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Genetic Determinants of *ABCB1* Promoter Activity and Their Effects on Environmental Exposure

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Genetic Determinants of *ABCB1* Promoter Activity and Their Effects on Environmental Exposure

by

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Dedicated to my wife and partner, Bethany Jo Baker, and to my family. This incredible journey would have been much more difficult without their love and support.

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Genetic Factors of *ABCB1* Promoter Alter its Activity and Response to Environmental Toxins

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Abstract

In utero exposure to xenobiotics, including medications and environmental agents, can lead to developmental and behavioral problems as well as cancer later in life. Efflux transporters, which are highly expressed in the human placenta, are key regulators of placental xenobiotic transfer from the maternal to the fetal circulation. A major placental efflux transporter is P-gp, which is encoded by the *ABCB1* gene. Many medications prescribed to pregnant women, such as antibiotics for acute illness or medications used to treat chronic diseases including asthma and diabetes, are P-gp substrates. Variability in placental P-gp expression and activity could, therefore, pose a challenge to physicians as it could significantly impact maternal and fetal exposure to medications that are P-gp substrates. There are many SNPs in the *ABCB1* promoter but their effect on *ABCB1* transcription and subsequent P-gp expression remains unclear. In the genome, SNPs seldom exist as independent variants, but rather form specific combinations or haplotypes due to linkage disequilibrium (correlation) between them. Importantly, recent data from our laboratory indicates the phenotypic effects of individual SNPs are not always

consistent but instead are haplotype dependent. The data generated from the studies herein offer important information on the role of genetic variability on the activity of the *ABCB1* promoter. These studies provide a detailed sequence information for the *ABCB1* promoter haplotypes in a mixed ethnic/racial population and demonstrate the effects from an individual SNP are not always consistent but differ in a haplotype-specific manner. One potential mechanism driving the promoter activity was explored, and we determined that transcription factors bind to the *ABCB1* promoter in a haplotype-dependent manner. Finally, it was identified that *ABCB1* promoter activity in response to both acute and chronic bisphenol exposures is haplotype-dependent. This information clearly demonstrates that *ABCB1* haplotypes, rather than individual SNPs, affect its promoter activity and could thus play a significant role in the expression of placental P-gp, ultimately having significant public health implications, particularly for pregnant women treated with P-gp-substrate medications.

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ABCB1;	ATP-binding cassette family, subfamily B member 1
ATP;	adenosine triphosphate
BPA;	Bisphenol A, 4,4'-(propane-2,2-diyl)diphenol
BPS;	Bisphenol S, 4,4'-Sulfonyldiphenol
DNA;	deoxyribonucleic acid
LD;	linkage disequilibrium
MDR1;	multiple-drug resistance 1
MRP;	multidrug resistance-associated protein
P-gp;	p-glycoprotein
PCR;	polymerase chain reaction
RLU;	relative light units
RNA;	ribonucleic acid
siRNA;	small interfering RNA
SNP;	single nucleotide polymorphism
TF;	transcription factor

**Abbreviations in italics are genes, i.e.; *ABCB1* is the gene encoding the protein P-gp.

Chapter 1: INTRODUCTION

General Background

Long before we understood what DNA was, and that each organism contained its own unique sequence of genetic information, there was an understanding that something must transfer information from one generation to the next. The first observations into heredity were published in the mid-19th century when Darwin and Wallace identified directed variability in natural selection (Darwin and Wallace, 1858). Later, the Austrian monk Gregor Mendel published his findings on dominant and recessive traits in pea plants (Mendel, 1865). In *S. pneumoniae* in the 1940s, DNA was identified as the genetic material (Avery et al., 1944). Later, with the assistance of Rosalind Franklin's data from X-ray crystallography scattering data, Watson and Crick first solved the double-helical structure of DNA (Watson and Crick, 1953). With the identification that DNA contained all of the information necessary to create the proteins necessary for an entire organism, it became a widely-held belief among geneticists that once the genome was sequenced, all of the question about what makes us human would be answered.

The first look into the entire genome came at the beginning of the 21st century, when the first human genome was sequenced (Venter et al., 2001). Unfortunately, the sequence alone did not provide all of the answers geneticists had hoped. Instead, the sequence has opened many new areas of study including epigenetics, gene-splicing variation, RNA biology, and numerous others. By sequencing more partial and complete genomes, scientists were able to identify single nucleotide polymorphisms (SNPs), variations in a nucleotide occurring in greater than 1% of the population, that were enriched within certain populations. These subpopulations of people could then be studied, identifying genetic factors that lead these groups to be either susceptible or resistant to disease, pharmaceuticals or environmental exposure. This has led to further understanding of the roles of both the biological and environmental factors in disease risk. By combining the inherent genetic properties as well as environmental factors

(gene-environment interactions), scientists and clinicians are better able to identify an individual's or a population's risk for disease or potential response to therapy.

Single Nucleotide Polymorphisms and Haplotypes

Single nucleotide polymorphisms (SNPs) are variations in one adenine (A), thymine (T), guanine (G), or cytosine (C) at one location within the DNA, occurring at a frequency of 1% or greater in the population. SNPs can occur in many locations in the genome, and often, multiple SNPs will be found within each gene. If a SNP occurs in an exon, or coding region of the DNA, similarly to a mutation, a SNP can: be a silent SNP, where no amino acids were changed or the amino acid substitution causes no alteration is protein structure or function, or the SNP can change an amino acid that can cause an increase or decrease in protein function. There are several SNPs that occur in the coding region of *ABCB1*, of which, C3435T and G2677T/A are the most studied. However, there are discrepancies regarding the effects of these SNPs on the expression and/or the activity of P-gp (Hemauer et al., 2010; Hitzl et al., 2004; Salama et al., 2006; Xuan et al., 2014).

In addition to SNPs in the coding regions of genes, SNPs are also found in non-coding regions of genes, including the promoters, introns (non-coding regions between exons), in the 5'-untranslated region before the gene, and in the 3'-untranslated region after a gene. In the promoters of different genes, SNPs have been shown to cause no change, increases, or decreases in gene expression (Faniello et al., 2006; Kim et al., 2006; Palikhe et al., 2017; Rouault et al., 2011). The conflicting results between the studies are not surprising because SNPs rarely occur individually, but rather, with various degrees of linkage disequilibrium (LD) with one or more other SNPs, form defined haplotypes. In population genetics, LD is the non-random association of alleles at different loci.

In some instances, results from multiple studies evaluating the same promoter lead to controversial results. As with coding SNPs, different studies examining the same promoter SNPs have shown an increase, decreases, and no effect on expression (Lourenço et al., 2008; Takane et al., 2004; Tanabe et al., 2001). This irreproducibility

between studies is likely not due to experimental error but most likely a result of the SNPs being present in haplotypes, where another SNP or SNPs may be driving the phenotype, masking the effect of the SNP when evaluated individually. Thus, promoter haplotypes can modify expression relative to individual SNPs through different/multiple mechanisms including alteration of transcription factor binding sites and/or alteration of methylation patterns in the promoter region. By employing haplotypes rather than individual SNPs, trends can be observed which could potentially explain the discrepancies between studies.

The Placenta

There are many important physiological barriers in the human body including: the intestines, kidneys, blood-brain-barrier and, during fetal development, the placenta. The placenta is a temporary organ, found only in mammals, which is used to transfer nutrients from the maternal circulation to the developing fetus, eliminate waste from the fetus, and regulate hormones to maintain the pregnancy. The outer, maternal side of the placenta is made of a layer of the uterine endometrium called the decidua basalis. The fetal placenta begins to form early after implantation when the outside of the blastocyst develops into the trophoblast, forming the fetal side of the placenta. The trophoblasts secrete enzymes that break down the layer of the uterus outside of the decidua basalis, the stratum spongiosum. Here the cells tap into the circulatory system of the uterus drawing blood from the maternal spiral arteries and returning it to the uterine veins (Rai and Cross, 2014).

Between the spiral arteries and uterine veins, the blood flows into a newly formed cavity, the intervillus space, where the fetal trophoblasts form the outer-layer of the placental villi. The layer of the trophoblasts in contact with the maternal blood fuses to form a large, multinucleated syncytiotrophoblast. To maximize the surface area for nutrient and gas exchange, the apical membrane of the syncytiotrophoblast forms a brush border with long, finger-like projections. This syncytiotrophoblast allows for passive

diffusion and also has numerous proteins to facilitate active transport of substrates into and out of the cells. These transporters include organic cation and anion transporters, solute carrier transporters, multiple resistance proteins, and many others (Iqbal et al., 2012; Joshi et al., 2016). These transporters are generally broken down into two groups, dependent on functional direction of substrate transport; if the substrate is moved into the cell it is an influx transporter; if the substrate is moved out of the cell it is an efflux transporter (Ganapathy and Prasad, 2005).

Efflux Transporters

Efflux transporters play a key role in the mechanism by which physiological barriers prevent the transfer of molecules from crossing. One of the largest families of efflux transporters is the ATP-Binding Cassette family. This family includes P-glycoprotein, breast cancer resistance protein (BCRP), the multidrug resistance proteins 1-9 (MRP 1-9), and many others. These ATP-dependent efflux transporters are responsible for a decrease in the intracellular concentration of their substrates. These transporters are very promiscuous, meaning that each one will have multiple substrates, and often, multiple transporters will have the same substrate, although at differing concentrations. Though each transporter has multiple substrates, they each tend to transport substrates with certain characteristics e.g., cations, anions, hydrophobicity (Joshi et al., 2016).

Efflux transporters are found on both the apical and basal membranes of cells, and many of the same transporters are found on either the basal or apical membrane in a tissue dependent manner, e.g. MRP4 and MRP5 (encoded by the *ABCC4* and *ABCC5* genes, respectively) are found on the apical membrane of brain endothelial cells and on the basolateral membrane of hepatocytes (Borst et al., 2007; Nies et al., 2004). Although they are on the opposing membranes, their function remains the same: in the brain capillary endothelium, these transporters work to keep the majority of their substrates in the blood and out of the brain, similarly, in the hepatocyte, the efflux transporters work to

transport their substrates out of the hepatocytes, into the biliary ducts for elimination from the body. Of all of the transporters studied, the first of these efflux transporters found, P-gp, has been the most thoroughly studied (Ambudkar et al., 2003).

P-Glycoprotein

P-gp was first isolated in 1976 from colchicine-resistant Chinese hamster ovaries, where it was named "P" glycoprotein because it was a glycoprotein that affected colchicine's membrane permeability (Juliano and Ling, 1976). A few years later, the homologous human protein was identified in vinblastine-resistant lymphoblasts (Kartner et al., 1983). As shown in Figure 1A, the 170kDa protein has two transmembrane and two nucleotide-binding domains (Jin et al., 2012). Each transmembrane region is comprised of six transmembrane helices. It is thought that upon ATP-binding, there is a scissor-like motion of the two transmembrane regions which actively moves the substrate from inside of the cell, or the cell membrane, extracellularly (Rosenberg et al., 2003). The crystal structure of P-gp from *c. elegans* (Figure 1B) depicts this conformational change in the transmembrane domains after nucleotide binding. Like other ABC family transport proteins, P-glycoprotein is a very promiscuous. However, P-gp preferentially transports substrates that are hydrophilic, cations including numerous pharmaceuticals and endogenous compounds like polysaccharides (Wang et al., 2003; Zhou, 2008). Though it was identified as the cause for drug-resistance in both human and other mammalian cell lines, because P-gp has numerous other substrates and is one of the major efflux transporters in the placenta, it is thought to be one of the major defenses protecting the fetus from exposure to numerous xenobiotics.

Role of P-gp in Placental Therapeutics

In the placenta, P-gp, along with other members of the ABC family of proteins, is expressed early in pregnancy and continues to have high expression for the duration of the pregnancy (Gil et al., 2005). This high expression is very important as these efflux

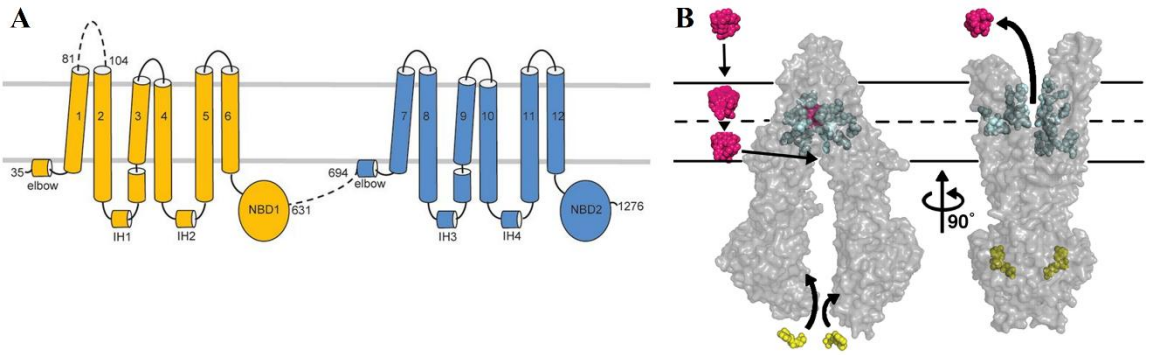


Figure 1: Structure of P-glycoprotein. P-gp structure depicted in **a)** the domain form showing its two nucleotide (ATP) binding domains and 12 transmembrane helices, and **b)** space filling model with substrate (red) interacting with inward facing P-gp drug binding pocket (cyan) and being effluxed out of membrane with a P-gp conformation change to the outward facing form after the binding of two ATP molecules (yellow). Reproduced with permissions (Appendix 1).

transporters play a major role in the regulation, transport and metabolism of xenobiotics and hormones which reach the fetus (Ceckova-Novotna et al., 2006). To this end, a study has demonstrated an inverse correlation between enterocyte P-gp protein expression and the area under the plasma drug concentration-time curve (AUC) for Digoxin (Greiner et al., 1999). Currently, there are numerous known pharmaceuticals that are substrates, inhibitors and/or inducers of P-gp (Kim, 2002). A thorough understanding of the dynamic regulation of the expression of the *ABCB1* gene, which encodes P-gp, is therefore necessary to further evaluate the safety of pharmacotherapy for pregnant mothers as well as potentially for required transplacental therapy for the developing fetus.

Transcription Regulation

The first insights into transcriptional regulation were discovered in bacteria in the 1960s (Jacob and Monod, 1961). The benefit of using bacteria to identify the regulatory agents of transcription is that eukaryotic genomes have much more DNA than prokaryotes, resulting in much more non-coding DNA than coding DNA. However, this abundance of DNA allows for many eukaryotes to have much more regulation of gene

expression (Alberts, 2014). To regulate gene expression, there are two primary regulatory components: the cis-acting elements (DNA sequences) and the trans-acting elements (proteins i.e. transcription factors). These components have been extensively studied and many transcription factors, co-factors, and their DNA cis-elements have been identified (Fuda et al., 2009; Roeder, 1998, 2005; Spitz and Furlong, 2012).

The cis-element is primarily made up of two regions: the proximal region which contains the promoter and other regulatory elements located ~1kb around the transcription start site, and the distal elements comprised of enhancers, silencers, and other regulatory elements which can be located both upstream and downstream of the proximal region and can be many kb away. The promoter contains the RNA polymerase binding site, the transcription start site (TSS), as well as binding sites for transcription regulating proteins that define when and where RNA polymerases may bind and commence mRNA transcription. One common cis-element is the TATA box (5'-TATAAA-3') which is located 25-35 bases upstream of the TSS in many human genes.

In addition to these core elements, many other cis-elements are present for induction, repression and other types of regulation. Some of these DNA regulatory regions are common in multiple genes, having the same ligand induce/repress them. For instance, estrogens are able to regulate numerous genes through their canonical pathway by binding to receptors on the membrane or in the cytosol. After binding, the receptor travels to the nucleus and binds to the cis-acting estrogen response element sequence in the DNA. There are several of these "response elements" which have one common ligand regulating tens or hundreds of genes including DNA damage response elements, the vitamin-D response element, etc.

On top of response elements, there are other cis- and trans-factors which regulate transcriptional activation or repression. One example of these types of transcription regulatory proteins are the specificity proteins 1 and 3 (Sp1, Sp3). These transcription factors bind to GC-rich regions in the DNA called the GC-box, and have been shown to have an important role in embryonic and early postnatal development (Zhao and Meng,

2005). Within a promoter and enhancer, the cis- and trans-factors as well as the response elements work in concert to regulate the expression of a gene.

ABCB1 Transcriptional Regulation and SNPs

The gene encoding P-gp has two common nomenclatures: *ABCB1* (ATP-binding cassette subfamily B member 1) and *MDR1* (multiple drug-resistance protein 1). The 210kb *ABCB1* gene locus is on the reverse strand of the long arm of chromosome seven (7q21.1). The gene contains 29 exons, of which two are fully or partially noncoding, and two promoters (Figure 2). The distal promoter is located 112k bases upstream of exon 1. Transcription from the distal promoter appears to only occur in diseased states such as cancer (Raguz et al., 2004; Rothenberg et al., 1989). The proximal promoter is the primary promoter used to drive *ABCB1* expression in most non-diseased cells and is located between the second and third exons.

Regulation of the proximal promoter has been well-studied, and numerous regulatory regions have been identified. Important regulatory transcription factors include Sp1, AP-1 and p53 (Reviewed in Labialle et al., 2002). Sp1 was demonstrated to bind to

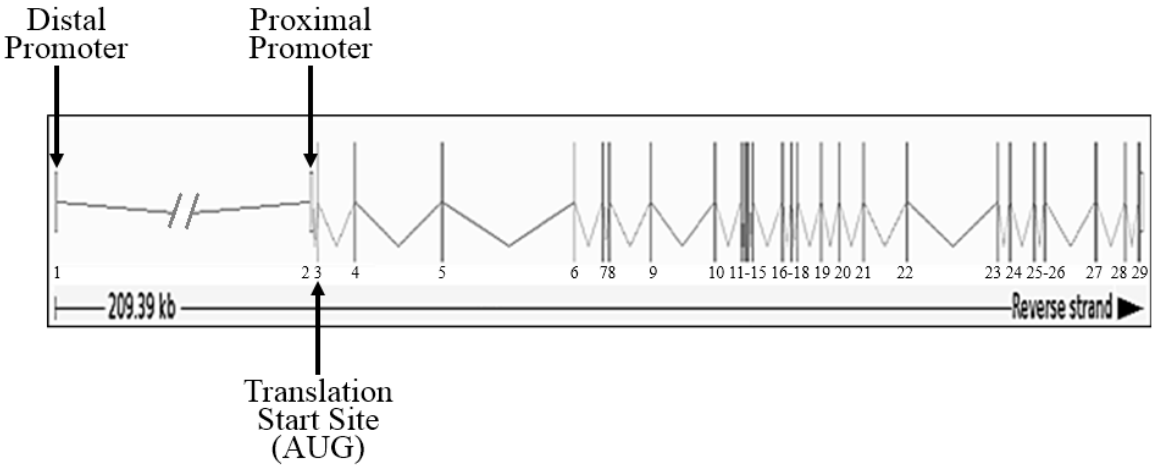


Figure 2: Map of *ABCB1* gene. Map showing the 209.39 kb *ABCB1* gene. The distal promoter is 115 kb upstream of the proximal promoter. The proximal promoter is in the second exon, and the translation start site is located partway through the 3rd exon.

two different regions in the *ABCB1* promoter. Depending on the binding site, Sp1 had either a transcriptional-activation or repression role (Cornwell and Smith, 1993). The promoter also contains both estrogen-response and heat-shock elements.

To date, over 300 SNPs have been identified in *ABCB1* but only a few have been extensively studied. Studies on these SNPs have been mainly focused in the coding region, and primarily on the C1236T, C3435T, and G2677T/A SNPs. These SNPs have been evaluated in relation to P-gp expression and transport activity with considerable controversy about their functional significance. In addition to these coding SNPs, there are many others in the promoter region which could affect the expression of *ABCB1* and thus P-gp. A few studies have evaluated some of these promoter SNPs focusing on the Japanese population, with conflicting results as to the impact of the SNPs on *ABCB1* transcription (Ito et al., 2001; Sai et al., 2006, 2010; Takane et al., 2004; Tanabe et al., 2001).

There have been numerous studies concerning the transcriptional regulation of the *ABCB1* gene. This regulation involves many factors including both intrinsic and inducible transcription factors, underlying genetic modifications such as SNPs, and promoter silencing or activation via promoter methylation, and histone modifications (Labialle et al., 2002; Lourenço et al., 2008; Prins et al., 2008).

