Regulation, Function, and Clinical Relevance of ABCG2 in Prostate Cancer

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ABCG2 is known to efflux folates and dihydrotestosterone, both of which are important for prostate tumor growth. Recently the Q141K variant of ABCG2, which decreases the function of ABCG2, was found to have an impact on survival time in prostate cancer (PCa) patients. In addition, ABCG2 may be involved in cellular resistance to docetaxel, a chemotherapeutic used to treat late-stage PCa. Because ABCG2 function appears to be correlated with survival times and may transport molecules important in prostate growth and cancer treatment, it is crucial to understand its expression, function, and regulation in PCa.

An observed CpG island in the ABCG2 promoter indicates the potential for regulation by DNA methylation. Accordingly, transcription of ABCG2 was increased after treatment with a DNA methyltransferase inhibitor. Furthermore, PCa cell lines grown in a folate deficient environment increased the expression of ABCG2, which was due to hypomethylation of the ABCG2 promoter.

Patients with recurrent PCa carrying the Q141K variant of ABCG2 had a significantly shorter time to PSA recurrence post-prostatectomy than patients carrying the wild-type allele. Transport studies showed that the Q141K variant expressing HEK293 cells retained more folic acid than their wild-type counterparts, and surprisingly, patients carrying the Q141K variant had decreased *systemic* folate levels. Furthermore, while it was known that docetaxel-treated PCa patients carrying the Q141K variant have a longer survival time, we found this is likely because ABCG2 plays a role in docetaxel efflux. PCa cell lines were highly resistant to forced expression of exogenous ABCG2. This was due in part to induced expression of two miRNAs known to repress ABCG2 in other cancer cell lines.

In conclusion, the Q141K variant appears to play opposing roles in prostate cancer. By decreasing folate efflux, intracellular folate levels and cell proliferation increase, but docetaxel

treatment can increase tumor-cell drug sensitivity, which increases patient survival time. Our enhanced understanding of the varied docetaxel response among patients as well as evidence that ABCG2 is an as of yet unforeseen, major player in regulation of systemic folate levels may have implications in determining a personalized treatment plan for a cancer patient based on their ABCG2 genotype.

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PREFACE

ACKNOWLEDGEMENTS

This work is dedicated to my mom, Betsy Gonyea and my grandma, Marjorie Gonyea. These two women inspired me to understand the intricacies of cancer. Their cancer diagnoses prompted me to pursue a career in which I might have a slight effect on how cancer patients are treated.

I would not be here today if not for my advisor, Dr. Denise O'Keefe. Thank you for all of the encouragement you have given me since I joined your lab. You have encouraged me to apply for pre-doctoral fellowships, attend conferences, test out new techniques, and to think for myself and not rely on what others have done in the past. You pushed me to become an independent scientist and let me guide the direction of this research. Words cannot fully express how appreciative I am to you for all the sacrifices and work you have put into mentoring me.

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LIST OF ABBREVIATIONS

5-hmC: 5-hydroxymethylcytosine

5-mC: 5-methylcytosine

ABCB1/P-gp: P-glycoprotein

AdrVp: Adriamycin/verapamil

AML: acute myeloid leukemia

ATP: adenosine triphosphate

BCRP/ABCP/MXR: ABCG2

Bp: base pair

BSA: bovine serum albumin

Ct: Threshold cycle

DEPC: diethylpyrocarbonate

DHFR: dihydrofolate reductase

DHT: dihydrotestosterone

FPGS: folylpolyglutamate synthase

GEO: Gene Expression Omnibus

IC50: half maximal inhibitory concentration

LB: Luria Broth

L. casei: *Lactobacillus casei*

MBD: methyl binding domain

miR: microRNA

MR: mitoxantrone

MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBD: nucleotide binding domain

