 cell lines were tested for significance ($P < 0.05$) from empty pGL4.11b vector with an analysis of variance (ANOVA) followed by a Bonferroni's multiple-comparison *t* test. Basal promoter activity in the HCT116 and MCF-7 cell lines was tested for significance ($P < 0.05$) from the empty pGL4.11b vector with a Student's *t* test. Variant *ABCG2* promoter sequences selected for in vivo testing were significantly different ($P < 0.05$) from the reference promoter in three of four cell lines. Variant promoter plasmids tested in vitro or in vivo were tested for significance ($P < 0.05$) from the reference *ABCG2* promoter with an ANOVA followed by a Bonferroni's multiple-comparison *t* test. The reference promoter and the *ApoE* enhancer were tested for difference from the empty vector sequence in vivo using an unpaired Student's *t* test. All statistics were run using the GraphPad Prism 5 program (GraphPad Software, San Diego, CA).

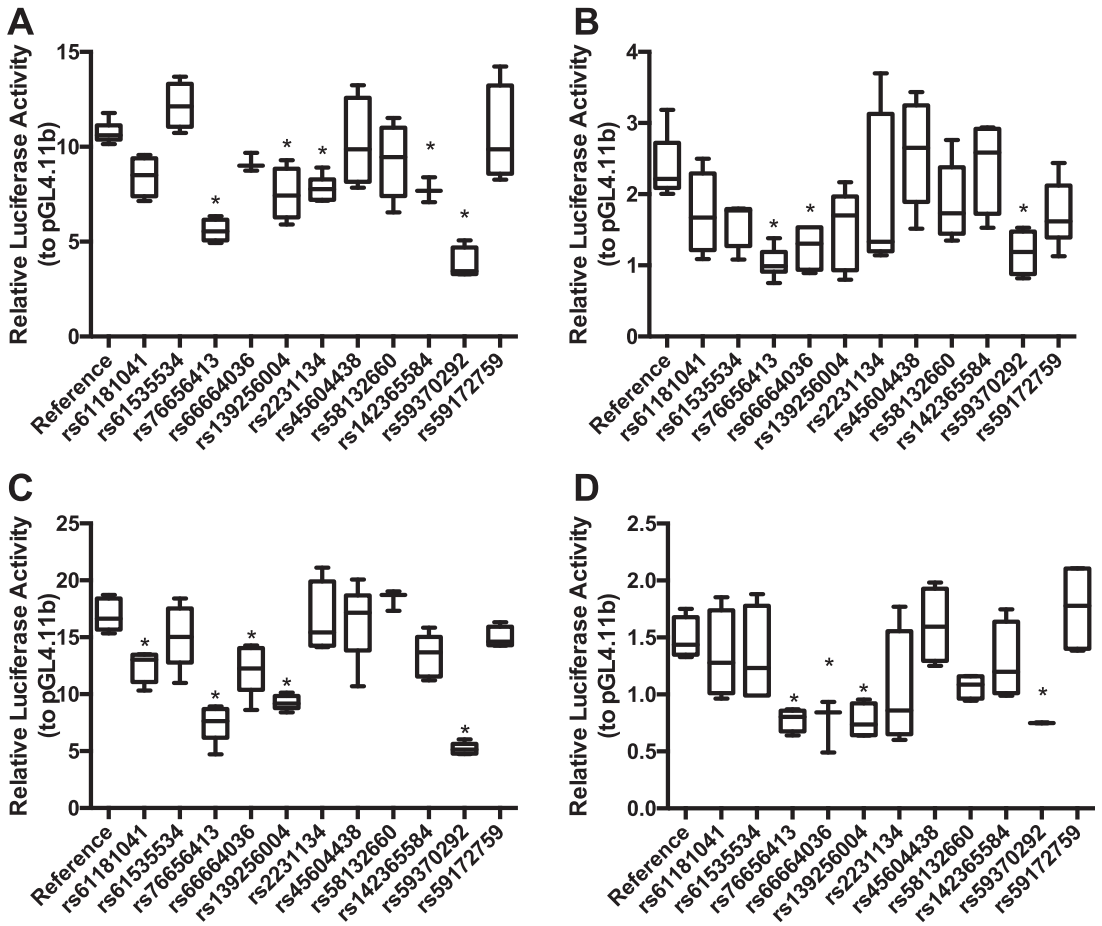

Fig. 2. Effect of promoter variants in vitro. Luciferase assay of *ABCG2* reference and variant promoter sequences was measured in transiently transfected kidney (HEK293T) (A), liver (HepG2) (B), intestine (HCT116) (C), and breast (MCF-7) (D) cell lines. Promoter activity is expressed as the ratio of firefly to *Renilla* luciferase activity normalized to empty pGL4.11b. Data are expressed as box-and-whisker plots of mean values from multiple experiments ($N = 4$–8 biologic replicates with three to six wells per construct). Differences between reference and variant promoter constructs were tested by an ANOVA followed by a post hoc Bonferroni multiple-comparison *t* test: $^*P < 0.05$.

Results

Genetic Polymorphisms of the *ABCG2* Promoter. Twelve variants (Table 1) were obtained for the *ABCG2* promoter region from publicly available databases and from sequencing of the SOPHIE cohort. Of the 12 variants, rs57327643, rs2231134, rs45604438, and rs59172759 had a minor allele frequency (MAF) above 4% in at least one ethnic population (Table 1). The *ABCG2* promoter variants included the single nucleotide insertion rs66664036 and two multiple–base pair deletions, rs57327643 and rs139256004. Attempts to construct the rs57327643 variant promoter plasmid were unsuccessful, and this variant was not evaluated in the functional assays. There was no notable linkage disequilibrium between variants in the *ABCG2* promoter.