Gene Environment Interactions

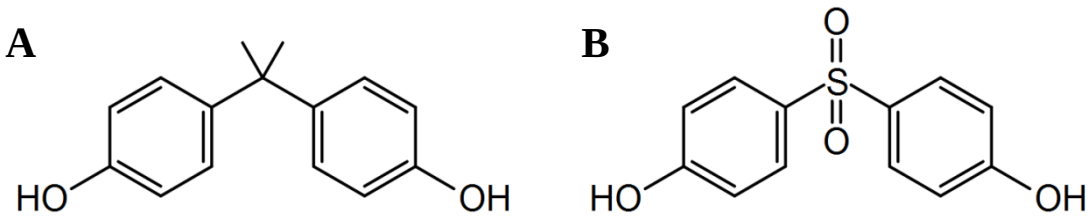
Anthropogenic activities have had many impacts in all aspects of our environment including the air, water and land. Air quality is primarily a concern in large cities or industrial areas. Although air quality standards have greatly improved the air quality since the 1980s, there are still numerous “ozone days” where the ozone levels are above the recommended levels (Cooper et al., 2012). Waterways inside and outside of the

United States contain measurable amounts of pharmaceuticals, wastewater treatment byproducts, fertilizers, pesticides, etc. (Kostich et al., 2014; Sousa et al., 2017). Persistent organic pollutants including polychlorinated biphenyls have severely contaminated the ground, have permeated the food chain, and are found in measurable amounts in almost every person (Bae et al., 2018). Environmental conditions include many other important considerations beyond the air we breathe and the water we drink: it is also imperative that diet, fitness, stress, pharmaceutical use and other toxin exposure like tobacco consumption are considered part of a person's environment.

When the consequences of genetic variation depend upon environmental exposure, this is called a gene-environment interaction (Comstock and Moll, 1963). With some instances of gene-environment interaction, there are strongly significant differences in response to environmental exposures between individuals. For example, workers who have genetic variants for low microsomal epoxide hydrolase (mEH) activity, and are exposed to 1,3-Butadiene have increases in mutation frequencies compared with subjects with wild type mEH (Abdel-Rahman et al., 2003). With renal cell carcinomas, it has been observed that alcohol exposure decreases the risk of renal cell carcinoma, but the degree of the reduction is determined by allelic variation (Antwi et al., 2018). There have been numerous gene-environment interactions that have been identified, but with the large number of xenobiotics in the environment, human exposure to compounds over extended periods of time often results in the identification of a genetically susceptible subpopulation presenting a diseased state. The bisphenol family of compounds is an example of a group of xenobiotics that is being actively researched for its potential effects.

Bisphenols and Human Exposure

Bisphenols are a group of compounds which contain two phenol groups (two hydroxyphenyl groups). The nomenclature for the differing bisphenols comes from the name of one of the reactants used to generate the bisphenol, i.e. bisphenol A is a product



Bisphenol A (BPA) Bisphenol S (BPS)

of phenol and acetone, and bisphenol F is a product of phenol and formaldehyde. These compounds have been synthesized for a long time. In fact, the most well-known of the

Figure 3: Chemical structures of A) bisphenol A and B) bisphenol S.

bisphenols, bisphenol A [4,4'-(propane-2,2-diyl)diphenol, BPA, Figure 3A], was first synthesized by A.P. Dianin in 1891. It is a white, crystalline solid that when polymerized, forms the very rigid and strong plastic polycarbonate. Today, the majority of BPA is used in the manufacture of polycarbonate plastics and epoxy resins (Flint et al., 2012). BPA is very highly produced chemical, with a current annual global consumption over 7 million tons, and demand increasing annually (Industry Experts, 2016; Vandenberg et al., 2007). Even though BPA is the most ubiquitous of the bisphenols, other bisphenols are becoming more prevalent including Bisphenol S (4,4'-Sulfonyldiphenol, BPS, Figure 3B), and are being produced in large quantities leading to their presence in many of the consumer goods in which BPA is found.

Humans come into contact with BPA and BPS multiple times per day by three main routes of exposure: oral ingestion, inhalation, and dermal exposure. For most people the majority of exposure to bisphenols is through dietary intake, but additional exposure occurs through dermal contact, as well as from ambient air and dust where BPA has also been found (Vandenberg et al., 2007; Wilson et al., 2007). Because of its ubiquitous presence in our food and environment, over 90% of the population have measurable BPA and/or BPS concentrations in both the urine and blood, where blood concentrations are in the range of nanomoles/liter (Zhang et al., 2013). Though these concentrations are

seemingly nominal, femtomolar concentrations have been demonstrated to disrupt the timing and/or magnitude of extracellular signal-regulated kinase and c-Jun-N-terminal kinase activation in rat pituitary cells (Viñas and Watson, 2013a, 2013b).

Bisphenols as Xenoestrogens

The potential effects of BPA and BPS are a subject of continued controversy; several nations, including the United States, have begun to limit the use of BPA in products. The U.S. FDA has banned the use of BPA in any product designated for neonates, infants and toddlers (FDA.gov). These recent bans along with an increasing public demand to remove BPA have inadvertently led to an increase in BPS use, as "BPA free" polycarbonate plastics often substitute BPS for the ousted BPA. The call for change within the plastic industry is largely due to an increased number of studies demonstrating that BPA is an endocrine disruptor, meaning that it interferes with endogenous hormone synthesis, secretion, transport, receptor binding, or signaling (Choi et al., 2004; Deodutta et al., 1997; DeRosa et al., 1998). This signaling includes, but is not limited to, nuclear localization and activation of transcription for genes with estrogen response elements including *ABCB1* (Viñas et al., 2013; Zampieri et al., 2002). Interestingly, this phenomenon was identified as early as 1936 when Dodds and Lawson determined that BPA was a xenoestrogen in that it was able to induce estrous in laboratory rats (Dodds and Lawson, 1936).

Besides modifying transcription by dysregulating receptor mediated signaling, BPA has been shown to alter methylation patterns in genes, thereby changing their subsequent epigenetic regulation (Kundakovic and Champagne, 2011). This change in methylation leads to aberrant expression of genes which has been linked to breast and prostate cancer (Doherty et al., 2010; Soto et al., 2013; Tang et al., 2012). The alteration of methylation has also been directly demonstrated *in vivo* through the use of Agouti mice. By feeding dams a diet high in BPA, *in utero* BPA exposure caused a significant decrease in methylation in the promoter for a coat-color regulating gene within the pups.

The decrease in methylation caused a significantly higher incidence of yellow Agouti mice as compared to the brown control Agouti mice (Dolinoy et al., 2007). Based on this information, a potential mechanism by which BPA/BPS could alter P-gp expression indirectly is through epigenetic modifications. Alternatively, BPA/BPS through other mechanisms could directly alter P-gp through different mechanisms, including the modification of transcription factor binding.

Effects of Exposure to Bisphenols on P-gp and *ABCB1* activity and expression

Whether BPA or any of its metabolites are substrates of P-gp is currently debatable. BeWo cells, a human choriocarcinoma cell line, have been used to demonstrate verapamil dependent BPA transfer in an *in vitro* trans-well plate assay (Mørck et al., 2010). In a different study, a colorimetric assay was used with membranes containing P-gp isolated from humans as well as two P-gp isoforms from rats. The membrane bound transporters were exposed to BPA as well as the primary BPA metabolite, BPA-glucuronide (BPA-G). Through this assay it was demonstrated that human P-gp ATPase activity was not stimulated by a varied concentrations of BPA or BPA-G (Mazur et al., 2012). Using the same methodology as Mazur and colleagues, our laboratory performed a similar analysis which verified the results of that laboratory, finding BPA unable to stimulate ATP hydrolysis. Additionally, our lab repeated the same experiment using BPS and found that it was also unable to stimulate ATP hydrolysis from P-gp.

Not only should the direct effects of bisphenols on P-gp be evaluated, but the effects on *ABCB1* regulation should also be considered. The endogenous ligands for androgen and estrogen receptors are steroids, e.g. testosterone, estradiol, estrone. After binding with their ligands, these receptors utilize multiple signaling routes which ultimately result in changes in transcriptional activity (Rosner et al., 1999). This phenomenon occurs primarily in genes that contain either androgen receptor binding sequences or estrogen response elements in their promoters. However, because of cell-

membrane bound steroid receptors, alternative , non-genomic signaling can also occur (Rosner et al., 1999; Viñas and Watson, 2013a). Xenoestrogenic compounds are chemicals structurally or functionally similar to estrogens that can act as agonists or antagonists of estrogen receptors thereby disrupting gene regulation activation (Davis et al., 1993). The compounds BPA and BPS have been found to have xenoestrogen characteristics (Ben-Jonathan and Steinmetz, 1998), and have been demonstrated to have differential agonistic and antagonistic signaling through estrogen receptors alpha and beta in a tissue dependent manner (Kurosawa et al., 2002).

Gene Expression Assays

Luciferase Assay Systems

With the development of the luciferase assay system, the understanding of gene regulation and expression grew drastically (Gould and Subramani, 1988), and bioluminescence became one of the most used assays for determining a gene's transcriptional activity. The firefly luciferase protein from the common eastern firefly (*Photinus pyralis)* was first purified in 1956 (Green and McElroy, 1956). The first complete cDNA sequence for firefly luciferase was generated in λgt11 bacteriophages and subsequently expressed in E. coli (De Wet et al., 1985). Observing the high potential for luciferase protein luminescence to replace the chloramphenicol acetyltransferase (CAT) assay in determining promoter function, De Wet et al. (1987) generated a full-length cDNA luciferase gene which they transfected into African green monkey kidney cells. The experiment proved the usefulness of the technique, in that it reduced the overall experiment time, reduced the amount of materials used, increased the sensitivity (relative to the CAT assay), and had a higher protein stability compared to the acyltransferase (De Wet et al., 1987; Williams et al., 1989).

After the development of firefly luciferase, the usefulness of other luciferases was considered and modifications of these luciferase enzymes have occurred. One other commonly used luciferase is the Renilla luciferase from *Renilla reniformis,* a sea pansy.

While firefly luciferase produces a green-yellow light at a wavelength of ~560nm (Seliger and McElroy, 1964), *Renilla* luciferase produces 480nm blue light. Firefly luciferase requires ATP as a cofactor, while *Renilla* uses only oxygen and coelenterazine. Additionally, there is a large size difference of the proteins, with firefly luciferase having a mass of 61kDa while *Renilla* is 36kDa. One other advantage of the *Renilla* luciferase is it has a longer half-life at 37°C compared with firefly luciferase, with half-lives of 99 minutes and 7 minutes in HEK-293 cell lysates respectively (Hall et al., 2012).

More recently, scientists at Promega Corporation have engineered a smaller luciferase, called NanoLuc, modified from a luciferase found in a deep sea shrimp (Hall et al., 2012). This modified NanoLuc is half the size of the Renilla luciferase and is almost 100x brighter than both the Firefly and *Renilla* luciferases. Additionally, the NanoLuc has a much higher stability than both *Renilla* and Firefly luciferases, having a half-life at 37°C of over 7 days (Hall et al., 2012). Most marine luciferase enzymes, *Renilla* luciferase included, use the substrate coelenterazine in and ATP-independent manner to produce light. NanoLuc was engineered to use the molecule furimazine, which is very structurally similar to coelenterazine, in an ATP independent manner. The NanoLuc is still capable of utilizing coelenterazine as a substrate, making dual-luciferase assays with NanoLuc and *Renilla* luciferases currently impossible.

In many cases, one experimental control used for luciferase assays is a second reporter plasmid with a constitutively active promoter. Because there are two active luciferase genes, these assays are referred to as dual-luciferase or dual-reporter assays. In the past, these usually consisted of the *Renilla* and Firefly luciferases being paired together, with either luciferase plasmid used for the control or the gene of interest. With the development of the NanoLuc, which is capable of producing much more light than equivalent amounts of Firefly and *Renilla* luciferases, the NanoLuc reporter can be used to examine both very small differences in promoter activity as well as the activity of very lowly expression promoters.

Besides identifying new enzymes for use in molecular biology assays, additional research is focused on modifying the currently known enzymes. Some of the current enzyme modifications include destabilizing the protein by the addition of a proline, glutamic acid, serine and threonine (PEST) domain, which was found to signal proteasomal degradation (Gilon et al., 1998; Li et al., 1998), and the fusion of an N-terminal secretion signal, which allows for the enzyme to be found in the cell media. In this study, we utilized a destabilized NanoLuciferease enzyme. The benefit of having a destabilized protein is when compared with the normal, stable version of the luciferase, the destabilized version allows a researcher to determine more dynamic effects of a promoter. For example, one could expose cells to a substance and then identify the promoters, which respond either more quickly or with greater magnitude to the insult by measuring the luciferase activity after minutes or hours, rather than days, and correlate the differences in luminescence with changes in transcriptional activity.

In the current study, we used a dual luciferase assay system to determine the effects of SNPs, in the context of haplotypes, on the promoter activity of *ABCB1*. Briefly, DNA fragments of the *ABCB1* promoter were inserted into NanoLuciferase (Nluc) plasmid pNL1.1 (Promega Corporation, Madison, WI). These reporters were then co-transfected into placental cells with a second, firefly luciferase containing the constitutively phosphoglycerate kinase (PGK) promoter (pGL4.53) as a transfection control. The Nluc plasmid was also used to characterize the effects of chronic bisphenol (BPA and BPS) exposure on multiple *ABCB1* promoter haplotypes, and to determine any haplotype-dependent differences in response. In addition to the Nluc plasmid, a second NanoLuciferase plasmid, NanoLuc-PEST (NlucP), was used to characterize the effects of acute bisphenol (BPA and BPS) exposure on multiple *ABCB1* promoter haplotypes, and to determine any haplotype-dependent differences in response. DNA fragments of the promoter for *ABCB1* were inserted into the destabilized NanoLuc-PEST (NlucP) plasmid pNL1.2 (Promega Corporation, Madison, WI) and a second firefly luciferase with the

constitutively active pGL4.53 Firefly luciferase with a phosphoglycerate kinase (PGK) promoter was used for transfection control.

Site-Directed Mutagenesis

The molecular biology technique of site-directed mutagenesis was initially developed in 1974, and has been used millions of times since then. Site-directed mutagenesis enables a researcher to deliberately modify the DNA by changing a single DNA base, or group of bases in close proximity to each other, while keeping the remaining portion of the DNA constant. This technique is used by many scientists to determine the effect of single or multiple amino acid substitutions regarding alterations in protein structure and function changes. Other researchers use this technique, as we have in this study, to determine the effect of single base substitutions on promoter activity. In the current study, site-directed mutagenesis was used to generate Nluc reporter constructs for the determination of the effects from individual SNPs whose haplotypes were not present in the study population.

Objectives of the Present Study

Several SNPs have been identified in the promoter region of *ABCB1,* but their effects when studied individually have been inconsistent. This could be attributed to effects of other, additional SNPs that are present with the evaluated SNP as part of a larger haplotype grouping. Therefore, the other SNP (or SNPs) may be the driver of the observed phenotype. The haplotype DNA sequences encompassing the *ABCB1* promoter SNPs are unknown, and the effect of these haplotypes on *ABCB1* and placental P-gp expression remains elusive. Furthermore, the mechanism(s) through which *ABCB1* promoter haplotypes exert their effects on *ABCB1* promoter activity is not fully understood. Additionally, the interaction between *ABCB1* promoter haplotype activity

and exposure to environmental agents is not known. The present study was designed to address these important gaps in knowledge by elucidating the role of genetic variability in *ABCB1* promoter transcriptional regulation by testing the hypothesis that *ABCB1* promoter haplotypes, rather than individual SNPs, regulate *ABCB1* expression in the human placenta, and that the promoter activity of these haplotypes is influenced by exposure to environmental agents.

The first aim of this study was to define the *ABCB1* promoter haplotype sequences and to determine the effect of the different haplotypes on *ABCB1* promoter activity. To achieve this goal, DNA sequencing was used to identify all of the SNPs present in the sequence of the proximal *ABCB1* promoter from 100 placental DNA samples. Based on self-reported ethnicity, the subjects were 50% Hispanic, 25% African American and 25% Caucasian, which is representative of the population seen at the UTMB clinic in Galveston, Texas. We then defined the promoter haplotype sequences using bioinformatics implemented in the PHASE (phylogenetic and sequence evolution) software, which utilizes Monte Carlo simulation and Bayesian statistics. Luciferase constructs were then generated for each of the haplotypes present in our population. In addition to these constructs, additional reporter-constructs were generated using site-directed mutagenesis to determine the effect of some of the individual SNPs present in haplotypes. Promoter activity for all of the haplotype reporter constructs were measured *in vitro*, using a dual-luciferase reporter assay.

The second aim of this study was to identify potential mechanistic explanations to our findings which indicated haplotype-dependent variability in promoter activity. We hypothesized that the differences in *ABCB1* promoter activity are the result of haplotype-specific alteration in TF binding. To test this hypothesis, we first used a TF profiling array to determine if there were TF binding differences across the promoter haplotypes. TFs that showed differences, together with others identified using an *in silico* analysis and from a review of the literature were selected for further mechanistic studies. These studies were used to determine the regulation of *ABCB1* promoter activity by individual

TFs through the use of siRNA mediated knockdown of individual TFs in cultured human placental cells.

The third aim of this study was to determine the effect of *ABCB1* haplotypes on the response of the promoter activity to xenobiotic exposures. This aim tested the hypothesis that exposure to BPA and BPS, individually or as a mixture, affect *ABCB1* promoter haplotype activity in a haplotype-dependent manner. *ABCB1* promoter haplotype luciferase reporter constructs were generated using recombinant DNA and cloning techniques. These constructs were then transiently transfected into cultured human placental cells and the effects of chronic and acute bisphenol exposure, individually or in combination, on *ABCB1* promoter activity was determined using the dual-luciferase assay.

Collectively, the data generated from our studies, presented in detail in the following sections, provide important information on the role of genetic variability on *ABCB1* promoter activity. The data clearly demonstrate that haplotypes, rather than individual SNPs, better represent the effect of genetic variability on the evaluated phenotype. Our studies elucidate the haplotype-specific differential transcriptional regulation of *ABCB1* and provide a stepping stone for further studies on transcriptional regulation of this important transporter.

From a public health perspective, there are a number of acute and chronic conditions that can occur during pregnancy requiring pharmacotherapy. In fact, the use of pharmacotherapy during pregnancy has steadily increased since the 1980s, with current studies showing that over 80% of pregnant women take at least one medication during gestation, and around 50% taking four or more medications (Mitchell et al., 2011). Therefore, the data generated from our studies have important public health and translational implications for pregnant women and their fetuses. Because P-gp influences the concentration and distribution of many medications in maternal and fetal circulation, understanding the effects of *ABCB1* promoter haplotypes and their interaction with environmental exposures will ultimately help improve the accuracy of assessing the

risks/benefits from certain treatments of pregnant mothers and/or their developing fetuses.

Chapter 2: Determination of *ABCB1* Promoter Haplotype Sequences

INTRODUCTION

The growing use of medications during pregnancy (Mitchell et al., 2011) emphasizes the urgent need to develop approaches which would maximize therapy for the mother while minimizing risks to her fetus. A better understanding of the mechanisms regulating drug transfer across the human placenta is an important step toward this goal. Efflux transporters, which are highly expressed in the human placenta, are key regulators of placental drug transfer from the maternal to the fetal circulation. P-glycoprotein (P-gp), encoded by the *ABCB1* (MDR1) gene, is one of the most important placental efflux transporters and is highly expressed in the apical membrane of the placental trophoblast (Young et al., 2003). P-gp interacts with many compounds (Ceckova-Novotna et al., 2006) and, in the placenta, it uses an ATP-dependent process to actively extrude its substrates from the trophoblasts back into the maternal circulation, thus limiting their entry into the fetal circulation (Nakamura et al., 1997). Variability in placental P-gp expression and activity therefore poses a potential challenge to treating physicians as it can significantly influence maternal and fetal exposure to prescribed medications that are P-gp substrates.