NCBI: National Center for Biotechnology Information

ObsCpG/ExpCpg: Observed CpG dinucleotide/Expected CpG dinucleotide

O.C.T.: optimum cutting temperature

PBS: phosphate buffered saline

PCFT: proton coupled folate transporter

PSA: prostate specific antigen

PSMA: prostate specific membrane antigen

RFC: reduced folate carrier

RT-PCR/qPCR: reverse transcription quantitative polymerase chain reaction

SNP: single nucleotide polymorphism

STR: short tandem repeat

TMD: transmembrane domain

TS: thymidylate synthase

UTR: untranslated region

1.0 INTRODUCTION

1.1 ATP-BINDING CASSETTE SUB-FAMILY G MEMBER 2 (ABCG2)

1.1.1 Discovery of ABCG2 as a multi-drug resistance gene

The failure of many chemotherapeutics is due to multidrug resistance developed by the cancer cell. The cells can develop resistance to the therapy by upregulating the expression of molecular pumps that efflux the drug from the cell and protect the cell from the drug's toxicity. The first multidrug resistance gene discovered was P-glycoprotein (P-gp) or ABCB1 [1, 2]. Other genes associated with the multidrug resistance phenotype have subsequently been discovered after selection of resistant cells with the drug of interest while also inhibiting P-gp. Chen et. al. [3] treated the human breast cancer cell line, MCF-7 (known to overexpress P-gp) with Adriamycin/Doxorubicin, and verapamil, a calcium channel blocker and P-gp inhibitor. The resulting cell line, MCF-7/AdrVp is resistant to adriamycin, does not express P-gp, and expresses a novel membrane protein.

Breast cancer resistance protein (BCRP) was first identified as the novel membrane protein after Doyle et. al. cloned the cDNA in 1998 from the MCF-7/AdrVp cell line [4]. They found that the BCRP sequence was homologous to one-half of the P-gp sequence and was later confirmed to be a half-transporter that must dimerize in order to function [5]. The clones from

the MCF-7 cells transfected with the BCRP cDNA were resistant to mitoxantrone, daunorubicin, and doxorubicin compared to non-transfected MCF-7, vector-only MCF-7, or non-BCRP expressing clone-transfected MCF-7 [4]. BCRP was concurrently cloned from the placenta and named ABCP [6] and from a mitoxantrone-resistant human colon carcinoma cell line and named MXR [7].

Further characterization of the BCRP cDNA confirmed that BCRP is a 655 amino acid protein (~72kDa). The BCRP gene is located on chromosome 4q22 and contains 16 exons and 15 introns. It is part of the ATP-binding cassette transporter superfamily and part of the G family due to its structural homology to the other proteins within that family (e.g. *Drosophila* white, brown, and scarlet genes and human ABCG1 gene). The G family proteins are half-transporters because they contain only one nucleotide binding domain (NBD) and one transmembrane domain (TMD) with six helices. ABCG2 also contains Walker A, B, and C motifs, which are important for nucleotide binding (**Figure 1-1**).

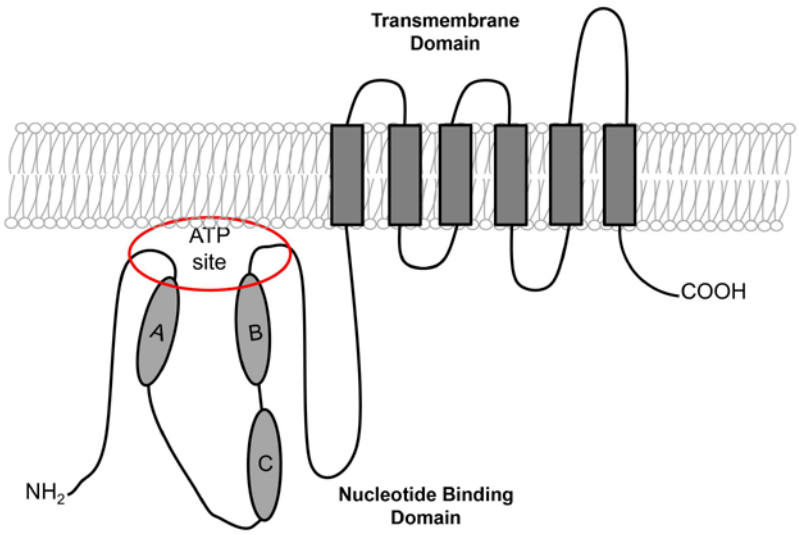

Figure 1-1. Schematic of ABCG2 structure.