Activity of the *ABCG2* Promoter In Vitro. The activity of the *ABCG2* promoter sequence (chr4:89079947-89080567, hg19), cloned into the firefly luciferase reporter vector pGL4.11b, was investigated in transiently transfected HEK293T, HepG2, HCT116, and MCF-7 cell lines (Fig. 2). This region includes the basal promoter of *ABCG2* and the structural elements displayed in Fig. 1. The reverse *ABCG2* promoter activity was strongest in HEK293T and HCT116 cells, with average activation of 11-fold and 17-fold, respectively; in the HepG2 and MCF-7 cells promoter activity was weaker, with average activation of 2.4-fold and 1.5-fold, respectively (Fig. 2).

Variant *ABCG2* Promoter Activity In Vitro. The effect of 11 variants (Table 1) on the basal *ABCG2* promoter activity was investigated in all four cell lines. Five variants had significantly decreased activity in HEK293T cells; two of them (rs59370292 and rs76656413) had over a 50% decrease in promoter activity ($P < 0.05$, Fig. 2A). Of the three variants with decreased activity in HepG2, only rs76656413 had over a 50% decrease ($P < 0.05$, Fig. 2B). In HCT116 cells, five variants had decreased activity, including a 50% decrease with rs76656413 and rs139256004 and >75% decrease with rs59370292 ($P < 0.05$) (Fig. 2C). All four variants with significant decrease in activity in MCF-7 cells, (rs76656413, rs66664036, rs139256004, and rs59370292) had at least a 50% decreased activity ($P > 0.05$, Fig. 2D). The rs66664036 and rs139256004 variants both had 25%–50% decreased activity in three of the four cell lines and the rs76656413 SNP had over a 50% decreased activity in all four of the cell lines. The rs59370292 SNP was the most detrimental variant in both HEK293T and HCT116 cell lines with an almost 75% decreased activity; it also had 25%–50% decreased activity in HepG2 and MCF-7 cells. Because of their decreased promoter activity in at least three of four cell lines, rs66664036, rs139256004, rs76656413, and rs59370292 were chosen for follow-up in the in vivo hydrodynamic tail vein injection assay.