Previous studies from our laboratory (Hemauer et al., 2010) and others (Hitzl et al., 2001; Tanabe et al., 2001) have demonstrated wide individual variability in placental P-gp expression and activity. The mechanisms underlying this variability are not well understood, but there is evidence that single nucleotide polymorphisms (SNPs) of the *ABCB1* gene could affect P-gp expression and function. A few *ABCB1* SNPs in the coding region, primarily the C1236T (rs1128503), C3435T (rs1045642), and G2677T/A (rs2032582), have been extensively evaluated for their effect on P-gp expression and function. However, there is considerable discrepancy in the results, as there have been observations of increased, decreased, and similar expression/activities reported (Hitzl et

al., 2001, 2004; Hoffmeyer et al., 2000; Kim et al., 2001; Kroetz et al., 2003; Mölsä et al., 2005; Salama et al., 2006; Tanabe et al., 2001; Taniguchi et al., 2003). In addition to coding SNPs, there are other SNPs in the *ABCB1* promoter that could affect P-gp expression by affecting *ABCB1* transcription. While few studies evaluated some of them, conflicting data were reported where the same SNPs were associated with increased and decreased expression, or with no effect (Ito et al., 2001; Lourenço et al., 2008; Sai et al., 2006, 2010; Takane et al., 2004; Tanabe et al., 2001).

This lack of reproducibility is not surprising and is likely a result of haplotype effects rather than effects of individual SNPs. Within the genome, SNPs do not occur individually, but rather form defined combinations or haplotypes due to varying degrees of linkage disequilibrium (LD). Thus, several SNPs create identifiable haplotypes and act in concert to provide the biological basis for genetic variability in response to an exposure. Interestingly, recent studies from our laboratory indicate that the phenotypic effect of a SNP is not always consistent but varies depending on the SNP presence as part of a specific haplotype (Xu et al., 2014, 2016). Therefore, genotyping of a single or a few individual SNPs, which may or may not be driving the phenotype, may, by chance, either capture or fail to capture true functional variants. Racial/ethnic variability in both frequency and LD of SNPs may also contribute to disparate results.

The haplotype sequences encompassing all SNPs of the *ABCB1* promoter is currently unknown. Furthermore, the functional and biological significance of these haplotypes (i.e. whether they differentially affect *ABCB1* transcription) remains elusive. In the current investigation, we comprehensively identified the SNPs found in the *ABCB1* promoter in 100 full term placentas collected from patients delivering at the Ob/Gyn Department at the University of Texas Medical Branch (UTMB) and determined the haplotypes encompassing these SNPs. We then determined the effect of these haplotypes on promoter activity. Our working hypothesis is that promoter haploytpes, rather than individual SNPs, alter *ABCB1* promoter activity and thus alter placental P-gp levels. The approach described in our study is more efficient and biologically more plausible since it

involves the evaluation of the effects of multiple, coexisting SNPs, which jointly influence human placental P-gp, ultimately affecting placental transfer of many xenobiotics.

MATERIALS AND METHODS

Placenta Collection

One hundred term placentas (38-41 weeks) were collected from study volunteers upon delivery in the Ob/Gyn department at UTMB by trained research nurses according to a protocol approved by the UTMB Institutional Review Board. Volunteers were recruited without regard to age or ethnicity and all participants signed a written informed consent document describing the purpose of the study. Collected placentas were immediately transported to our laboratory after delivery. Along with each placenta, a de-identified data sheet was provided which included information on maternal age, self-reported race/ethnicity, health conditions (if any), current medications, gestational age and type of delivery. Placentas were excluded if there were pregnancy complications, pre-term delivery, documented drug abuse during pregnancy, intake of medications that are known P-gp substrates, or infection with HIV or hepatitis. 100 mg of tissue from the fetal side of each placenta was collected for DNA isolation using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Inc., Valencia, CA) for genotyping of the promoter SNPs.

ABCB1 promoter region amplification and SNP determination

To provide a full coverage of the SNPs existing in the *ABCB1* promoter region, specific primers were designed for PCR amplification of the known downstream *ABCB1* promoter region (Chen et al., 1990). The forward primer CTCGGTACCCTTAAGGAG AACAGCCAAG with a KpnI restriction site and reverse primer GATGCTAGCCAGTG CCACTACGGTTT with an NheI restriction site were designed to bind to a sequence 2050bp upstream and 300bp downstream of the transcription start site, respectively, thus

allowing the amplification of the 2357bp *ABCB1* promoter region. The PCR cycling conditions were 95°C for 20 seconds, annealing at 63°C for 30 seconds, and extension at 68°C for 150 seconds. Full-length amplicons were purified for sequencing by QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) after gel verification. For sequencing, four sets of primers were designed, but only seven were used due to sequencing errors generated by the eighth (Table I). Automated DNA sequencing on an ABI Prism™ 3130XL sequencer at the UTMB Molecular Genomics Core Facility generated sequences from purified PCR amplicons. The generated DNA Sequences were assembled into contigs using DNA Baser version 4.16 (Heracle Biosoft S.R.L, Romania) as we previously described (Xu et al., 2014).

Table I: Primers used for *ABCB1* promoter sequencing.

Forward Primers	Reverse Primers
5' - CAGAAGTGGTGGTGGCAC - 3'	5' – ATGCTAACTCACATCAGAGC - 3'
5' - ACGTGTAGCTCACGCCTG R - 3'	5' – GACACGTCTTTCAAAGTTCAC - 3'
5' - GATTGCACGTACTTTTCCTC - 3'	5' – CTACGGTTTGGGCGCTGC - 3'
5' – CCCTTAACTACGTCCTGTAG - 3'	

Haplotype Inference

SNPs identified were determined by comparing the generated sequences to the reference *ABCB1* promoter sequence (Entrez GeneID: 5243, GenBank accession NT_007933) using DNA Baser. Composite genotypes were used to infer haplotypes by Bayesian statistics using the Phylogenetic and Sequence Evolution (PHASE) program (http://c4c.uwc4c.com/expresslicensetechnologies/phase). From the generated information, haplotype pairs were assigned to each placenta, and their frequencies were determined.

***ABCB1* Promoter Haplotype Luciferase Constructs**

To investigate the differential effects of the various haplotypes on *ABCB1* promoter activity, we generated a total of 17 reporter constructs. These represented

fourteen haplotypes that were found in our population (see Results), including two haplotypes not inferred by PHASE, and three additional constructs which served to assist in revealing the effects of specific SNPs in haplotype combinations on promoter activity.

To generate vectors containing the different *ABCB1* haplotypes evaluated, *ABCB1* promoter regions with different haplotypes were purified, digested and ligated into a NanoLuc™ Luciferase pNL1.1 vector (Promega, Madison, WI). Reporter plasmids with the desired haplotypes were then transformed into 5α E. coli (New England Biolabs, Ipswich, MA) and plated on 100 μg/ml ampicillin LB agar plates. Colonies were selected and grown in LB broth with 100μg/ml ampicillin for 18-24 hours. Plasmids were isolated using the endofree ZR Plasmid Miniprep™ - Classic kit (Zymo Research Corp, Irvine, CA) and quantified at 260nm using a DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE). The plasmids were subsequently sequenced to verify the plasmid carried the desired haplotype sequence and to ensure no additional mutations were introduced during the preparation. Isolated plasmids were stored at -20ºC to maintain plasmid integrity until transfection into human 3A trophoblast cells. The effect of the haplotypes on *ABCB1* promoter activity in placental cells was determined using the manufacturer's protocol for the Nano-Glo® Dual-Luciferase® Assay System (Promega, Madison, WI).

Site-directed Mutagenesis

To create supplemental *ABCB1* haplotypes needed for understanding of the role of individual SNPs within the haplotypes, we utilized the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Site-Directed mutagenesis primers were designed according to the principles described in the manufacturer's protocol, as well as by utilizing QuikChange Primer Design Tool (www.genomics.agilent.com) available from Agilent Technologies. The mutagenesis and subsequent transformation were performed according to the manufacturer's recommendations. For the mutant strand synthesis reaction, 100ng double-stranded DNA

template in the pNL1.1 plasmid was used for each construct with an extension time of three minutes. Subsequent DPNI restriction enzyme digestion was extended beyond the recommended five minutes to thirty minutes to remove all residual template DNA. The DNA was subsequently used to transform 5α bacterial cells as described above, and positive mutant colonies were confirmed by DNA sequence analysis.

Cell Culture and Transfection

The human trophoblastic 3A placental cell line (CRL-1584) was purchased from American Type Culture Collection (ATCC, Manassas, VA) and used as the host cell for haplotype construct transfection. Cells were maintained in 75cm2 flasks with Minimal Essential Medium with Earle's Salts and L-Glutamine (Gibco Cat. 11095-080, Grand Island, NY) supplemented with 10% FBS in 5% CO2 at 37°C. Cells were passaged at 85% confluency (2-3 days) and subcultured at a 3:1 ratio. A solution of 0.25% (w/v) Trypsin – 0.53mM EDTA was used to detach the cells for subculture or transfer to 6-well plates for transfection. Transfections were performed between passages 6-8 with cells at low confluency (≤ 40%). For each transfection, the cells were treated with a mixture of 0.6μg promoter haplotype plasmid DNA, 0.06μg firefly luciferase control plasmid pGL4.53 PGK (Promega, Madison, WI) and 2μL Fugene 6 transfection reagent (Promega, Madison, WI). After transfection, cells were allowed to recover for 36-48h before harvest.

NanoGlo® Dual-Luciferase® Assay to determine the effect of the different haplotypes on *ABCB1* promoter activity

The NanoGlo Dual-Luciferase Reporter Assay (Promega, Madison, WI) was performed according to the manufacturer's instructions. Briefly, 3A cells were harvested using 500μL Passive Lysis Buffer 36-48 hours after transfection. The lysates were then either used immediately or stored at -80°C for later analysis. Luciferase activity was measured according to the manufacturer's recommendations, and luminescence was

measured in triplicate using a Tecan GenIOS Pro plate reader (Tecan, Durham, NC). Luminescence was measured as relative light units, by normalization, against the co-transfected Firefly luciferase. Each experiment was repeated at least six times.

Statistical Analysis

Genotype frequencies for each SNP were estimated from our sample of 100 placentas and tested for deviation from Hardy-Weinberg equilibrium (HWE) by the standard Chi-square test as well as a permutation test, both implemented in the Linkage Disequilibrium Analyzer version 1.0 program25. Deviations from HWE were considered significant at $P< 0.05$ for either test. None of the SNPs identified deviated from HWE and thus none were excluded from haplotype inference and further analyses. Frequencies of haplotypes inferred from the PHASE analysis were used to determine haplotype frequencies. The nonparametric Kruskal-Wallis One Way ANOVA was used to compare luminescence values corresponding to the different haplotypes to determine their effect on *ABCB1* promoter activity. Post-Hoc analysis using the rank based Dunn's Method was used to compare the luminescence values for the individual haplotypes against the ancestral haplotype. Expression levels were presented as mean + SE. P-values <0.05 were considered significant.

RESULTS

ABCB1 promoter SNPs and haplotype Inference

In our study population, we identified a total of 12 SNPs. The 1000 genome project reported 23 SNPs in this region (Table I) (Auton et al., 2015). These SNPs had minor allele frequencies (MAF) ranging from 5% for the most frequent SNP (T-1017aC) to 0.5% for the least frequent SNPs. In our population, some SNPs occurred at frequencies comparable to the 1000 genome project (A-1572aT, G-1459aA, T-1017aC, T-129C and A-43G), others were observed at frequencies 2 to 8 times higher (A-684aT,

Table II: SNPs found in *ABCB1* Promoter in 1000 genome project.

SNP[b]	rs number	Database Reported MAF (Allele, Count)	Our MAF (Allele, Count)
A-1572aT	rs28381795	0.0134 (T,67)	0.015 (T,3)
T-1517aC	rs28381796	0.0116 (C,58)	0.005 (C,1)
A-1503aG	rs149353773	0.0006 (G,3)	ND
G-1459aA	rs12720464	0.0581 (A,291)	0.035 (A,7)
C-1360aT	rs181041839	0.0004 (T,2)	ND
C-1167aT	rs191072244	0.0002 (T,1)	ND
G-1157aA	rs28381797	0.0236 (A,118)	0.01 (A,2)
C-1073aA	rs185837535	0.0008 (A,4)	ND
T-1017aC	rs28746504	0.0543 (C,272)	0.05 (C,10)
T-824aC	rs182495914	0.0006 (C,3)	ND
A-684aT	rs28381798	0.001 (T,5)	0.005 (T,1)
G-496aA	rs138442576	0.004 (A,2)	ND
A-297aG	rs140872872	0.0004 (G,2)	ND
G-274aA	rs35462624	0.0022 (A,11)	0.005 (A,1)
C-223aT	rs34762047	0.0012 (T,6)	ND
A-131aG	rs148824712	0.0008 (C,4)	ND
G-68aA	rs145617883	0.0006 (A,3)	ND
A-60aT	rs28381800	0.0078 (T,39)	ND
A-41aG	rs2188524	0.0214 (G,107)	0.005 (G,1)
G-240A	rs35265821	0.0006 (A,3)	0.005 (A,1)
T-129C	rs3213619	0.0543 (C,272)	0.04 (C,8)
A-43G	rs28381801	0.0048 (G,24)	0.005 (G,1)
C133A	rs28381802	0.0156 (A,78)	0.005 (A,1)

MAF – Minor Allele Frequency; ND – Not Detected

a. 1000 genome project.

b. Small "a" nomenclature represents nucleotide before the non-transcribed Exon 1. Without the a are within or after Exon 1 before the transcription start site. Derived from Takane et al., 2004.

G-274aA and G-240A) or 2 to 4 times lower (T-1517aC, G-1157aA, A-41aG and C133A).

Bayesian statistics implemented in PHASE were then used to infer the haplotypes encompassing these 12 SNPs. PHASE analysis inferred 28 potential haplotypes, of which 12 were found in our population with frequencies ranging from 0.5% to 88% (Table III).

Table III: Haplotypes and frequencies inferred by PHASE

Haplotype #	*ABCB1* Promoter Haplotype Structure	PHASE Frequency	Sample Frequency	Pairs	Number of Subjects (Frequency)
1	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.8817	0.880	1,1	80
2	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/**133A**	0.0004	ND	1,3	1
3	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/**-43G**/133C	0.0050	0.005	1,4	1
4	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/**-240A**/-129T/-43A/133C	0.0050	0.005	1,7	1
5	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/**-41aG**/-240G/-129T/-43A/133C	0.0004	ND	1,8	1
6	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/**-41aG**/-240G/-129T/-43A/**133A**	0.0004	ND	1,9	2
7	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/**-274aA**/-41aA/-240G/-129T/-43A/133C	0.0050	0.005	1,10	2
8	-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/**-684aT**/-274aG/-41aA/-240G/-129T/-43A/133C	0.0050	0.005	1,15	1
9	-1572aA/-1517aT/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0150	0.010	1,16	6
10	-1572aA/-1517aT/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/-41aA/-240G/**-129C**/-43A/133C	0.0210	0.025	10,27	3
11	-1572aA/-1517aT/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/-41aA/-240G/**-129C**/-43A/**133A**	0.0004	ND	13,19	1
12	-1572aA/-1517aT/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/**-41aG**/-240G/**-129C**/-43A/133C	0.0004	ND	29,30	1
13	-1572aA/-1517aT/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/**-41aG**/-240G/**-129C**/-43A/**133A**	0.0004	0.005		
14	-1572aA/-1517aT/-1459aG/**-1157aA**/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0144	ND		
15	-1572aA/-1517aT/-1459aG/**-1157aA**/**-1017aC**/-684aA/-274aG/-41aA/-240G/**-129C**/-43A/133C	0.0004	0.005		
16	-1572aA/-1517aT/**-1459aA**/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0293	0.030		
17	-1572aA/-1517aT/**-1459aA**/-1157aG/**-1017aC**/-684aA/-274aG/-41aA/-240G/**-129C**/-43A/133C	0.0002	ND		
18	-1572aA/-1517aT/**-1459aA**/**-1157aA**/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0005	ND		
19	-1572aA/**-1517aC**/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0004	0.005		
20	-1572aA/**-1517aC**/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133A	0.0004	ND		
21	-1572aA/**-1517aC**/-1459aG/-1157aG/-1017aT/-684aA/-274aG/**-41aG**/-240G/-129T/-43A/133C	0.0004	ND		
22	-1572aA/**-1517aC**/-1459aG/-1157aG/-1017aT/-684aA/-274aG/**-41aG**/-240G/-129T/-43A/**133A**	0.0013	ND		
23	-1572aA/**-1517aC**/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/-41aA/-240G/**-129C**/-43A/133C	0.0004	ND		
24	-1572aA/**-1517aC**/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/-41aA/-240G/**-129C**/-43A/**133A**	0.0004	ND		
25	-1572aA/**-1517aC**/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/**-41aG**/-240G/**-129C**/-43A/133C	0.0004	ND		
26	-1572aA/**-1517aC**/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/**-41aG**/-240G/**-129C**/-43A/**133A**	0.0012	ND		
27	**-1572aT**/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	0.0003	0.015		
28	**-1572aT**/-1517aT/-1459aG/-1157aG/**-1017aC**/-684aA/-274aG/-41aA/-240G/**-129C**/-43A/133C	0.0097	ND		
29*	-1572aA/-1517aT/**-1459aA**/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/**-129C** /-43A/133C	ND	0.005		
30*	-1572aA/-1517aT/-1459aG/**-1157aA**/**-1017aC**/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C	ND	0.005		

Two additional haplotypes that were not inferred by PHASE (Haplotype 29, 30; Table III) were identified in the study population. The ancestral haplotype 1 (-1572aA/-1517aT/-1459aG/-1157aG/-1017aT/-684aA/-274aG/-41aA/-240G/-129T/-43A/133C) was the most common (frequency of 88%) and was considered the reference haplotype for subsequent promoter activity comparisons. Within the study population, there were six haplotypes which were found only one time with a MAF of 0.5%. The 14 haplotypes identified formed 12 paired haplotype combinations. We evaluated the effect of the 14 haplotypes found in our population on promoter activity, as well as 2 additional haplotypes that we generated using site-directed mutagenesis (M1 and M4) to determine effects of individual SNPs found in specific haplotypes.

These SNPs were found in two haplotypes: one in haplotype 30 conferring decreased expression and the other in haplotype 29 conferring increased expression. Haplotype 30 is composed of two SNPs (G-1156aA; rs28381797 and T-1017aC; rs28746504). To determine the effect of each SNP individually on promoter activity, two constructs would have to be available, each containing one of these two SNPs. SNP T-1017aC is found individually as haplotype 9. To determine the effect of G-1156aA individually, we used site-directed mutagenesis to generate haplotype M4. Similarly, haplotype 29 is composed of two SNPs (G-1459aA; rs12720464 and T-129C; rs3213619). SNP G-1459aA is found individually as haplotype 16. To determine the effect of SNP T-129C individually, we created construct M1 using site-directed mutagenesis.

Effect of different *ABCB1* haplotypes on promoter activity

Each of the 16 haplotype sequences (14 observed in our population and 2 generated by site-directed mutagenesis) were ligated into pNL1.1 luciferase reporter plasmids. The luciferase reporter gene is driven by the inserted *ABCB1* haplotype promoter, allowing the promoter activity to be determined from luciferase chemiluminescence. Promoter haplotype NanoLuc plasmids were co-transfected into

placental 3A trophoblast cells with a firefly luciferase containing plasmid to control for transfection efficiency.

Using this assay system, we found significant haplotype-dependent variations in luciferase activity. As shown in Figure 4, compared to the ancestral haplotype (haplotype 1), haplotype 30 showed a 94.4% decrease in promoter activity ($p<0.01$), while haplotypes M4, 29 and 4 produced significant increases ($p<0.05$) in promoter activity with 107.4, 247.4 and 290.2% increases, respectively. Other haplotypes evaluated showed differential effects on promoter activity, however the differences were not statistically significant.