Variant *ABCG2* Promoter Activity In Vivo. Four variants (two SNPs, one single-base insertion, and a four-base deletion) were screened for their effect on in vivo *ABCG2* promoter activity using the hydrodynamic tail vein injection assay. In this assay, the *ApoE* liver enhancer (Simonet et al., 1993) positive control had more than 200-fold

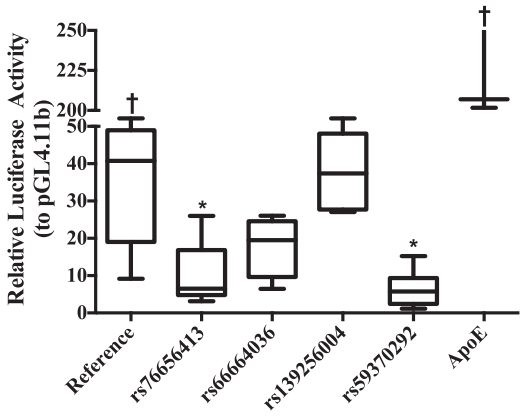

Fig. 3. Effect of promoter variants in vivo. The luciferase activity in mouse liver homogenates was measured 24 hours after plasmid injection. Promoter activity is expressed as the ratio of firefly to *Renilla* luciferase activity normalized to empty pGL4.11b activity. An enhancer for *ApoE* was used as the positive control (Simonet et al., 1993). Data are expressed as box-and-whisker plots for four to five mice. Differences between reference promoter or *ApoE* and pGL4.11b were tested by an unpaired Student's *t* test, †$P < 0.05$; reference and variant promoter sequences were compared using a one-way ANOVA followed by a Bonferroni's multiple-comparison *t* test, *$P < 0.05$.

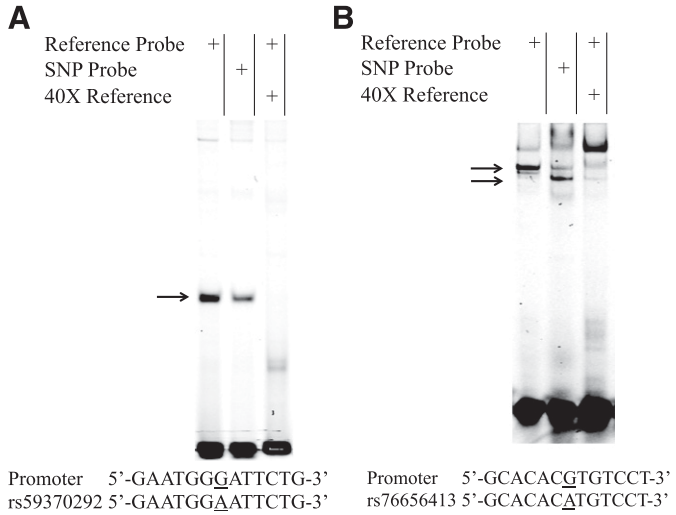

Fig. 4. Effect of rs76656413 and rs59370292 on DNA-protein binding. Representative electrophoretic mobility shift assay using HepG2 nuclear extracts incubated with IRDye 700–labeled probes for reference and rs76656413 (A) or rs59370292 (B) sequences. Competition assays were performed with 40-fold excess unlabeled oligonucleotides, with arrows indicating specific DNA/protein bands. Reference and variant DNA sequences surrounding each nucleotide mutation (underlined) are shown below their respective gel.

increase in activation over empty vector. The *ABCG2* promoter plasmid exhibited a strong 35-fold activation over pGL4.11b. Two of the four promoter variants significantly decreased promoter activity in vivo ($P < 0.05$, Fig. 3). The rs59370292 SNP decreased promoter activity over 80%, whereas rs76656413 resulted in a 70% decrease in promoter activity in vivo.

Variant ABCG2 Promoter DNA Binding to Nuclear Protein. The *ABCG2* variants rs59370292 and rs76656413 were tested for alteration in binding to nuclear proteins via EMSA. Reference DNA probes at these SNP locations showed strong HepG2 nuclear protein binding with specific DNA/protein bands susceptible to competition by unlabeled oligonucleotide probes (Fig. 4). The rs59370292 SNP showed reduced binding to HepG2 nuclear proteins compared with its reference DNA sequence (Fig. 4A). The rs76656413 SNP lost the specific DNA/protein binding of the reference sequence, but gained a separate DNA/protein binding interaction (Fig. 4B).