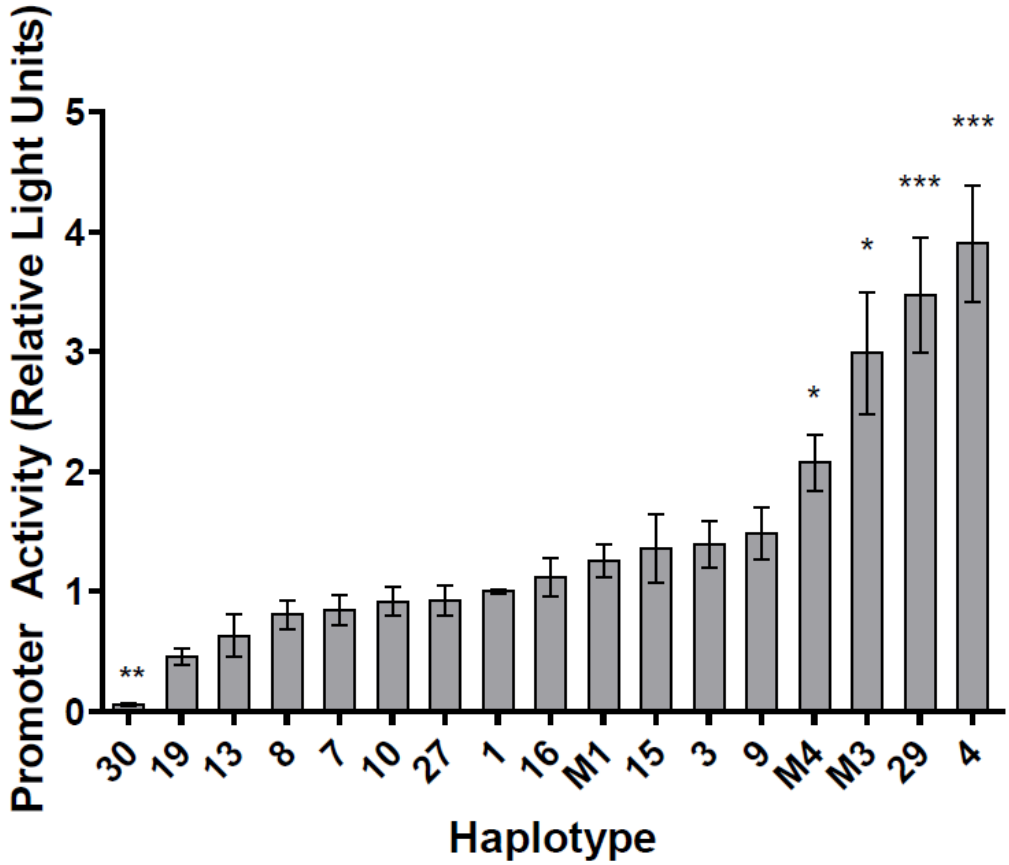


Figure 4: Promoter Haplotype Luciferase Activity. *ABCB1* promoter haplotype nanoluciferase activity was measured and normalized in-well with firefly luciferase for each of the 14 haplotypes observed in the population as well as for the 3 generated haplotypes. The resulting activity was then normalized to the activity of the ancestral promoter (no SNPs) for comparison, data are represented as the mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Differential effects of SNPs in the context of haplotypes

Our data indicate the effects from individual SNPs on promoter activity depends on their presence in a specific haplotype. A linear representation of the individual SNPs is found in Figure 5. For example, the T-1017aC SNP is found in several haplotypes. When this SNP is found with the variant G-1157aA (haplotype 30), the promoter activity was dramatically reduced (94% reduction; $P< 0.01$). However, when this variant is found individually (haplotype 9), an increase in promoter activity of 48.8% relative to haplotype 1 was observed (Figure 4). When the effect of the G-1157aA SNP alone was tested (haplotype M4), the promoter activity was significantly increased to 107.4% of haplotype 1 ($p<0.05$).

The only haplotype containing the G-240A variant is haplotype 4. This haplotype exhibited a significant increase in activity (300%; $P<0.001$). Thus, this increase could be attributed to this specific variant. Haplotype 29, associated with a significant 250% increase in promoter activity, is composed of the G-1459A and T-129C SNPs. When T-129C was found in conjunction with another SNP (as in haplotype 10), the promoter activity was not significantly affected (reduced by 9% compared to haplotype 1). However, when the T-129C SNP was tested alone using haplotype M1 generated by site-directed mutagenesis, only a slight non-significant increase in activity (13% compared to haplotype 1) was observed. It seemed plausible to assume that the G-1459A variant is responsible for that dramatic increase observed with haplotype 29 based on the T-129C data. However, when G-1459A is present alone (haplotype 16), a non-significant increase (~12%) in activity was observed compared to haplotype 1.

Figure 5: Linear Map of identified SNPs in *ABCB1* proximal promoter. Location of the 12 SNPs identified in the 2657bp proximal promoter region. The distance between SNPs is drawn to scale.

DISCUSSION

We determined the haplotype encompassing the SNPs present in the *ABCB1* promoter region and investigated their effects on promoter activity. *ABCB1* promoter haplotype DNA sequences have been previously described for a Japanese (n=115) and a Caucasian (n=96) population (Takane et al., 2004). In that study, Takane et al. described 10 SNPs/deletions in the *ABCB1* promoter, identifying 8 SNPs in Japanese and 2 additional SNPs in Caucasians. They reported the SNPs segregating into 10 haplotypes varying in frequency between Japanese and Caucasians.

In our investigation, we identified 12 SNPs but none of the insertions/deletions they reported (Takane et al., 2004). Moreover, we found 14 haplotypes of which three were reported by Takane et al.: haplotype 1 and our haplotypes 10 and 16. However, the frequencies of these haplotypes varied considerably. In our study, the frequency of haplotype 1 was 88.1% compared to 66.5% and 96.4% for Japanese and Caucasians (Takane et al., 2004), respectively. Similarly, haplotype 10 was not found in the Japanese, but was found at a frequency of 1.6% in Caucasians (Takane et al., 2004) compared to 2.5% in our population. Haplotype 16 was found at a frequency of 19.1% in Japanese but was not found in Caucasians (Takane et al., 2004) and its frequency was 3.0% in our population. The discrepancy between our study and Takane et al. could be attributed to the different racial/ethnic backgrounds and the number of subjects evaluated within each population (Table IV). In our study, PHASE predicted 28 possible haplotypes in our population, however we only observed 12 in addition to two that were not predicted. This could be attributed to the relatively small number of subjects we evaluated (n=100), and missing racial/ethnic groups, notably people of Eastern Asian descent.

Consistent with our previous studies (Rondelli et al., 2010; Xu et al., 2014), our data indicate that evaluation of SNPs in the context of haplotypes more accurately determines their functional and biological effects. A case in point is the G-1157aA, which

Table IV: Selected demographic information for the study population.

Ethnicity[a]		
	Non-Hispanic White	13
	African American	26
	Hispanic	59
	Unreported	2
Newborn Gender		
	Female	42
	Male	52
	Unreported	6
Maternal Age		
	≤ 27 Years	49
	≥ 27 Years	49
	Unreported	2
Gestational Age		
	< 37 Weeks	38
	≥ 37 Weeks	60
	Unreported	2

(a) Ethnicity of child as self-reported by newborn's mother.

when found in haplotype 30, produced a significant decrease in promoter activity. However, when G-1157aA is found alone (haplotype M4), the activity was significantly increased indicating that single SNPs are not always the phenotype drivers. This also corroborates our earlier observations involving the O-6-methylguanine DNA methyltransferase (*MGMT*) gene (Xu et al., 2014, 2016). In these studies, we found the *MGMT* promoter SNP C575A (rs113813075) to have no effect in one haplotype but dramatic effects on *MGMT* expression when found in another haplotype. Our data, therefore, provide explanations for discrepant results of studies evaluating effects of the same SNP. For example, when considering the pharmacogenomic effects of the *ABCB1* coding SNP C3435T in the context of total body digoxin exposure, this SNP was reported to be associated with an increased, a decreased, and with no effect on digoxin AUC (Pauli-Magnus and Kroetz, 2004). The differences observed could be due to the lack of consideration of *ABCB1* promoter haplotypes in these studies which could potentially increase or decrease P-gp expression, thus masking the effect of the coding SNP evaluated.

While there was some concordance between our study and that of Takane et al. with respect to haplotypes sequences, there were discrepancies in the biological effects observed. For example, while Takane et al. reported a 30% increase with haplotype 10, we found no significant change in promoter activity. This could be attributed to several factors including experimental differences and the use of different host cells used for the expression assays. In their studies, Takane et al. used human liver HepG2 hepatoma cells for promoter expression assays while we used human 3A placental cells. In fact, when we tested our same *ABCB1* haplotype constructs in U87MG glioblastoma cells, we observed differences in the activity of some promoter haplotypes compared to those observed with the 3A trophoblasts (data not shown). This effect was also observed by (Wang et al., 2006) when they were evaluating the effects of *ABCB1* promoter SNPs on expression in multiple cell lines. Differences observed in a protein's expression between cell lines is not surprising and could be due to differences in transcription factors (TF) known to exist between cells (Wilhelm et al., 2014).

The mechanisms underlying the differential *ABCB1* haplotype effects on promoter activity remain elusive. One potential mechanism may involve non-cis-acting mechanisms. For example, it has been reported that SNPs can alter DNA methylation patterns within gene promoters (Kerkel et al., 2008). Therefore, similarly to individual SNPs, haplotypes could alter methylation patterns, especially when SNPs occur in CpG rich regions. In the proximal *ABCB1* promoter, there are 2-3 CpG islands (Li et al., 2015; Takai and Jones, 2003) and varying levels of promoter methylation have been observed (Wu et al., 2015). Takane et al. demonstrated that methylation pattern variability within the *ABCB1* promoter resulted in differential expression of the gene. However, the effects of haplotypes on the *ABCB1* promoter methylation pattern needs further investigation.

Another potential mechanism is allelic imbalance, which is a deviation from the expected equal expression from each individual allele. In studies addressing multiple genes, it was found that this ratio can deviate from the expected equal ratio of 1:1 up to an expression ratio of 4.3:1 (Yan et al., 2002). In studies specifically involving *ABCB1*,

this phenomenon appears to favor the expression of an allele containing C3435T if present in the gene (Loeuillet et al., 2007). However, these studies evaluated only two SNPs in the distal promoter region and six tagging SNPs within the introns and exons but did not address the SNPs in the proximal *ABCB1* promoter.

In summary, we provided detailed haplotype DNA sequences for the *ABCB1* promoter in a mixed ethnic/racial population. We demonstrated the effect of an individual SNP is not always consistent but differs in a haplotype-specific manner, indicating that *ABCB1* haplotypes, rather than individual SNPs, affect its expression and could thus play a significant role in the expression of placental P-gp. Our study underscores the need for future mechanistic investigations to elucidate the multiple cis- and trans-acting effects of promoter haplotypes to better understand how they affect promoter activity and subsequent protein expression. We have initiated studies in this direction as described in more detail in Chapter 3 below.

Chapter 3: Transcription Factor Regulation of *ABCB1* Promotors

INTRODUCTION

P-glycoprotein (P-gp), encoded by the *ABCB1 (MDR1)* gene, is a promiscuous efflux transporter that interacts with numerous structurally diverse substrates (Ceckova-Novotna et al., 2006; Kim, 2002; Schinkel and Jonker, 2003). P-gp was first discovered in cancer cells, associated with the phenomenon of multiple drug resistance (Juliano and Ling, 1976). However, it is now known that P-gp is highly expressed in normal tissues including liver, lower GI tract, kidneys, endothelial cells that make up the blood brain barrier, and in other tissue-blood barriers such as the testes and placenta (Kim, 2002). In the placenta, P-gp is found on the apical membrane of the syncytiotrophoblasts (Young et al., 2003). By utilizing ATP hydrolysis, P-gp actively extrudes its substrates from the trophoblasts back to the maternal circulation, thus limiting their entry into the fetal circulation (Nakamura et al., 1997). As such, variability in placental P-gp expression/activity could significantly influence the maternal and fetal distribution of xenobiotics, including many prescribed medications and environmental agents that are P-gp substrates.

There is a large interindividual variability in placental P-gp levels/activity (Hemauer et al., 2010) which could be a result of variability in *ABCB1* expression levels. The *ABCB1* promoter region contains many single nucleotide polymorphisms (SNPs). We, and others, have shown that these SNPs form specific haplotypes, which are defined SNPs combinations, that differentially affect *ABCB1* promoter activity (Speidel et al., 2018; Takane et al., 2004). However, the exact mechanism(s) by which these haplotypes exert their effect on *ABCB1* promoter activity remain to be elucidated.

The *ABCB1* promoter region is rich with regulatory regions and many cis-elements have been identified, including a heat-shock element and a partial estrogen-response element (Chin et al., 1990; Shi et al., 2014). In addition, other binding sites for

important transcription factors (TFs) that regulate *ABCB1* promoter, including the transcriptional regulatory proteins Sp1, AP-1 and p53 have been identified (Reviewed in Labialle *et al.*, 2002). Previous work has shown that Sp1 binds to two different regions of the *ABCB1* promoter and, depending on which of the two sites it occupies, it has either a transcriptional-activating or repressing role (Cornwell and Smith, 1993). In a recent study, we performed *in silico* analyses of the *ABCB1* promoter region which further confirmed the presence of these binding sites and identified additional putative TF binding sites in the promoter (Speidel et al., 2018). These sites included glucocorticoid receptors, vitamin D receptors, aryl hydrocarbon receptors and many more (Speidel et al., 2018).

To date, a comprehensive evaluation of the effects on the *ABCB1* promoter activity of TF binding has not been conducted. Additionally, the effect of *ABCB1* promoter haplotypes on TF binding and their regulatory effect on *ABCB1* promoter activity have not been considered. Several *ABCB1* haplotypes include SNPs within or in close proximity to known or predicted TF binding sites. For example, the G-1157aA SNP found in a specific haplotype (Speidel et al., 2018) is in a binding domain for the TF Sp1. Similarly, the G-1459aA SNP found in another haplotype (Speidel et al., 2018) is in a shared binding domain of the TFs GATA1 and GATA2. Given that *ABCB1* haplotypes have been shown to differentially alter its promoter activity (Speidel et al., 2018; Takane et al., 2004), it is plausible that these haplotypes induce 3D-structural changes capable of modifying the recruitment and/or binding of different transcriptional regulators, resulting in altered *ABCB1* expression.

We therefore hypothesized that the haplotype-dependent differences in *ABCB1* promoter activity are due to haplotype-specific alterations in TF binding. We tested our hypothesis using four different *ABCB1* promoter haplotypes with different promoter activities that we identified from a previous study from our laboratory (Speidel et al., 2018). These haplotypes included the ancestral haplotype – two haplotypes with significantly increased promoter activity and one with significantly lower activity

compared with the ancestral haplotype (Speidel et al., 2018). To test our hypothesis, we first used a TF binding profile array to determine if differences in TF binding across the haplotypes exist. TFs which showed significant differences in binding to the different haplotypes were identified and, together with others we identified from bioinformatics analysis of the *ABCB1* promoter and from a review of the literature were selected for further *in vitro* mechanistic studies. Their role in regulating *ABCB1* promoter activity was evaluated using small interfering RNA (siRNA) in cultured human placental cells. Our data indicate that TF binding, as well as their regulatory effect on promoter activity, is haplotype dependent.

MATERIALS AND METHODS

In Silico Analysis of the *ABCB1* Promoter Region

The putative transcription factor binding site predictor PROMO (ALGGEN Research Software http://alggen.lsi.upc.edu) (Messeguer et al., 2002; Nec Farré et al., 2003) was used for in silico confirmation of cis-binding elements as reviewed by Labialle et al., 2002, as well as to predict additional potential transcription factor binding sites within the *ABCB1* promoter region. A map of the known and predicted binding sites and the SNPs existing on the proximal promoter sequence found in our study was then generated (See Figure 6).

Cell Culture

The human trophoblastic 3A placental cell line (CRL-1584) was purchased from American Type Culture Collection (ATCC, Manassas, VA). Nuclear extracts containing TFs were isolated from cultured cells and used to determine the effect of the different haplotypes on TF binding. The 3A cells were also used in additional siRNA studies as the host for *ABCB1* promoter haplotype luciferase reporter construct transfection. These studies were conducted to further characterize the effect of individual TFs on *ABCB1* promoter activity. Cells were maintained in 75cm^2 flasks with Minimal Essential Medium

with Earle's Salts and L-Glutamine (Gibco, Grand Island, NY) supplemented with 10% FBS in 5% CO_2 at 37°C. Cells were passaged at 85% confluency (2-3 days) and subcultured at a 3:1 ratio. A solution of 0.25% (w/v) Trypsin – 0.53mM EDTA was used to detach the cells for subculture or transfer to 6-well plates for transfection. Nuclear extracts were collected from 3A cells following the manufacturer's protocol with the Nuclear Extraction Kit (Signosis, Santa Clara, CA).

ABCB1 promoter haplotype luciferase reporter generation

We generated luciferase reporters using the NanoLuc™ Luciferase vector system (Promega, Madison, WI) to determine the effects of *ABCB1* promoter haplotypes on promoter activity as we had done previously (Speidel et al., 2018). Briefly, four *ABCB1* promoter haplotype constructs; two representing high basal promoter activity (haplotypes 4 and 29), one representing low basal promoter activity (haplotype 30), and the ancestral haplotype (haplotype 1) were generated by inserting the appropriate promoter sequences into the NanoLuc™ pNL1.1 vector (Promega, Madison, WI) after double digestion with the restriction enzymes KpnI-HF and NheI-HF (New England Biolabs, Ipswich, MA). The reporter constructs were then used to transform competent 5α E. coli (New England Biolabs, Ipswich, MA) and plated on 100 μg/ml ampicillin LB agar plates. Individual colonies were selected and grown in LB media containing 100 μg/ml ampicillin for 18-24 hours at 37ºC.

Plasmids were isolated using the endotoxin free ZR Plasmid Miniprep™ - Classic kit (Zymo Research Corp, Irvine, CA) and quantified at 260nm using a DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE). The isolated plasmids were sequenced to verify the presence of the proper promoter haplotype in the reporter plasmid and to ensure no additional mutations were introduced during the preparation. Isolated plasmids were stored at -20ºC to maintain plasmid integrity until transfection.

Transcription factor binding assay

TF binding to the *ABCB1* promoter haplotypes was determined with the Promoter Binding TF Profiling Plate Array I (Signosis, Santa Clara, CA) using nuclear extracts isolated from placental 3A cells. This array provides an assay for quickly determining the binding of 48 TFs as detailed in the product manual. Briefly, an *ABCB1* promoter DNA fragment, corresponding to a specific haplotype, competes with biotin-labelled DNA oligos for TFs present in the nuclear extract from placental 3A cells. If a TF binding site is present in the *ABCB1* promoter DNA fragment tested, a decrease in the formation of biotin-labelled probe/TF-complex for that TF occurs. Using streptavidin conjugated with horseradish peroxidase and a chemiluminescent substrate, a luminescence signal is detected. The intensity of the luminescence correlates to the degree of TF binding to the corresponding *ABCB1* promoter, where a strong luminescence signal indicates low binding between the TF and the tested *ABCB1* promoter while a weak luminescence signal indicates strong binding between the TF and the tested *ABCB1* promoter.

Transcription factor knockdown with siRNA

To further characterize the effect of TFs on promoter activity, the effect of selected TFs (Table VII) on *ABCB1* promoter activity was investigated using siRNAs. The siRNAs were co-transfected into 3A cells with luciferase reporter constructs representing the haplotypes tested. Transfections were performed between passages 6-8 with cells at low confluency (≤ 40%). For each transfection, cells grown in a 24-well plate were treated with a mixture of 600ng promoter haplotype plasmid DNA, 60ng firefly luciferase control plasmid pGL4.53 PGK (Promega, Madison, WI), 2.5pmol siRNA, and 2μL Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA). After transfection, cells were allowed to recover for 40 hours before harvest.

NanoGlo® Dual-Luciferase® Assay to determine the effect siRNAs on activity of *ABCB1* promoter haplotypes

The NanoGlo Dual-Luciferase Reporter Assay (Promega, Madison, WI) was performed according to the manufacturer's instructions. Briefly, 3A cells were harvested using 400µL Passive Lysis Buffer 36-48 hours after transfection. Lysates were then either used immediately or stored at -80°C for later analysis. Luciferase activity was measured according to the manufacturer's recommendations, and luminescence was measured in triplicate using a Tecan GenIOS Pro plate reader (Tecan, Durham, NC). Luminescence was measured as relative light units by normalization against the co-transfected Firefly luciferase. Each experiment was repeated at least three times.

Statistical analysis

The nonparametric Kruskal-Wallis one-way analysis of variance was used to compare luminescence values corresponding to the effects of different siRNA knockdowns on *ABCB1* haplotype promoter activity. Post-hoc analysis using Dunnett's method was used to compare each siRNA TF knockdown against control within the same haplotype. P values less than 0.05 were considered significant.

RESULTS

In Silico Analysis of the *ABCB1* Promoter Region using PROMO

We utilized the software PROMO to identify haplotype-dependent alterations in putative TF binding sites which could result in differential TF binding and/or modification of cofactor recruitment (Lee and Young, 2000; Orphanides, 2002; Roeder, 1998). The *in silico* analysis of the *ABCB1* promoter predicted many putative binding sites for more than 80 TFs. Several putative sites are depicted in Figure 6. All SNPs identified in this study, with the exception of T-129C, were predicted to form or eliminate one or more putative TF binding site(s). Therefore, it is plausible to hypothesize that the observed expression changes are driven by SNPs-induced sequence alterations in close

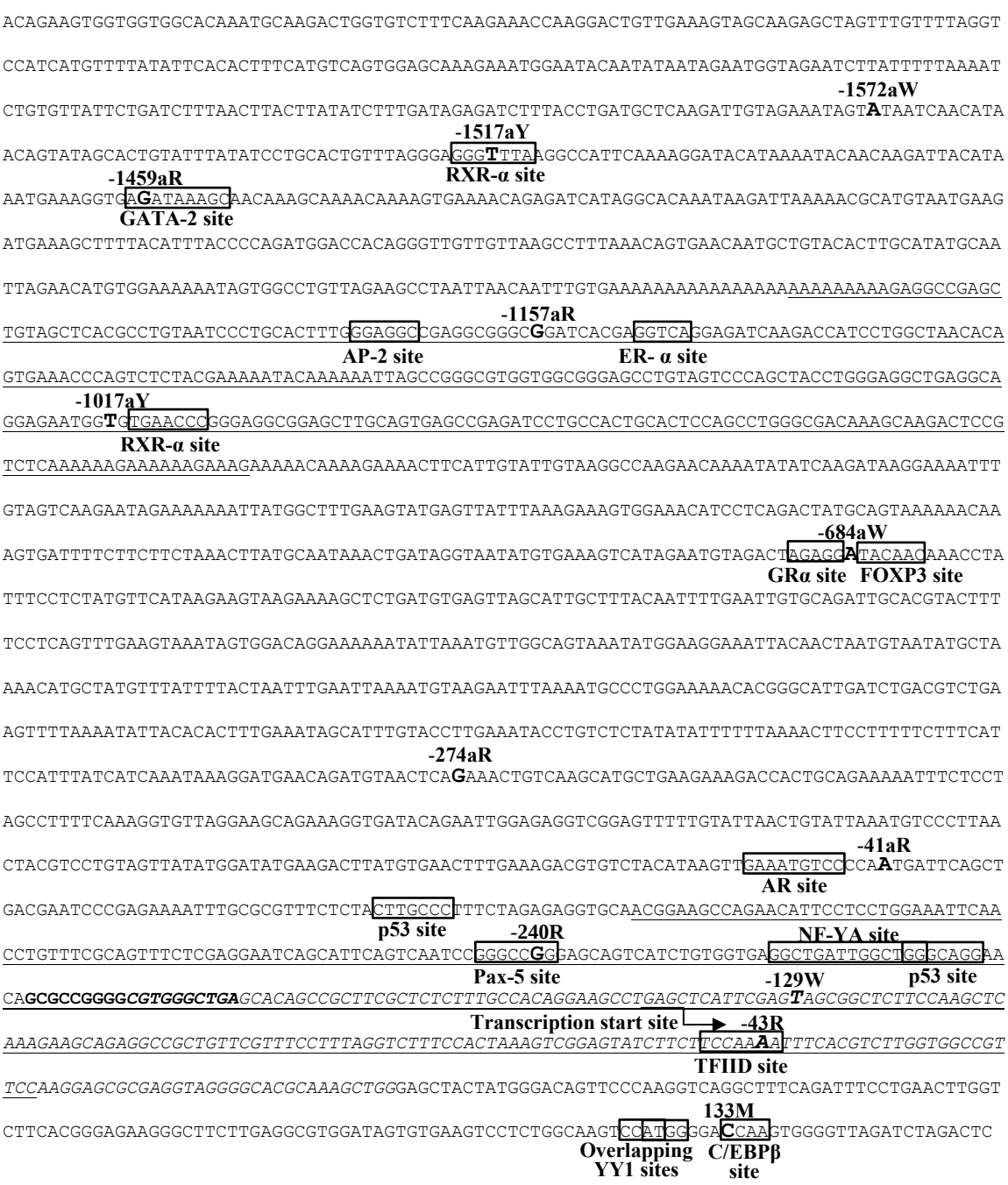


Figure 6: Proximal Promoter sequence, SNPs and TF binding sites. This map shows the 2357bp *ABCB1* proximal promoter region sequenced in this study. The grey nucleotides represent the 12 SNPs identified in this study. Boxes represent some of the PROMO predicted transcription factor binding sites near SNPs. Underlined nucleotides represent CpG islands predicted by CpG Island Searcher (Takai and Jones, 2003), while the italicized bases are CpG islands predicted by EMBL-EBI (Li et al., 2015). For SNP variants: R = A or G, Y = C or T, W = A or T, M = A or C.

proximity or within TF binding motifs. Among the TFs binding sites predicted to be altered are GR-α, GR-β, C/EBPβ, AhR, GATA-1, NF-1, and several others (Table V).

Determination of *ABCB1* promoter haplotype transcription factor binding profiles

Nuclear extracts were isolated from placental 3A cells, which are known to express P-gp. *ABCB1* promoter DNA fragments representing the four haplotypes tested were then individually used in the Promoter Binding TF Profiling Plate Array.

The *ABCB1* promoter haplotype 1 was used as a reference to identify the binding profile of the 48 TFs present in the array to the *ABCB1* promoter region. The TF binding activity, measured by chemiluminescent signal, is inversely correlated to the binding activities of the TFs evaluated where strong signals indicate weak TF-DNA binding and weak signals indicate strong DNA-TF complex formation. The TF binding activity expressed as relative light units (RLU) for haplotype 1 are presented in Table VI. The

Table V: PROMO predicted transcription factor binding sites gained or lost for each of the *ABCB1* promoter haplotypes.

Haplotype	Transcription Factors Lost	Transcription Factors Gained
1	-	-
3	GR, GR-β, HNF-3α, TFII-D	-
4	-	GR-α, ENKTF-1
7	c-Myb	C/EBPβ, GR
8	GATA-1, TFII-I	-
9	-	AhR, ENKTF-1, RAR-β:RXR-α, XBP-1
10	-	AhR, ENKTF-1, RAR-β:RXR-α, XBP-1
13	C/EBPα, C/EBPβ, GR-β, NF-1, NF-Y, NFI/CTF, XBP-1,	AhR, c-Ets-1, ENKTF-1, PR-A, PR-B, RAR-β:RXR-α, STAT4, XBP-1
15	E2F-1, Sp1	AhR, ENKTF-1, RAR-β:RXR-α, XBP-1
16	GATA-1, GATA-2	GR-β
19	FOXP3, RAR-β:RXR-α	Pax-5, p53
27	-	FOXP3
29	GATA-1, GATA-2	GR-β
30	E2F-1, GATA-1, GATA-2, Sp1	AhR, ENKTF-1, GR-β, RAR-β:RXR-α, XBP-1
M1	-	-
M2	E2F-1, GATA-1, GATA-2, Sp1	AhR, ENKTF-1, GR-β, RAR-β:RXR-α, XBP-1
M3	E2F-1, GATA-1, GATA-2, Sp1	GR-β
M4	E2F-1, Sp1	-

signal ranged from 417 RLU for GR/PR, indicating a strong TF binding, or multiple binding sites for this TF, to 5125 RLU for TFIID demonstrating weak binding or lack of a binding site for this TF in the *ABCB1* haplotype 1 promoter.

To evaluate the effect of the different *ABCB1* promoter haplotypes on TF binding, the TF Profiling Array was also used with the other three haplotypes evaluated. The differences observed in TF binding are shown in Table VI and visually in the heatmap presented in Figure 7. In Table VI, the haplotype effect on TF binding activity is expressed as the ratio of RLU of the tested haplotype over the RLU of haplotype 1. A ratio less than 1.0 indicates stronger binding activity for a TF with the evaluated haplotype compared to that observed with haplotype 1, while a ratio greater than 1.0 indicates lower haplotype binding activity for a TF than that observed with haplotype 1. In Figure 7, high binding is represented by the red color, and weak binding is denoted by green. It should be noted that a high binding activity reflects a high affinity of the TF to one or more binding sites on the promoter.

Our data indicate clear haplotype-dependent differences in TF binding. For example, C/EBP has a 12.5-fold lower signal when binding with haplotype 4 compared to haplotype 1, indicating much stronger binding activity with haplotype 4 compared to haplotype 1. Alternatively, the signal from AP2 binding activity is increased four-fold for haplotype 30 relative to haplotype 1, indicating decreased binding activity with haplotype 30 compared to haplotype 1.

Effect of TF binding on *ABCB1* promoter activity

To investigate the potential regulatory effects of specific TFs on *ABCB1* expression, we used Ambion® Silencer® Select siRNAs to target 20 individual TFs. These TFs were chosen for more in-depth evaluation based on the results obtained from the TF binding array data, as well as from our *in silico* bioinformatic analysis using PROMO software (Speidel et al., 2018). Additional TFs were also identified from the

Table VI: Haplotype transcription factor binding activities.[a]

	Haplotype Construct			
TFs	**Haplotype 1**	**Haplotype 4**	**Haplotype 29**	**Haplotype 30**
TFIID	5125	0.22	0.63	0.55
TR	4703	0.16	1.04	0.51
NF-1	4686	0.29	1.62	0.87
ER	4499	0.13	0.63	0.52
Stat3	4254	0.36	0.49	0.60
TCF/LEF	4061	0.31	0.36	0.40
Brn-3	3974	0.14	0.62	0.48
C\EBP	3851	0.08	0.20	0.33
Myb	3438	0.18	0.58	0.40
YY1	3345	0.32	1.38	0.53
NF-E2	3233	0.25	1.02	0.35
Pax-5	3216	0.23	2.10	0.48
OCT4	3169	0.26	0.62	0.83
FAST-1	2940	0.20	0.41	0.45
GAS/ISRE	2856	0.66	0.83	0.35
GATA	2855	0.63	1.21	0.39
SATB1	2849	0.45	0.26	0.65
PXR	2733	0.33	1.76	0.67
Sp1	2473	0.22	0.31	1.02
CAR	2408	0.22	1.14	0.42
Pit	2394	0.61	1.04	0.68
Ets	2366	0.18	0.81	0.63
MEF2	2202	0.16	0.44	0.69
Stat1	2083	0.72	1.05	0.20
E2F-1	2076	0.38	0.72	0.42
p53	2028	0.24	1.11	0.54
CDP	1950	1.00	1.11	2.20
Stat6	1898	0.45	0.60	1.07
CREB	1653	0.29	1.32	2.89
IRF	1646	0.51	0.56	0.65
SMAD	1552	0.34	0.69	1.14
CBF	1429	0.50	0.64	0.55
NFAT	1418	0.31	0.75	1.04
ATF2	1380	0.51	0.66	0.72
HNF4	1361	0.39	0.80	0.94
SRF	1335	0.24	2.99	1.17
EGR	1276	0.69	0.74	0.92
HIF	1272	0.56	0.66	0.57
Pbx1	1218	0.84	1.70	0.71
NFkB	1217	0.45	0.61	0.88
Stat5	1178	0.93	1.18	1.25
PPAR	1133	0.51	0.77	0.74
Myc-Max	1091	0.57	1.69	1.01
AP1	1058	1.96	2.05	1.66
Stat4	826	1.32	1.30	1.97
AR	762	1.04	2.08	1.51
AP2	471	0.97	1.45	4.24
GR/PR	417	1.13	1.12	3.04

a. The change in binding activity is expressed as the ratio of RLU of the tested haplotype over RLU of haplotype 1. A ratio less than 1.0 indicates stronger TF promoter binding than to haplotype 1 while a ratio greater than 1.0 indicates lower promoter binding for the TF than to haplotype 1.

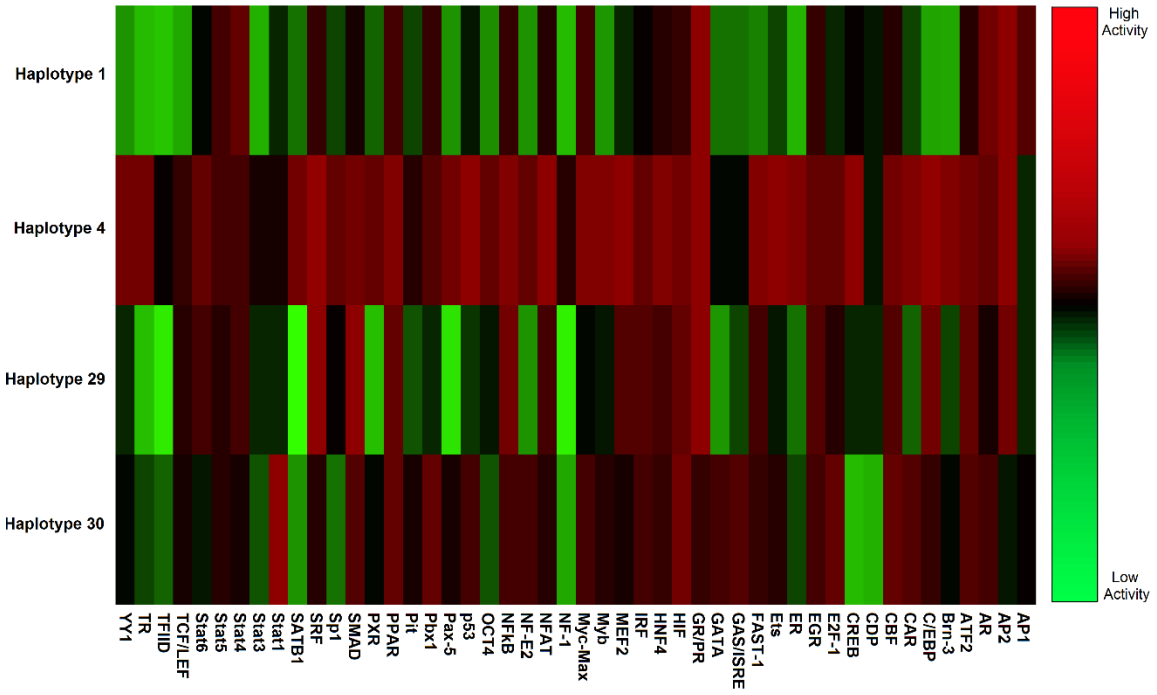


Figure 7: Heatmap representing the TF binding profiles for the four *ABCB1* promoter haplotypes evaluated. The heatmap was generated using Heatmapper (Babicki et al., 2016) from the RLU values representing the binding activity for the 48 TFs to the *ABCB1* promoter haplotypes evaluated. Each color-tile represents the average RLU value from 2 independent assays for TF binding to an *ABCB1* promoter fragment.

review of the literature on TFs regulating *ABCB1* expression (Gromnicova et al., 2012; Labialle et al., 2002; Speidel et al., 2018). The complete list of the 20 siRNAs and their gene targets are listed in Table VII. The siRNAs, which target specific TFs, were co-transfected into 3A cells with the various haplotype reporter constructs and a luciferase transfection control plasmid. The effect of individual siRNAs on the haplotype-dependent promoter activity was then evaluated by comparing the activity to the corresponding basal promoter activity (without siRNA treatment).

The siRNAs studied induced significant haplotype-dependent changes in *ABCB1* promoter activity (Table VIII and Figure 8). In Table VIII the activity of the promoter with a value greater than 1.0 indicates higher promoter activity than the control while a value less than 1.0 indicates a promoter activity lower than the control. A value equal to 1 indicates no effect for the tested siRNA resulting in promoter activity equal to that of the control.

Table VII: List of siRNAs and their corresponding targets evaluated in this study.

siRNA ID	Gene symbol[a]	TF[b]	Gene Name
s19772	CEBPZ	C/EBP	CCAAT/Enhancer Binding Protein (C/EBP), Zeta
s3489	CREB1	CREB	cAMP Responsive Element Binding Protein 1
s5594	GATA1	GATA1	GATA Binding Protein 1
s10064	PAX5	Pax-5	Paired Box Gene 5
s279	STAT1	Stat1	Signal Transducer and Activator of Transcription 1
s13531	STAT4	Stat4	Signal Transducer and Activator of Transcription 4
s13318	SP1	Sp1	Specificity Protein 1
s14778	VDR	VDR	Vitamin D Receptor
s4825	ESR1	ERα	Estrogen Receptor 1, Alpha
s9528	NFYα	NFYα	Nuclear Transcription Factor Y, Alpha
s5596	GATA2	GATA2	GATA Binding Protein 2
s9501	NFIX	NF-1	Nuclear Factor I/X (CCAAT-Binding Transcription Factor)
s743	STAT3	Stat3	Signal Transducer and Activator of Transcription 3
s14006	TFAP2B	AP2	Transcription Factor AP-2 Beta
s21070	NFAT5	NFAT	Nuclear Factor of Activated T-cells 5
s16909	NR1I2	PXR	Pregnane X Receptor
s3494	ATF2	ATF2	Activating Transcription Factor 2
s605	TP53	p53	Tumor Protein p53
s12479	SATB1	SATB1	Special AT-Rich Sequence Binding Protein 1
s13826	TBP	TFIID	TATA-Box Binding Protein

a. Genes targeted by the siRNA listed according to Thermo Fisher Scientific.
b. Name of the TF targeted by siRNA (Signosis, PROMO).

While several of the siRNAs tested induced significant changes in promoter activity, the effect was not consistent across the haplotypes. For example, the siRNA knockdown of Pax-5 produced a significant increase in the promoter activity for haplotype 1. However, knockdown of Pax-5 caused a significant decrease in the promoter activity for haplotype 29. For haplotypes 4 and 30, Pax-5 knockdown produced only a slight, non-significant decrease in promoter activity. With haplotype 29, which has a high basal promoter activity, the knockdown of CREB, GATA1, Pax-5, Sp1, NFYA, and ATF2 with siRNA significantly decreased the promoter activity (Table VIII). Overall, our data indicate that siRNA knockdown of certain TFs results in up-regulating *ABCB1* promoter activity, while knockdown of others leads to a down-regulation of promoter

Table VIII: Differential effects of siRNAs on *ABCB1* promoter haplotypes.

	HAPLOTYPE 1[a]	HAPLOTYPE 4[a]	HAPLOTYPE 29[a]	HAPLOTYPE 30[a]
C/EBP	0.80 ± 0.17	0.67 ± 0.06	0.65 ± 0.09	2.40 ± 0.61
CREB	0.95 ± 0.10	0.53 ± 0.04	**0.44 ± 0.02**	1.44 ± 0.38
GATA1	1.28 ± 0.25	1.44 ± 0.39	**0.43 ± 0.04**	0.99 ± 0.12
Pax-5	**1.72 ± 0.08**	0.86 ± 0.07	**0.47 ± 0.06**	0.88 ± 0.10
Stat1	1.20 ± 0.25	0.94 ± 0.12	0.64 ± 0.09	1.08 ± 0.13
Stat4	1.35 ± 0.11	1.01 ± 0.17	0.49 ± 0.04	0.99 ± 0.14
SP1	1.04 ± 0.27	0.52 ± 0.07	**0.49 ± 0.08**	0.76 ± 0.12
VDR	1.41 ± 0.26	1.02 ± 0.11	0.72 ± 0.04	1.43 ± 0.18
ER1	1.44 ± 0.09	0.89 ± 0.04	0.89 ± 0.05	1.10 ± 0.18
NFYα	1.00 ± 0.08	0.68 ± 0.08	**0.37 ± 0.04**	1.25 ± 0.18
AP2	**2.33 ± 0.32**	1.59 ± 0.24	1.15 ± 0.12	0.96 ± 0.10
ATF2	1.27 ± 0.15	1.02 ± 0.11	**0.44 ± 0.03**	1.01 ± 0.12
GATA2	1.11 ± 0.15	1.14 ± 0.12	0.68 ± 0.08	1.80 ± 0.30
NF-1	1.15 ± 0.06	1.13 ± 0.19	0.54 ± 0.05	1.16 ± 0.11
NFAT	**2.00 ± 0.17**	1.06 ± 0.09	0.51 ± 0.06	1.54 ± 0.25
p53	0.73 ± 0.16	0.68 ± 0.09	0.50 ± 0.04	0.82 ± 0.12
SATB1	1.41 ± 0.11	0.60 ± 0.08	0.92 ± 0.15	1.20 ± 0.11
Stat3	1.35 ± 0.17	1.06 ± 0.12	0.56 ± 0.03	1.35 ± 0.19
TBP	**1.85 ± 0.16**	0.92 ± 0.06	1.46 ± 0.16	1.46 ± 0.20
PXR	1.48 ± 0.07	1.07 ± 0.08	0.71 ± 0.05	1.22 ± 0.16
Control	1.00 ± 0.09	1.00 ± 0.14	1.00 ± 0.10	1.00 ± 0.13

a. The change in promoter activity is expressed as the ratio of RLU for the siRNA over the RLU of the control within the same haplotype. A ratio less than 1.0 indicates a decrease in promoter activity with the TF knockdown, whereas a ratio greater than 1.0 indicates an increase in promoter activity after TF knockdown.
b. Bold values indicate significant difference ($P < 0.05$) for tested siRNA vs Control.

activity. Importantly, the effect of an individual siRNA was not always consistent across the haplotypes tested, but rather haplotype-dependent.