Discussion

Previous research has highlighted the role of the *ABCG2* promoter in the regulation of BCRP expression (Robey et al., 2009). Although the *ABCG2* proximal promoter has many transcription factor response elements, variants within the *ABCG2* promoter have not yet been associated with its mRNA levels. In this study, the activity of the *ABCG2* promoter and the effects of variants on that activity were investigated in liver, kidney, intestine, and breast cell lines. Variants with a consistent in vitro effect on ABCG2 promoter activity were tested for their effect in the in vivo mouse tail vein assay. The *ABCG2* promoter was highly active in intestinal and kidney cell lines, and had medium activity in liver and low activity in breast cell lines. Despite modest in vitro liver activity, the *ABCG2* promoter had strong in vivo liver activity. These results correlate with high expression of ABCG2 in intestine and liver and more moderate expression of ABCG2 in kidney (Maliepaard et al., 2001). In contrast, the low promoter activity in the MCF-7 cells is inconsistent with the high expression of ABCG2 in breast tissue (Maliepaard et al., 2001).

In earlier work, luciferase assays on the −628/+362 *ABCG2* promoter segment indicated suppressed activity in MCF-7 cells, whereas the −312/+362 promoter segment was highly active. It is possible that a suppressor element within the −499 to −312 segment reduces the activity of our *ABCG2* promoter construct in MCF-7 cells (Bailey-Dell et al., 2001). An interferon-gamma activated sequence at −448/−422 has been shown to increase the *ABCG2* promoter activity upon stimulation of the Janus kinase 2/signal transducer and activator of transcription 5 (STAT5) pathway by prolactin (Wu et al., 2013). STAT5 is well documented for its importance in regulating the expression of genes essential for mammary development and lactogenesis (Watson, 2001). Naturally occurring dominant-negative isoforms of STAT5 have also been shown to suppress the transcriptional activity of the estrogen receptor in MCF-7 cells (Yamashita et al., 2003). Previous research has shown discordant results in the ability of the *ABCG2* promoter to be upregulated in MCF-7 cells when treated with 17β-estradiol (Imai et al., 2005; Yasuda et al., 2009). Therefore, it is possible that without stimulation of the STAT5 pathways, there is a suppressive factor bound between −499 and −312 of the *ABCG2* promoter in MCF-7 cells that inhibits promoter activity. This indicates a fragile and complex network of transcription factors that bind to the *ABCG2* promoter and regulate its expression in a cell/tissue contextual manner.

Two of the *ABCG2* promoter variants (rs76656413 and 59370292) decrease the hepatic activity of the *ABCG2* promoter in vitro and in vivo. A third variant, rs66664036, significantly decreased in vitro promoter activity in a hepatic cell line and showed an almost 50% decrease in in vivo hepatic promoter activity that did not reach significance. Similar to in vitro and in vivo analysis of enhancer variants in *ABCG2* and other pharmacogenes (Kim et al., 2011; Eclov et al., 2017a,b), there was not a complete concordance between promoter assays in cell lines and results from the in vivo assay. Discordance between in vitro and in vivo results might reflect differences between human and murine transcription factors, and highlights one of the limitations of the tail vein assay. This assay is also restricted to analysis of only hepatic transcriptional activity, and additional studies are needed to determine whether variants that alter in vitro *ABCG2* promoter activity in renal, intestinal, and breast cell lines affect in vivo ABCG2 expression in those tissues.

Three of four variants in the *ABCG2* promoter that altered promoter activity in vitro have reported low minor allele frequencies. This is in concordance with a large analysis of ABC and SLC (solute carrier) gene

promoter variation that found the proximal promoters of these gene families had low nucleotide diversity (Hesselson et al., 2009) and global genome analysis showing enrichment in promoters for rare variants (Li et al., 2017). Due to the low frequency of these variants, it is difficult to correlate them with the expression of ABCG2 and, thus, whether they contribute significantly to population variability in ABCG2 expression cannot be determined. Nonetheless, consistent with similar global rare variant observations, these rare variants could have large effects on expression within individuals (Li et al., 2017). Further studies are needed to examine in more detail the association of these variants with the expression of ABCG2 and the function of the BCRP transporter in both the liver and extrahepatic tissues important for xenobiotic disposition.