DISCUSSION

The efflux transport protein P-gp, located on the apical membrane of the placental trophoblasts, plays a major role in the transfer of xenobiotics across the placenta. Alteration in P-gp expression can therefore have serious consequences for the fetus if the mother is being treated with a P-gp substrate medications or alternatively, if trans-placental fetal treatments with a P-gp substrate medication is required. While variability

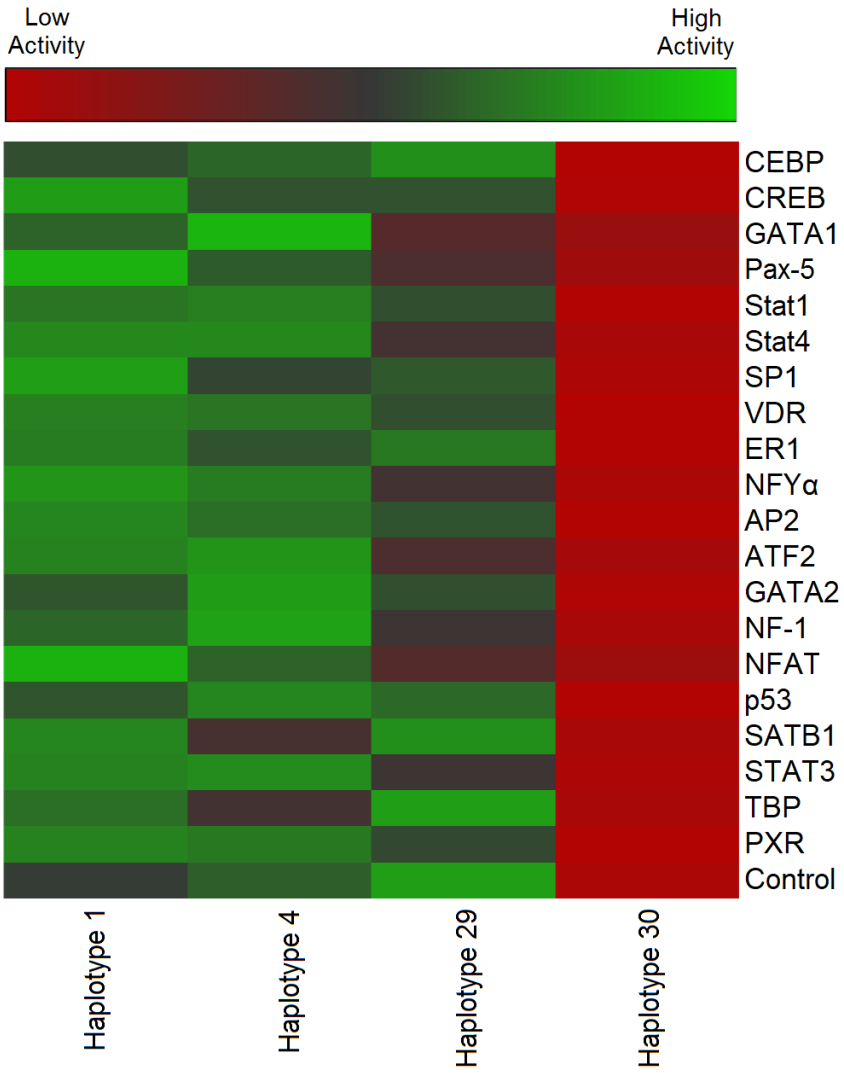


Figure 8: Heatmap representing *ABCB1* promoter activities before and after treatment with siRNA. The heatmap represents *ABCB1* promoter activity after siRNA knockdown of 20 different TFs. The heatmap was generated from the RLU values before (control) and after treatment with the different siRNA. Each tile represents the average RLU value from 3 independent assays for the promoter activity following treatment with a specific siRNA.

in P-gp could be due to a number of factors, genetic variability in the promoter of the *ABCB1* gene could alter its expression and consequently, P-gp levels (Speidel et al., 2018; Takane et al., 2004). Because genetic variants (SNPs) are arrayed as combinations forming specific haplotypes, it is plausible to assume that haplotypes rather than individual SNPs drive the phenotype. Recently, we comprehensively identified the haplotype DNA sequences encompassing the common promoter SNPs of the *ABCB1*

gene and demonstrated that the activity of the *ABCB1* promoter is haplotype-dependent (Speidel et al., 2018).

One potential mechanism for alterations in TF binding sites, resulting in differential TF binding and/or modification of cofactor recruitment (Lee and Young, 2000; Orphanides, 2002; Roeder, 1998). Consistent with the hypothesis, changes in *ABCB1* promoter activity was observed after introducing mutations in promoter Sp1 binding sites (Kerkel et al., 2008). Importantly, one Sp1 binding domain was found to have a repressive role and alteration of this site led to higher promoter activity (Cornwell and Smith, 1993). This region contains the G-240A SNP found in haplotype 4, which could affect the repressive Sp1 regulation, possibly explaining our observed significant increase in activity with this haplotype. Alternatively, the G-240A variant could generate a higher affinity-binding site for a transcription activator or alter the secondary structure of the DNA. Additional support to this hypothesis is provided by our previous work with *MGMT* (Xu et al., 2014, 2016). In these studies, we observed haplotype-dependent TF binding profiles that significantly correlated with *MGMT* promoter activity (Xu et al., 2016). In an effort to define the underlying mechanisms, in the current study we investigated whether the variability in promoter activity is due to alterations in TF binding.

To determine the putative TFs involved in regulating *ABCB1* promoter activity, we used the Signosis Promoter-Binding TF Profiling Array I plate, which provides the ability to evaluate the binding activity of 48 common TFs known to affect expression of many genes. The Signosis profiling assay indicated a haplotype-dependent difference in TF binding to the *ABCB1* promoter. Certain TFs that demonstrated strong binding activity to haplotype 1, as expressed by the low RLU values in Table VI, exhibited lower binding affinity with the other haplotypes (e.g. AP1; Table VI and Figure 7). Other TFs had low binding affinity to haplotype 1, but strong binding to one or more of the other haplotypes evaluated as indicated by the low ratio values (Table VI) and as depicted in the heatmap (Figure 7). Examples include TFIID, TR, ER, Stat3, TCF/LEF and others.

Other TFs appeared to exhibit a strong binding activity to the *ABCB1* promoter regardless of the haplotype tested (e.g., PPAR; Table VI and Figure 7), suggesting that these TFs may be essential for regulating *ABCB1* promoter activity, a possibility that needs to be confirmed or refuted with additional studies in the future.

An interesting observation from our study was the variability in binding affinity of the glucocorticoid/progesterone (GR/PR) receptor depending on the haplotype evaluated. While there are numerous GR/PR binding sites on the *ABCB1* promoter, as indicated by its strong binding observed with the ancestral haplotype 1, 4 and 29, its binding affinity with haplotype 30 was significantly decreased by 3-fold compared to that observed with haplotype 1. While there is no TATA box in the *ABCB1* promoter (van Groenigen et al., 1993), we observed a 5-fold stronger binding affinity for the TATA box-binding TFIID with haplotype 4 as compared to the minimal affinity observed with haplotype 1. The exact mechanisms for such variabilities remain to be elucidated; however, it is possible that structural changes due to SNPs forming different haplotypes resulted in changes in *ABCB1* promoter architecture and, consequently, differentially affected TF binding, including TFIID. It is known that the architecture of promoter DNA, dictated by its sequence, determines TFs binding to the promoter (Reményi et al., 2004). TFIID is a protein complex composed of TATA box Binding Protein (TBP) and several subunits called TATA-binding protein Associated Factors (TAFs) which add promoter selectivity, especially if there is no TATA box sequence for TBP to bind to (Louder et al., 2016). Therefore, it is possible that structural changes associated with haplotype 4 facilitated the binding of TFIID and/or its associated TAFs. Consistent with this possibility, our PROMO analysis revealed the presence of several putative TFIID binding sites on this promoter (Speidel et al., 2018).

The potential regulatory function of different TFs on *ABCB1* promoter activity across different haplotypes was evaluated using siRNAs that preferentially block individual TFs. Our strategy to identify the potential targets for siRNA involved several approaches. First, we used the data generated with the profiling array. We then used the

data from our PROMO analysis on the *ABCB1* promoter and identified additional putative TFs, particularly those with binding sites encompassing or in close proximity to SNPs identified (Speidel et al., 2018). Additionally, we performed a literature search to identify TFs known to bind to the *ABCB1* promoter (Labialle et al., 2002; Rigalli et al., 2015; Saeki et al., 2011). These combined approaches identified 20 TFs as potential targets for evaluation (Table VII).

While some siRNA-mediated TF knockdown demonstrated universal alterations in promoter activity across the different haplotypes evaluated, the knockdown effect of others was not always consistent across the haplotypes. For example, siRNA-mediated knockdown of p53 resulted in a decrease in *ABCB1* promoter activity with all haplotypes tested (Table VIII and Figure 8). However, AP2 siRNA knockdown led to a significant increase in promoter activity of haplotype 1 but no change in activity of any of the other 3 haplotypes. Knockdown of Pax-5 exhibited a differential effect depending on the haplotype where it led to a significant increase in activity of haplotype 1, a significant decrease of haplotype 29, and non-significant change with haplotypes 4 and 30, suggesting that Pax-5 can act as either an activator or a repressor, depending on the haplotype. Targeting TBP with siRNA induced a significant increase in promoter activity with haplotype 1 but had no effect with the other haplotypes tested. These findings are consistent with recent data from our laboratory, which indicated that different haplotypes alter the binding of TFs to the *MGMT* promoter and, subsequently, affect *MGMT* promoter activity and expression level (Xu et al., 2016).

The siRNA studies with haplotype 30, which has very low basal activity (Speidel et al., 2018), revealed that none of the tested siRNAs had an effect on promoter activity (Figure 8). Additionally, none of the tested siRNAs were able to bring the promoter activity to a level close to that of haplotype 1. A possible explanation may be that TFs other than those evaluated are involved in the regulation of the promoter activity of haplotype 30. Another explanation could be that other non-cis acting elements are driving the phenotype of this *ABCB1* promoter haplotype.

One additional, important observation is that the TF binding activity does not always parallel the regulatory function as determined by siRNA. For example, we found that knockdown of ER1 had no significant effect on promoter activity across the different haplotypes (Table VIII and Figure 8). While these observations are difficult to interpret, it is well-documented that eukaryotic gene expression regulation is combinatorial in nature involving multiple proteins and different signaling pathways (Pique-Regi et al., 2011; Vazquez-Santillan et al., 2015). Therefore, it is possible that changes in promoter sequence and the 3-D architecture, due to haplotypes effects, allow other TFs to compensate for the effect of the blocked TF and still drive the expression.

In conclusion, our data indicate that *ABCB1* promoter haplotypes can affect promoter activity by altering TF binding. Our data also show that the regulatory effects of TFs are haplotype-dependent. These results reinforce the need to further investigate the role of genetic variants in the context of haplotypes, rather than individual SNPs in order to provide better explanations for how genetic variants regulate genes and proteins expression. The information generated from our studies has significant translational implications, particularly for pregnant women treated with P-gp-substrate medications.

Chapter 4: Bisphenol A (BPA) and bisphenol S (BPS) differentially alter *ABCB1* promoter activity in a haplotype-dependent manner

INTRODUCTION

Bisphenol A is produced in mass quantities for use in polycarbonate plastics and many daily-use consumer products including water bottles, canned food linings, thermal receipt paper, and many more (Biedermann et al., 2010; Chen et al., 2016; Liao and Kannan, 2013). BPA is a xenoestrogen that is thought to mimic natural estrogens' action through binding to estrogen receptors and regulating expression of target genes (Bonefeld-Jørgensen et al., 2007; Richter et al., 2007). Evidence for the estrogenic effects of BPA was first identified in 1936 and, subsequently, health risks associated with chronic low-dose exposure have been reported (Dodds and Lawson, 1936; Krishnan et al., 1993; Rochester, 2013). BPA-exposure associated health effects have led to the emergence of many other bisphenol derivatives as BPA replacements and are now appearing at measurable quantities in many of the same goods (Liao and Kannan, 2013; Liao et al., 2012). One of the common substitutes is bisphenol S, with measurable amounts observed in surface waters, sewage effluent (Xuan et al., 2014; Yamazaki et al., 2015) and indoor dust at concentrations as high as 25% of that of BPA (0.34μg/g; 1.33μg/g) (Liao and Kannan, 2013). Chronic exposure to both of these chemicals has led to detectable, quantifiable amounts of BPA and BPS in human tissues and fluids, raising concern for potential health hazards resulting from exposure to these chemicals individually or as a mixture (Ikezuki et al., 2002).

During pregnancy, bisphenol exposure can significantly affect fetal development and increase the risk of adverse health consequences. Associations between BPA and reproductive dysfunction (Naderi et al., 2014; Sharpe and Skakkebaek, 1993), obesity (Vafeiadi et al., 2016; Valvi et al., 2013), developmental behavioral problems (Braun et al., 2011; Miodovnik et al., 2011; Wolstenholme et al., 2012) and cancer (Prins et al.,

2008; Sengupta et al., 2013) have been reported. Animal studies have also shown that BPA can diffuse across the placenta to the fetal circulation and into fetal tissue (Takahashi and Oishi, 2000). In humans, BPA has been detected at early gestation in maternal serum at ng/ml levels and in significantly higher concentrations (up to 5-fold and greater) in amniotic fluid, suggesting accumulation during fetal development (Ikezuki et al., 2002; Schönfelder et al., 2002).

As discussed previously, the human placenta serves as an interface for regulating xenobiotic distribution between the fetal and maternal blood. Here, the syncytiotrophoblast forms a physiological barrier that regulates drug and chemical transport between maternal and fetal circulations through a group of transporter proteins including P-gp encoded by *ABCB1* (St-Pierre et al., 2000). P-gp interacts with a wide variety of structurally diverse compounds (Ceckova-Novotna et al., 2006) and extrudes its substrates out from the trophoblasts back into the maternal circulation, thus limiting their entry into the fetal circulation (Nakamura et al., 1997). Of note is that steroid hormones, such as progesterone, although not transported by P-gp, are found to influence P-gp transport activity depending on concentration where both low inhibitory and stimulatory effects were observed (Orlowski et al., 1996; Shapiro et al., 1999). Because of their structural similarity to natural steroid hormones, it is conceivable that environmental estrogens such as BPA and BPS may also alter P-gp transport activity. Such an alteration would affect maternal and fetal exposure to many xenobiotics that are P-gp substrates. Consistent with this notion, using BeWo cells as a placental model, Jin and Audus (2005) reported that BPA indeed does have an influence on P-gp transport activity, where the found acute BPA exposure reduced calcein accumulation in BeWo cells.

Hemauer et al., 2010 and others (Hitzl et al., 2004; Tanabe et al., 2001) have previously demonstrated wide interindividual variability in placental P-gp expression and activity. While many environmental and physiological mechanisms could be involved, such variability was largely attributed to single nucleotide polymorphisms (SNPs) of the

ABCB1 gene which could affect P-gp expression and function (Hitzl et al., 2004; Hoffmeyer et al., 2000; Kim et al., 2001; Mölsä et al., 2005; Tanabe et al., 2001). There are many SNPs in the *ABCB1* promoter that could affect P-gp expression by affecting *ABCB1* transcription. While a few studies evaluated the effect of some of these SNPs on *ABCB1* expression, discrepant results were reported where the same SNPs were associated with increased and decreased expression and also with no effect (Ito et al., 2001; Lourenço et al., 2008; Sai et al., 2010; Takane et al., 2004; Tanabe et al., 2001). The lack of reproducibility between studies is not surprising because, in the genome, SNPs form defined haplotypes (combinations of SNPs). As discussed previously, we found that the phenotypic effect of a SNP is not always consistent but varies depending on its presence in different haplotypes (Speidel et al., 2018; Xu et al., 2014, 2016). As discussed previously, we determined the haplotype DNA sequences of the *ABCB1* promoter and found that haplotypes differentially regulate *ABCB1* promoter activity (Speidel et al., 2018). Here we investigate the interaction between BPA/BPS exposures with the different *ABCB1* haplotypes. Our working hypothesis is that BPA and BPS exposure, individually or in combination, affects *ABCB1* promoter activity in a haplotype dependent manner.

MATERIALS AND METHODS

Chemicals

4,4'-Isopropylidenediphenol (Bisphenol A) was purchased from Acros Organics (97%; Geel, Belgium). 4,4’-sulfonyldiphenol (Bisphenol S) was ordered from Sigma-Aldrich (98%; St. Louis, MO). All other chemicals and supplies were ordered from Fisher Scientific unless otherwise stated.

Bisphenol A and bisphenol S were dissolved in 50% ethanol (PHARMCO-AAPER, Toronto) and 50% Minimal Essential Medium with Earle’s Salts and L-Glutamine (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA). Serial dilutions were then performed which

allowed for a 10μM BPA or BPS working solution with a final ethanol concentration of 0.01%.

ABCB1 promoter haplotype construction

Previously, we generated luciferase reporters using the NanoLuc™ Luciferase vector system (Promega, Madison, WI) to determine the effects of *ABCB1* promoter haplotypes on promoter activity (Speidel et al., 2018). For the evaluation of acute exposure to BPA and BPS, we selected four of these previously generated *ABCB1* promoter haplotype constructs: haplotypes 4 and 29, representing high basal promoter activity; haplotype 30, representing low basal promoter activity; and haplotype 1, the ancestral haplotype, representing normal promoter activity (Speidel et al., 2018).

For the evaluation of chronic exposures to BPA and BPS, luciferase reporters for the same four haplotypes (1, 4, 29, and 30) were generated using the NanoLuc™ pNL1.2 vector (Promega, Madison, WI) as previously described (Speidel et al., 2018). Briefly, reporter constructs representing these haplotypes were generated by inserting appropriate promoter sequences into the pNL1.2 vector after double digestion with KpnI-HF and NheI-HF (New England Biolabs, Ipswich, MA). Then, 5α *E. coli* (New England Biolabs, Ipswich, MA) were transformed with the generated reporters and plated on 100 μg/ml ampicillin LB agar plates. Individual colonies were selected and grown in LB media containing 100 μg/ml ampicillin for 18-24 hours at 37ºC. Plasmids were isolated using the endotoxin free ZR Plasmid Miniprep™ - Classic kit (Zymo Research Corp, Irvine, CA) and quantified at 260nm using a DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE). The isolated plasmids were sequenced to verify the proper promoter haplotype was present in the reporter plasmid and to ensure no additional mutations were introduced during the preparation. Isolated plasmids were stored at -20ºC to maintain plasmid integrity until transfection.

Cell Culture

The human trophoblastic 3A placental cell line (CRL-1584) was purchased from American Type Culture Collection (ATCC, Manassas, VA) and used as the host cell for all bisphenol exposures and haplotype construct transfections. Cells were maintained in 75cm^2 flasks with Minimal Essential Medium (MEM) with Earle's Salts and L-Glutamine (Gibco Cat. 11095-080, Grand Island, NY) supplemented with 10% FBS in 5% CO_2 at 37°C. Cells were passaged at 85% confluency (2-3 days) and subcultured at a 3:1 ratio. A solution of 0.25% (w/v) Trypsin – 0.53mM EDTA was used to detach the cells for subculture or transfer to 6-well plates for transfection.

MTT assay for cytotoxicity and cell viability determination

The tetrazolium MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Acros Organics, Belgium) was dissolved in phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) at a concentration of 5mg/mL. The MTT solution was then filter-sterilized using a 0.22-micron syringe filter (Merck Millipore, Billerica, MA) and placed in a light-protected bottle. To each well of a 96-well plate containing human 3A placenta cells, 20μL of the MTT solution was added and the cells were incubated at 37°C for 3.5 hours. The media was removed and 150μL MTT solvent [4mM hydrochloric acid (Sigma-Aldrich, St. Louis, MO), 0.1% NP-40 substitute (US Biological, Salem, MA) in isopropanol (EMD Millipore, Billerica, MA)] was added. The plate was then covered with aluminum foil and incubated on a shaker at room temperature for 15 minutes. Absorbance at 590nm was measured in triplicate using a Tecan GenIOS Pro plate reader (Tecan, Durham, NC)

Acute Bisphenol Exposures and Transfection

Untreated placental 3A cells were trypsin digested and transferred into 6-well plates at low confluency (≤40%). After allowing the cells to adhere to the plate (14-18 hours), cells were transfected using a mixture of 600ng pNL 1.2 haplotype promoter

plasmid DNA, 66ng firefly luciferase control plasmid pGL4.53 PGK (Promega, Madison, WI) and 2μL Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA). After transfection, cells were allowed to recover for 36-48h and were then exposed to BPA, BPS or a mixture of both bisphenols dissolved in ethanol with a final concentration in the cell media 0.00003%. For the initial acute exposures, the experiment was carried out for 90 minutes with samples taken at t=0, 15, 30, 45, 60 and 90 minutes. At each time point, cells were lysed with 500μL Passive Lysis Buffer (PLB, Promega, Madison WI) and effects of acute exposure were measured using the NanoGlo Dual-Luciferase® Assay (Promega, Madison, WI). For all subsequent exposures, samples were collected after 15 minutes.

Chronic Bisphenol Exposures and Transfection

Human 3A placental cells were split into six T-10 flasks at low confluency ≤20% and chronically exposed to BPA or BPS individually or as a mixture of both bisphenols dissolved in a final culture medium ethanol concentration of 3×10^{-5} %. Complete MEM with BPA and/or BPS added was replaced every day for 12 days (~4 passages).

After 12 days of bisphenol exposure, cells were trypsin digested, and moved to 6-well plates. Bisphenols were then added to their respective wells, and after allowing 12-16 hours for cells to adhere, transfections were performed in the 6-well plates between passages 9-10 with cells at low confluency (≤ 40%). For each transfection, cells were treated with a mixture of 600ng haplotype plasmid DNA, 66ng pGL-PGK vector and 2μL Lipofectamine 3000 transfection reagent. Six hours after transfection, the media was replaced and bisphenols were added. Treatment continued with another media change/exposure occurring 24 hours after transfection. Cells were harvested 36-48 hours after transfection using 500μL PLB and effects of chronic exposure were measured using the NanoGlo Dual-Luciferase Assay.

NanoGlo Dual-Luciferase Assay to determine the effect of bisphenols on different *ABCB1* promoter haplotypes' activity

The NanoGlo Dual-Luciferase Reporter Assay was performed according to the manufacturer's instructions. Briefly, 3A cells were harvested using 500µL Passive Lysis Buffer and the lysates were then used immediately or stored at -80°C for later analysis. Luciferase activity was measured according to the manufacturer's recommendations, and luminescence was measured in triplicate using a Tecan GenIOS Pro plate reader (Tecan, Durham, NC). Luminescence was measured as relative light units after normalization against the co-transfected Firefly luciferase. Each experiment was repeated at least three times.

Statistical Analysis

The Shapiro-Wilk normality test was used to determine the distribution of the data for all subsequent statistical analyses. The non-parametric Kruskal-Wallis one-way analysis of variance was used to compare the absorbance values corresponding to the cellular viability of placental 3a cells exposed to bisphenols. Posthoc analysis using the rank based Dunnett's Method was used to compare the absorbance values for the exposed groups with the non-treated control. One-way analysis of variance was used to compare the effects of acute and chronic BPA and BPS exposure on *ABCB1* promoter activity. Posthoc analysis using the Holm-Šídák method was used to compare the exposed groups with their respective haplotype's ethanol control. P-values <0.05 were considered significant.

RESULTS

Effect of BPA and BPS on placental 3A cell viability

The human placental 3A cell line has not previously been used to test the effects of exposure to bisphenols. Furthermore, the effects of the solvents used to dissolve the bisphenols evaluated on the viability of the 3A cells are currently unknown. To test for

cytotoxicity, the well-established colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used (Mosmann, 1983). The MTT assay measures the activity of the mitochondrial reductase enzymes, which convert the yellow tetrazolium MTT to a purple formazan. This reduction only occurs when the mitochondrial reductase enzymes are active. Thus, the conversion of tetrazolium to formazan can be directly related to the number of viable cells.

Because of the low solubility of BPA and BPS in water, we tested two solvents (ethanol and dimethyl sulfoxide [DMSO]) at different concentrations to dissolve both compounds. We found a low final concentration of either ethanol or DMSO to be adequate for completely dissolving both compounds without a measurable effect on cell viability (Figures 7A and 7B. For subsequent experiments, ethanol was chosen since it has been used in previous studies (Viñas and Watson, 2013a, 2013b). To determine if BPA and BPS, individually or in combination, exert cytotoxic effects on placental 3A cells following acute and chronic exposures, cells were exposed to the bisphenols for 15 minutes or 12 days, respectively. Acute exposures were tested for both compounds at concentrations ranging from 0.5 to 500 nM, while chronic exposure was carried out at concentrations from 5 to 30 nM for both compounds.

Effect of acute exposure to BPA and BPS on *ABCB1* promoter activity

We conducted an initial time course study to identify a time point suitable for the determination of the effect of acute exposure to BPA and BPS on *ABCB1* promoter activity in placental 3A cells. Cells transfected with the Nanoluciferase reporter plasmid containing the ancestral *ABCB1* haplotype 1 were exposed to 50nm BPA or BPS and cell lysates were collected at time intervals over a period of 90 minutes (Figure 10A). The data indicated an initial increase in chemiluminescence, indicative of increased *ABCB1* promoter activity, at 15 min following exposure to either BPA or BPS followed by a decrease and another less prominent increase around 45 minutes. This multimodal curve

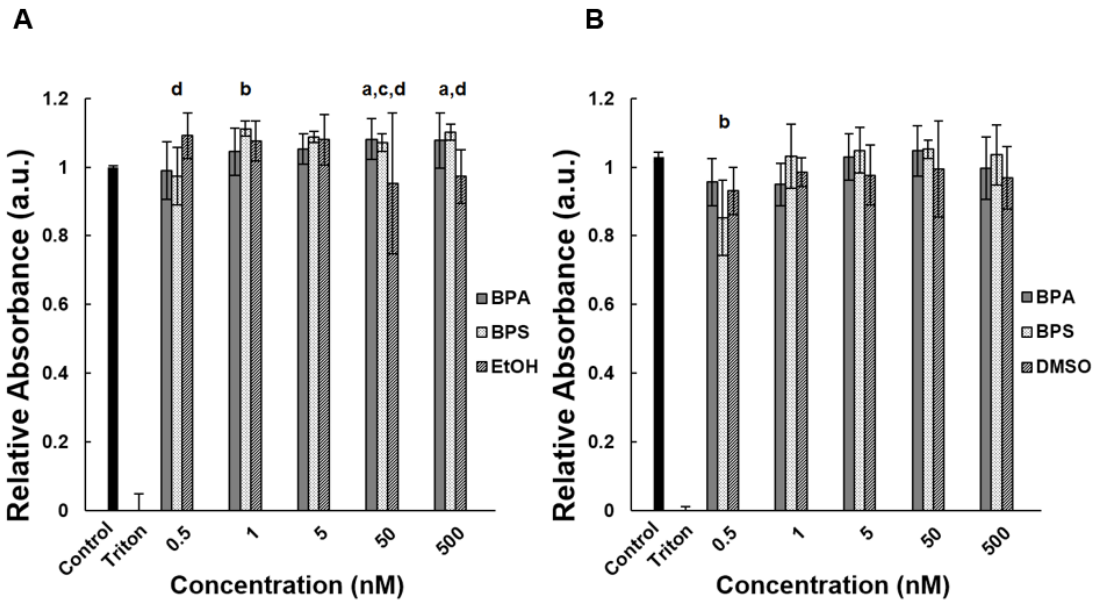


Figure 9: Solvent ctyotoxicity for placental 3A cells. The cytotoxicity of two solvents (A)Ethanol and (B)DMSO were tested in placental 3A cells using the MTT assay. a. $p<0.05$ for BPA vs Control, b. $p<0.05$ for BPS vs Control, c. $p<0.05$ for BPA vs solvent control, d. $p<0.05$ for BPS vs solvent control.

was observed for both xenoestrogens, with the maximum signal observed for both bisphenols at 15 minutes. To maximize the observable differences in response to bisphenols, the 15-minute time point was therefore chosen for subsequent experiments.

The concentration of BPA and BPS to be used in our investigation was determined by conducting a dose-response study with BPA and BPS concentrations ranging from 0.5nM to 500nM (Figure 10B). Cells transfected with the Nanoluciferase reporter plasmid containing the ancestral *ABCB1* haplotype 1 were exposed for 15 minutes to BPA or BPS and the effect of the exposures on *ABCB1* promoter activity was determined. Within this concentration range, there was a significant increase ($p<0.001$) in the luminescence (indicative of increased *ABCB1* promoter activity) when cells were exposed to 50nM BPA. In contrast, no statistically significant change in promoter activity was observed with any of the BPS concentrations tested. Based on these results, the concentration of 50nM for BPA was chosen for subsequent studies since it produced significant effect on promoter activity. For subsequent BPS studies, we chose the concentration of 0.5nM for BPS, the lowest concentration tested, since it is comparable to

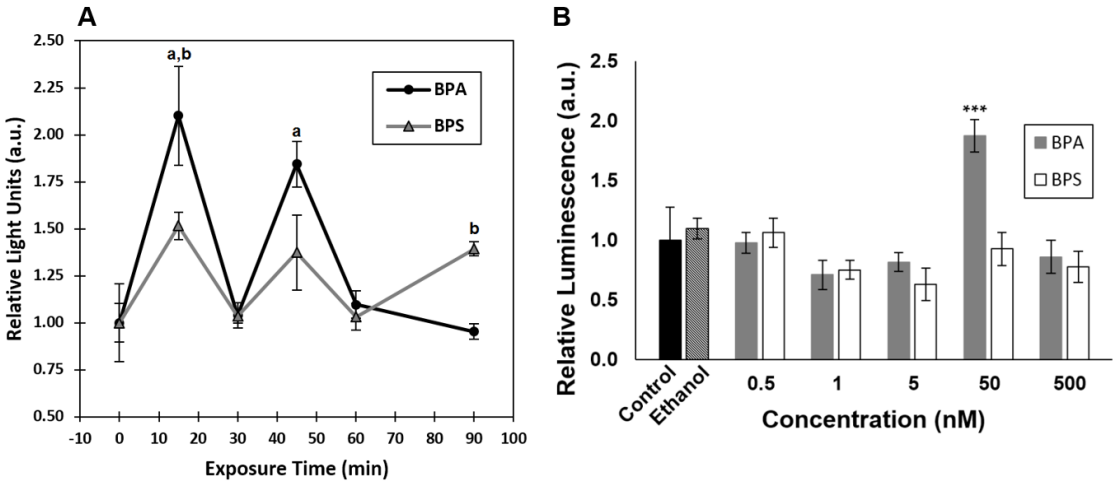


Figure 10: Effect of acute bisphenol exposure on *ABCB1* promoter luciferase activity. (A) Placental 3A cells were exposed to 50nm BPA or BPS to determine the exposrure time with highest sensitivity. **(B)** Concentration of BPA and BPS for exposure were varied over 1000x to find the concentrations yielding the highest sensitivity. Data are reported as mean ± SEM. a $P<0.05$ for BPA group vs. BPA time = 0 min, b $P<0.05$ for BPS group vs BPS time = 0 min, ***$P<0.001$.

physiological concentrations observed in humans.

To test for potential gene-exposure interaction on *ABCB1* promoter activity, we exposed the four different *ABCB1* promoter haplotypes to 50nM BPA alone or 0.5nM BPS alone. Exposure to 50nM BPA alone had no significant effect on promoter activity for all haplotypes tested (Figure 11A). In contrast, exposure to 0.5nM BPS alone caused a significant reduction in promoter activity that was haplotype dependent. As shown in Figure 11B, while haplotype 1 showed no difference in promoter activity, haplotypes 4, 29 and 30 all displayed significant decreases in *ABCB1* promoter activity.

Because human exposure to these xenoestrogens occurs more commonly as a mixture, we also evaluated the effect of combined acute exposure to both BPA and BPS on *ABCB1* promoter activity. Our data indicate that co-exposure to a mixture of BPA and BPS at the concentrations of 50 and 0.5nM, respectively, had no effect on promoter activity regardless of the haplotype (Figure 11C). An interesting observation was the observed effect of ethanol on haplotype 30 promoter activity. With this haplotype, $3x10^{-5}$ % ethanol induced a significant ($p<0.05$) 23% increase in promoter activity compared to the effect observed with haplotype 1, suggesting a haplotype-dependent effect not only in

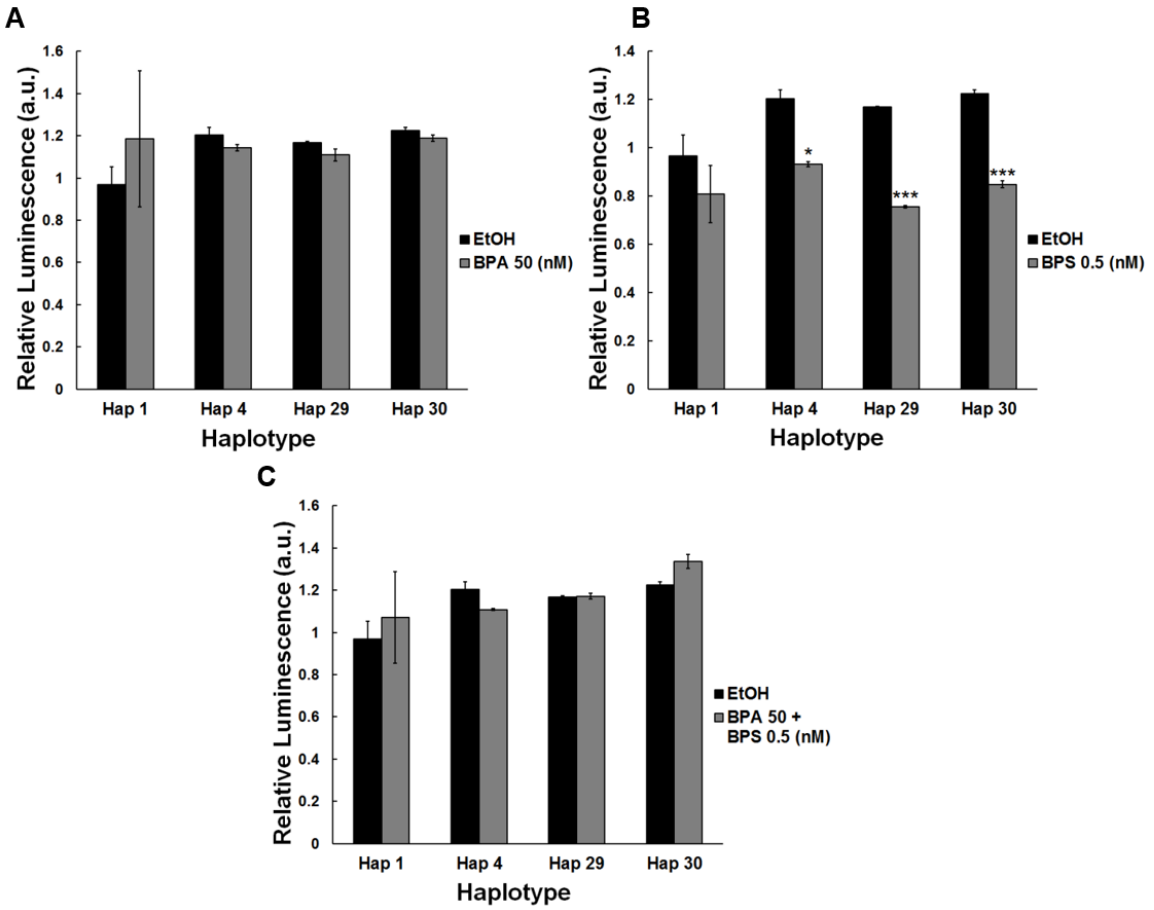


Figure 11: Haplotype specific response to acute BPA and BPS exposures. Luficerase reporters containing *ABCB1* promoter haplotypes were acutely exposed to (A) 50nM BPA, (B) 0.5nM BPS, or (C) a mixture of 50nM BPA and 0.5nM BPS. Data are reported as mean ± SEM. *P<0.05, ***P<0.001.

response to bisphenols but potentially in response to other chemicals such as ethanol.

Chronic BPA and PBS Exposure and *ABCB1* Promoter Activity

Since pregnant women are chronically exposed to BPA and BPS individually or as a mixture, we tested the effect of chronic exposure to BPA alone (5nM; Figure 12A), BPS alone (0.3nM; Figure 12B), and both as a mixture (5nM BPA:1.5nM BPS; Figure 12C).

Our data indicate a haplotype dependent response to these exposures. While exposure to BPA alone induced a significant increase in promoter activity of haplotypes 1, 4 and 29 ($p<0.05$), it did not affect the promoter activity of haplotype 30 (Figure 12A). Similarly, this haplotype-exposure interaction was observed with BPS. While chronic exposure to 0.3nM BPS induced the promoter activity of haplotypes 1, 29 and 30 ($p<0.05$), it did not affect the activity of haplotype 4 (Figure 12B). Exposure to the BPA and BPS mixture, however, induced a significant increase ($p<0.05$) in *ABCB1* promoter activity for all haplotypes tested (Figure 12C).

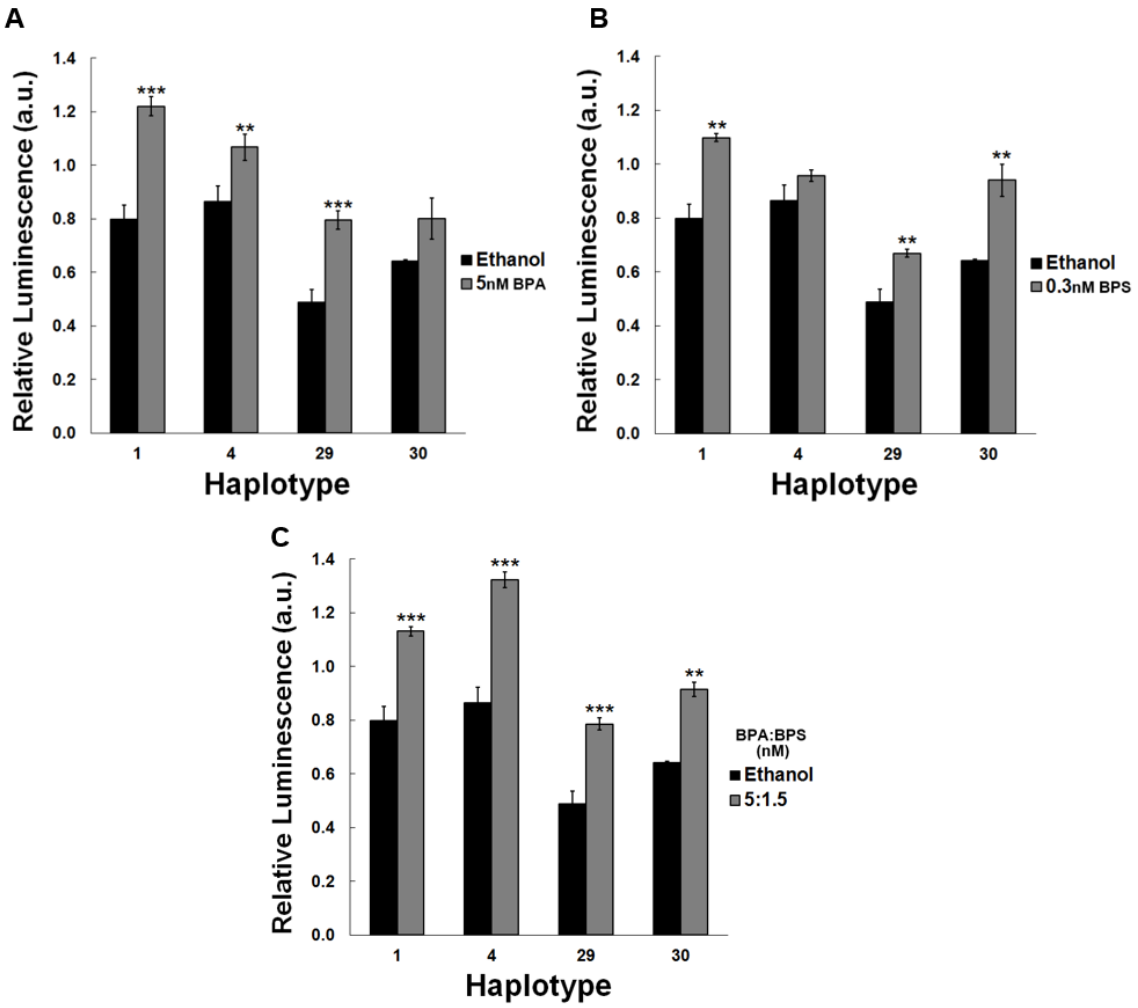


Figure 12: Haplotype specific response to chronic BPA and BPS exposures. Luficerase reporters containing *ABCB1* promoter haplotypes were chronically exposed to (A) 5nM BPA, (B) 0.3nM BPS, or (C) a mixture of 5:1.5nM BPA:BPS. Data are reported as mean ± SEM. **P<0.01, ***P<0.001.