The *ABCG2* promoter SNP rs76656413 had strong evidence for altering the transcriptional activity of the *ABCG2* promoter. It attenuated the relative luciferase activity of the *ABCG2* promoter by 50% in all four cells lines and decreased *ABCG2* liver promoter activity by 70% in vivo. Transcription factor binding site analysis predicted rs76656413 to have significant losses in upstream stimulatory factor-1 (USF-1), n-Myc, myc-associated factor X (Max), and Myc-Max binding (data not shown) that is consistent with its location in the middle of several USF-1 and c-Myc ChIP-seq peaks reported by ENCODE. Not only does the SNP fall in the middle of ChIP-Seq peaks, it is directly within a canonical motif for both Myc and Max. Additionally, c-Myc and Max have been reported to direct the transcriptional regulation of *ABC* genes, particularly the unmethylated *ABCG2* promoter, in human leukemic hematopoietic progenitor cells (Porro et al., 2011). Furthermore, the expression of ABCG2 is altered by the overexpression of c-Myc in human breast epithelial cells (Kang et al., 2009). Competition assays and transcription factor supershift experiments based on the predicted binding of transcription factors did not reveal changes in the binding of specific transcription factors by the rs76656413 SNP. Further studies are needed to confirm that USF-1, Max, and c-Myc transcription factors bind to the *ABCG2* promoter and that rs76656413 changes USF-1, Max, and/or Myc binding, thus altering the transcriptional activity of the *ABCG2* promoter.

The *ABCG2* promoter SNP rs59370292, located just upstream of the ARE, has the lowest reported MAF of the four variants that alter in vitro *ABCG2* promoter activity. It altered the relative luciferase activity of the *ABCG2* promoter in three of four cell lines and had the largest effect of any *ABCG2* promoter variant in vivo, decreasing the promoter activity by 80%. EMSA showed reduced binding of nuclear liver extract to the rs59370292 mutated probe. The transcription factor predicted to have the largest reduced binding due to rs59370292 is the vitamin D receptor (VDR; data not shown). Although VDR has not been directly linked to ABCG2 expression, VDR is important in regulating bile acid transporters, and its ligands include bile acid derivatives and steroids (Germain et al., 2006; Halilbasic et al., 2013). Since *ABCG2* encodes a bile acid transporter (Blazquez et al., 2012) and has been shown to be important for the pharmacokinetics and pharmacodynamics of statins (Generaux et al., 2011), VDR could be the link for statin regulation of ABCG2 expression. However, supershift or competition EMSAs were not able to demonstrate a role for VDR, and further studies are needed to determine whether VDR binding to the proximal promoter of *ABCG2* is involved in *ABCG2* transcription.

In summary, the *ABCG2* promoter (−499 to +21) had strong activity in HCT116 and HEK293T cell lines and in vivo in the liver. The rs76656413 and rs59370292 SNPs within the basal promoter of *ABCG2* affect its function both in vitro and in vivo. We found these SNPs to have altered transcription factor binding through EMSAs. Although their low allele frequencies limit their impact on the population-level expression of ABCG2, these and other rare variants in *ABCG2* could be important for regulating expression in individual patients.

Authorship Contributions

Participated in research design: Eclov, Kim, Smith, Ahituv, and Kroetz.

Conducted experiments: Eclov, Kim, and Smith.

Performed data analysis: Eclov, Kim, Ahituv, and Kroetz.

Wrote or contributed to the writing of the manuscript: Eclov, Kim, Smith, Ahituv, and Kroetz.

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Address correspondence to: Deanna L. Kroetz, University of California at San Francisco, 1550 4th Street RH584E, San Francisco, CA 94158-2911. E-mail: deanna.kroetz@ucsf.edu