DISCUSSION

Encoded by the polymorphic *ABCB1* gene, P-gp is one of the most important efflux transporters in human placenta. This transporter limits the entry of its substrates into fetal circulation by extruding them from the fetal trophoblasts back into the maternal circulation (Nakamura et al., 1997). Alteration in expression and/or activity of placental P-gp can, have serious health consequences on the fetus. Recently, we reported that *ABCB1* promoter haplotypes affect its promoter activity and could thus play a significant role in the expression of placental P-gp (Speidel et al., 2018). In the current investigation, we evaluated the interaction between *ABCB1* promoter haplotypes and acute and chronic BPA and BPS exposures.

We used physiologically relevant concentrations of both BPA and BPS in our studies. Data on serum levels of BPA in humans varies considerably between different studies, because the materials used for the analysis could have potentially contained trace amounts of BPA, increasing the potential for contamination (Calafat et al., 2013; Twaddle et al., 2010; Ye et al., 2013). In non-pregnant subjects, the observed serum BPA levels varied from 0.13nM to 10.95nM (Vandenberg et al., 2010, 2012). In maternal and fetal cord blood, the concentrations were much higher, ranging from 2.0 to 39.6nM (Vandenberg et al., 2010, 2012). In term human placenta, concentrations were found to be even higher, with concentrations reported from 4.4nM to 459.5 nM (Schönfelder et al., 2002; Vandenberg et al., 2010, 2012). A recent study evaluating BPA and BPS concentrations in urine of cashiers found that BPS levels were between 12% and 38% of urine concentrations of BPA (Thayer et al., 2016). Because of the structural and chemical property similarities of the two molecules, the ratio of BPS to BPA in the serum should be similar to the ratio in urine levels. In studies investigating serum BPS, concentrations were found much lower than BPA, and found at concentrations between 0.04nM and 0.45nM (Thayer et al., 2016). Based on these observations, we used a BPA concentration of 50nM for acute exposure and 5nM for chronic exposure (10% of the chronic dose). For

BPS, 0.5nM was used for acute exposure and the 0.3nM concentration for chronic BPS exposures. For mixed BPA/BPS exposures, we kept the ratio at a 5:1.5 to the ratio reported (Thayer et al., 2016).

Our data indicate that both acute and chronic BPA and/or BPS exposures were not cytotoxic to 3A placental cells at concentrations from 0.5 to 500nM. This is consistent with other reports on other placental cell lines and non-placental cell lines. In these studies, the cytotoxic effects of BPA were not observed until the concentrations tested were above 150μM, 300-fold higher than the concentrations used in our study (Jin and Audus, 2005; Lee et al., 2004). For BPS, the cytotoxic effects were not observed until concentrations reached above 100μM (Fic et al., 2013; Lee et al., 2013), 200-fold higher than the concentrations used in our study.

Due to the important role P-gp plays in protecting the fetus from exposure to xenobiotics during pregnancy, we evaluated the effect of BPA and BPS exposures on the promoter activity of its *ABCB1* encoding gene. The effect of acute exposures on *ABCB1* promoter activity were measured using the Nanoluciferase-PEST (NlucP) plasmid. This plasmid produces a luciferase protein with an attached c-terminal peptide sequence of Proline-Glutamic Acid-Serine-Threonine (PEST) which has been demonstrated to enhance proteosomal degradation (Li et al., 1998). The half-life of the NlucP protein varies between cell lines (~25-30 min), but this short half-life and strong signal result in the protein as a good candidate for acute exposure studies (Hall et al., 2012). The effect of BPA/BPS exposure on the activity of different *ABCB1* promoter haplotypes has not yet been evaluated. However, several other xenoestrogens have been evaluated for their effect on *ABCB1* mRNA expression and the resulting P-gp expression. Both the synthetic estrogen ethynyl estradiol and the phytoestrogen genistein were reported to alter the expression of *ABCB1* mRNA as well as the P-gp protein expression (Arias et al., 2014).

In our study, we did not observe a significant haplotype effect on *ABCB1* promoter activity in placental 3A cells following acute exposure to BPA. However, when placental 3A cells were acutely exposed to BPS alone, there were significant decreases

($p<0.05$) in promoter activity of 3 of the 4 *ABCB1* haplotypes tested (haplotype 4, 29, and 30). These data indicate that structurally similar xenoestrogens do not exert the same effects on *ABCB1* promoter activity. These data also indicate that the same concentrations of a bisphenol can induce a different response on promoter activity depending on the haplotype, strongly suggesting a possible haplotype-exposure interaction.

When cells were exposed to a mixture of the two tested bisphenols at the same concentrations as the individual tests, no significant difference in *ABCB1* promoter activity was observed depending on the haplotype. Although exposure to BPS alone had a significant effect on promoter activity, the difference in response could be a result of the presence of BPA in the mixture. At a concentration 100 times higher than that of BPS, it is possible that BPA could be saturating potential target sites thus masking the effect of BPS. BPA has been shown to be a substrate for P-gp in the intestine *in vitro* (Yoshikawa et al., 2002) and to stimulate the drug efflux mechanism in a human placental cell line (BeWo, a choriocarcinoma cell line), indicating the possibility of regulation through P-gp (Jin and Audus, 2005).

For chronic BPA and BPS exposures, the regular Nluc plasmid (no PEST domain) was used because the response would be measured after several days rather than hours. In contrast to acute BPA exposure, our data indicate that chronic exposure to 5nM BPA, a concentration 10 times lower than the acute concentration tested, induced a significant increase ($p<0.05$) in *ABCB1* promoter activity for three of the four haplotypes evaluated (haplotypes 1, 4 and 29). Chronic exposure to BPS also induced a significant increase in promoter activity that was also haplotype-dependent. An increase in activity was observed with haplotypes 1, 29 and 30 but not haplotype 4. The same significant increase in promoter activity ($p<0.01$) was observed with chronic exposure to the BPA and BPS mixture for all haplotypes evaluated. These data with chronic exposure contrasts those observed with acute exposure to the same compounds, indicating that promoter activity in

response to bisphenols is not only haplotype-dependent, but is also exposure-dependent (acute vs. chronic).

Our data suggest there may be a difference in the mechanisms by which acute and chronic exposures alter the promoter activity of *ABCB1* haplotypes. For example, it is possible that chronic exposure to BPA or BPS, alone or in mixture, differentially alter transcription factor (TF) binding profile of *ABCB1* promoter. We have previously identified several TF binding sites in the *ABCB1* promoter that differed depending on the haplotype (Speidel et al., 2018), and chronic exposure could induce the translation of select alternate/additional TFs that could alter *ABCB1* expression depending on the haplotype. Other potential mechanisms include potential epigenetic modifications, such as histone modification or alteration of methylation patterns on the *ABCB1* promoter. Within the *ABCB1* promoter, there are two or three CpG islands (Li et al., 2015; Takai and Jones, 2003), regions rich in cytosine and guanosine DNA residues, which are often targets for DNA methylation which results in decreased activity of the promoter.

Alternatively, the effect observed with acute exposures could be due to different mechanisms. For example, acute exposures could exert their observed effects by initiating non-genomic signaling events that activate modifying enzymes (kinase, acetylase, methyltransferase) that would in turn inactivate certain TFs that normally bind to *ABCB1* promoter to induce (or repress) expression or activate alternative transcription factors that could activate (or repress) expression in a haplotype-dependent manner. This non-genomic signaling has been demonstrated with BPA and BPS exposures in pituitary cells measuring c-Jun-N-terminal kinases as well as extracellular signal-regulated kinases (Viñas and Watson, 2013a, 2013b). To determine if non-genomic signaling through these kinases is responsible for the rapid response in *ABCB1* promoter activity after acute BPA and BPS exposure, inhibitors against the AP-1 TF complex (Fos and Jun) could be used to effectively eliminate the Jun and Fos signaling pathways.

In summary, we show the ability of both BPA and BPS to alter the activity of *ABCB1* promoter in human placental 3A cells, indicating they could significantly impact

placental P-gp levels. We also demonstrated that the effects of BPA and BPS on *ABCB1* promoter activity depend on the *ABCB1* promoter haplotype. The results of our study have important health implications for pregnant women and their fetuses. The significant decrease in *ABCB1* promoter activity resulting from acute BPS exposure observed with three of four haplotypes tested could indicate reduced placental P-gp levels with these haplotypes in mothers exposed to this compound. Such decreases could increase fetal exposure to xenobiotics that are P-gp substrates. In contrast, *ABCB1* increased expression observed with chronic exposure to BPA and BPS may lead to placental P-gp over-expression with certain haplotypes, ultimately altering the equilibrium of endogenous P-gp substrates which minimally but necessarily cross the placenta. The importance of P-gp in the placenta warrants further studies into the mechanisms by which exposure to bisphenols alter *ABCB1* expression in different haplotypes.

Chapter 5: Conclusions

The data generated from this study provides important information on the role of genetic variability on *ABCB1* promoter activity. We clearly demonstrated that the effect of an individual SNP on *ABCB1* promoter activity is not always consistent but differs in a haplotype-specific manner. This information validates our hypothesis that *ABCB1* haplotypes, rather than individual SNPs, affect its expression and could thus play a significant role in the expression of placental P-gp. Additionally, our results clearly underscore the need for further investigations to elucidate the additional mechanism(s) by which *ABCB1* haplotypes exert their effects on promoter activity.

In the first part of our study, we identified 12 SNPs in the *ABCB1* promoter region using our sample population of 100 placentas (Table I). Using the generated data, PHASE inferred 28 potential haplotypes (Table II). In our population, we found two additional haplotypes that were not predicted by PHASE (Table II). The lack of concordance is likely attributable to the small sample size used in our study. The results on the baseline activity of the different promoters demonstrated a high variability, and confirmed that individual SNPs are not the best method for predicting the phenotypic outcome of genetic variance. Rather, examining haplotypes would provide a more accurate assessment of genotypic effects. This is clearly demonstrated in Figure 4 where haplotype 29, which consists of two SNPs (G-1459aA; rs12720464 and T-129C; rs3213619), had a much higher activity than either SNP alone. When G-1459aA was found individually, as haplotype 16, or SNP T-129C was found individually (as the generated construct M1), each individually exhibited different effects on promoter activity compared to when both were found together, as haplotype 29. Additionally, SNP T-129C appears in multiple haplotypes, where the *ABCB1* activity was determined to be both increased and decreased compared to haplotype 1.

One potential mechanism to explain the differences in haplotype promoter activity is the haplotype-dependent alterations in TF binding we observed in our study. Results

from our *in silico* bioinformatics analysis using PROMO identified 80 different TFs that could putatively bind to the *ABCB1* promoter region. Additionally, these TFs often had multiple binding sites within the promoter, with some having more than 50 putative binding sites. Therefore, our results validated our hypothesis that haplotypes could alter a TF's binding. The results of our study demonstrate the complexity of the *ABCB1* promoter transcriptional regulation, establishes the need for further identification and validation of TF binding motifs, and reveals additional transcriptional regulation targets specific to *ABCB1*.

Results from the TF profiling array and the siRNA knockdown studies further validated the possibility of TF binding alterations as a mechanism through which haplotypes exert their effects. In Figure 7 and Table VI, our results clearly depict a haplotype-dependent difference in TF binding for 48 common transcription factors. This assay does not capture all of the TFs binding to the promoters, but it does give a reasonable snapshot into some of the TFs that would potentially regulate the *ABCB1* promoter. Knockdown of some of these TFs as well as others identified in the literature and from our *in silico* analysis with targeted siRNAs (Figure 8 and Table 7) demonstrated there are many TFs that bind the promoter, and knockdown of some TFs caused differential response depending on the haplotype. For example, Pax-5 caused an increase in promoter activity for haplotype 1, but it caused a decrease in activity for haplotype 29. This demonstrates the need for further in-depth studies into the regulation of the gene, and also identifies potential targets for modifying the activity of P-gp which could ultimately be used to induce or repress gene expression, an approach that could be used in pharmacotherapy in the future.

Our studies also showed that BPA and BPS have little to no cytotoxicity in placental 3A cells, with no negative effects observed up to our highest tested concentration of 500nM (Figure 9A and 9B). This is in agreement with the literature which shows a low cytotoxicity for BPA and BPS (Jin and Audus, 2005; Lee et al., 2004)j. Our results also demonstrated that with exposure to BPA and BPS, individually

or in mixtures, there was a haplotype-specific response in *ABCB1* promoter activity. These observations were detected with both acute and chronic exposures (Figures 8 and 9). The results also demonstrated a concentration dependence for the xenoestrogen's effects. This type of non-monotonic dose-response curve has also been observed in other test systems investigating very low-dose estrogenic-signaling (Viñas and Watson, 2013a, 2013b; Viñas et al., 2013). One of the potential mechanisms behind the non-monotonic dose response is that different receptors have varying binding affinity to the BPA and BPS, and that at different concentrations the bisphenols are able to bind to, and activate different receptors and their signaling cascades.

Interestingly, even though all of the haplotypes have the same half-estrogen response element and no SNPs were found to alter the ER binding site, the response to the xenoestrogenic bisphenols was haplotype dependent. Even though this regulation is complex, these effects could be potentially explained by the ER binding with the BPA/BPS and assembling with accessory proteins, such as Sp1. One of the Sp1 binding sites was shown to contain a SNP (Figure 6) and the Sp1 proteins have also been shown to regulate the *ABCB1* promoter. This work provides the basis for future studies into the mechanisms through which acute and chronic exposures to BPA dysregulate gene expression, and the genetic influences altering this expression which expands beyond *ABCB1* to many other important genes, that are not currently being examined.

Overall, the information from the studies performed herein could have substantial implications for future basic science research and translational use by clinicians. The mechanistic studies performed with the TF binding and TF knockdown demonstrate the extensive, redundant systems responsible for transcription regulation. This paves the way for future studies to investigate additional TF regulation as well as to examine the non-cis acting mechanisms that may be responsible for the differential, haplotype-dependent expression of *ABCB1*. For clinicians, understanding the genetic factors that regulate the expression of P-gp could enhance the efficacy of treating a pregnant woman. The results from the current studies identify the benefits from haplotyping the placental/fetal *ABCB1*

gene to define haplotypes with high expression or low expression before treating a pregnant woman with any P-gp substrate drug. Furthermore, we identified three promoter haplotypes that greatly affect *ABCB1* promoter activity. One of these haplotypes has nearly no activity, and could potentially result in no P-gp expression. This would predispose the fetus to high levels of exposure from many substrate medications prescribed to the mother. Alternatively, the two high activity promoters could potentially require exceedingly high and potentially dangerous doses of therapeutics needed for transplacental fetal therapy.

FUTURE STUDIES

Transfection of *ABCB1* promoter haplotype constructs into multiple cell lines

When comparing our results from transfection of *ABCB1* promoter haplotypes into placenta 3A cells to those obtained from using other cell lines, the results are drastically different. In our studies, we transfected the glioblastoma cell line U87-MG, and found that all of the haplotypes had very high activity, and that the promoter activity of a haplotype in one cell type did not correlate with the same haplotype promoter's activity in the other cell type. Additionally, this was also observed when we compared our results with a previous study that used human HepG2 liver cells. Based on the activities of the different *ABCB1* haplotype promoters in different cell types, some of the regulatory elements could potentially serve as therapeutic targets for *ABCB1* dependent chemotherapeutic resistance in cancer as well as for other P-gp associated diseases including Crohn's Disease, inflammatory bowel syndrome and ulcerative colitis (Brant et al., 2003; Daniel et al., 2007).

Stable *ABCB1* haplotype transfections

We successfully used transient transfection of a reporter construct carrying an *ABCB1* promoter haplotype to determine the effect of the haplotype on the basal activity

of the promoters, and we also used transient transfection of a short-lived reporter protein to observe the effects of acute and chronic bisphenol exposure. To determine if the *ABCB1* promoter haplotype activity results in changes in mRNA levels as well as levels of P-gp expressed, stable haplotypes need to be created. With the CRISPR methods now available for research, one could generate the different haplotypes in a cell culture. This would allow for similar tests to be performed as were conducted within this dissertation though instead of indirectly measuring luciferase activity, one would be able to use P-gp expression levels and *ABCB1* mRNA as endpoints, directly measuring the effect of the *ABCB1* promoter haplotype.

Haplotype-dependent *ABCB1* promoter epigenetic alterations

Active genes have many common epigenetic characteristics including low promoter DNA methylation (Tate and Bird, 1993) and increased histone acetylation (Brownell et al., 1996). Methylation of the *ABCB1* promoter has been shown to correlate with the transcriptional activity of the gene (El-Osta et al., 2002). In some diseases, alterations in the methylation status for the promoter of the *ABCB1* gene have been characterized, including neuroblastoma (Qiu et al., 2007), leukemias (Kantharidis et al., 1997; Nakayama et al., 1998) and ulcerative colitis (Tahara et al., 2009). Additionally, in Chinese subjects, there has been found a correlation between *ABCB1* promoter CpG island methylation and polymorphisms in the coding region (Jiang et al., 2008; Wu et al., 2015). However, the effects of haplotypes on the status of promoter DNA methylation and histone acetylation are unknown. Further study into the effects of the haplotypes is warranted, as DNA variants could potentially cause hyper- or hypomethylation, altering the expression of P-gp and the equilibrium of its substrates.

Next generation sequencing of whole *ABCB1* gene

With the advances that have come in DNA sequencing over the last 20 years, it has become easier to sequence much larger segments of DNA. For *ABCB1*, which is over

100kb in length, this allows for sequencing of the entire gene. Within *ABCB1*, there are several coding SNPs including C1236T, G2677T/A and C3435T which have been associated with alterations in P-gp expression or activity (Hitzl et al., 2004; Hoffmeyer et al., 2000; Xuan et al., 2014). We, and others have also demonstrated that several promoter haplotypes or SNPs are also associated with alterations in the promoter activity of *ABCB1* (Sai et al., 2006; Speidel et al., 2018; Takane et al., 2004). By utilizing the capabilities of high-throughput sequencing, haplotypes could be created that would represent the SNPs present in the whole gene. By performing this type of analysis, some of the discrepancies between studies could be ameliorated, a more accurate definition of the haplotypes could be inferred, and the information could be used to more individually treat disease in pregnant mothers, their developing fetuses, and diseases in non-pregnant individuals where P-gp expression/activity has been implicated in the severity of disease.

Appendix

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FIGURE 1A.

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Vita

Jordan Thomas Speidel was born to parents Donna Marie Speidel and Craig Thomas Speidel in Bismarck, North Dakota on July 10, 1986. The family relocated to South Dakota and Jordan graduated from Watertown High School. He then attended South Dakota School of Mines and Technology where he earned a Bachelor of Science degree in Chemistry in 2008. There, during his senior year, his research career began in the nano- and polymer chemistry laboratory of Dr. Zhangtao Zhu. After graduating, he continued to build on his research experience in the microbiology laboratory of Dr. Rajesh Sani. Jordan then went on to take a position as a laboratory technician for Microbac Laboratories, Inc. in Boulder, Colorado. In 2011, Jordan matriculated to Colorado State University in Fort Collins, Colorado, where he earned his Master of Science degree in Biochemistry and Molecular Biology in 2012. Jordan then joined the Graduate School of Biomedical Sciences at the University of Texas Medical Branch (UTMB) at Galveston. While there, Jordan joined the laboratory of Dr. Sherif Abdel-Rahman, PhD to study the influence of genetic variance on gene expression. At UTMB, Jordan received many honors. One of these honors occurred in 2013 when Jordan was awarded an NIEHS T32 pre-doctoral fellowship in environmental toxicology. During the fellowship, Jordan was afforded the opportunity to be a course leader and to teach an undergraduate toxicology course at Texas A&M University in Galveston. Additionally, Jordan has presented his work at numerous local and national conferences, receiving multiple accolades for his work.

Education

BS Chemistry May 2004, South Dakota School of Mines & Technology, Rapid City, SD
MS Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO

Publications

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