1.1.2 ABCG2 substrates, tissue distribution and functions

ABCG2 was originally discovered because it was able to efflux chemotherapeutics and confer multi-drug resistance in tumor cells. The anti-cancer drugs it transports include topoisomerase inhibitors (e.g. mitoxantrone [8]), anthracyclines (e.g. doxorubicin and daunorubicin [9]), camptothecin analogs (e.g. topotecan and SN-38 [10]), tyrosine kinase inhibitors (e.g. gefitinib [11] and erlotinib [12]), antimetabolites (e.g. methotrexate [13] and 5-fluorouracil [14]), and others (e.g. bicalutamide [15] and flavopiridol [16]). In addition, ABCG2 substrates also include xenobiotics (e.g. estrone 3-sulfate [17] and 17-beta estradiol sulfate [18]), photosensitizers (e.g. pheophorbide a [19]), natural compounds (e.g. folic acid [13] and urate [20]), fluorescent dyes (rhodamine 123 [21] and Hoechst 33342 [22]), anti-inflammatories (e.g. sulfasalanine [23]), antibiotics (e.g. erythromycin [24]), flavonoids (e.g. kaempferol [25]), alpha-1-adrenergic receptor agonists (e.g. prazosin [21]), hypoglycemic drugs (e.g. glyburide [26]), and amyloid-beta [27]. This range of substrates indicates that ABCG2 may be involved in causing broad-spectrum drug resistance and disease pathogenesis as well as other physiologic roles discussed below.

ABCG2 is expressed ubiquitously throughout the body. It is highly expressed in syncytiotrophoblasts of the placenta, epithelium of the small intestine and colon, liver canalicular membranes, and mammary ducts and lobules [28]. The presence of ABCG2 has also been detected in epithelial gallbladder cells [29], proximal tubule brush border in kidney cells [30] alveolar pneumocytes, sebaceous glands, transitional epithelium of the bladder, interstitial cells of the testes, prostate epithelium, endocervical cells of the uterus, squamous epithelium of the cervix, small and large intestinal mucosa/epithelial cells, islet and acinar cells of pancreas, zona reticularis layer of the adrenal gland, kidney cortical tubules and hepatocytes [31].

Because ABCG2 is located at the plasma membrane, its primary physiological function is believed to be to protect the organism from environmental toxins. ABCG2 is expressed at the apical membrane of the colon and in the liver canalicular membranes and is therefore able to efflux toxins and metabolites via the biliary pathway (reviewed in [32]). ABCG2 also plays an important role in the blood-brain barrier. ABCG2 and ABCB1 (P-gp) work in concert to protect the brain from xenobiotic toxicity, but also inhibit drugs targeted to treat brain tumors (reviewed in [32]). The high expression of ABCG2 in the placenta is believed to protect the fetus against toxins consumed by the mother (reviewed in [32]. The expression of ABCG2 in undifferentiated hematopoietic stem cells has led ABCG2 to be considered a stem cell marker. It is believed that ABCG2 protects the stem cells from xenobiotics and other toxins (reviewed in [32]). Similarly, ABCG2 has been proposed to protect cancer stem cells from chemotherapy treatment. Recently, induction of ABCG2 expression during pregnancy and lactation in the mammary gland was observed in mice, humans, cows, and sheep [33, 34]. It is hypothesized that ABCG2 pumps essential nutrients into the milk for the infant. However, ABCG2 would also be effluxing toxins and xenobiotics into the milk and more experiments are needed to define the role of ABCG2 in the mammary gland. Even though many of the substrates and functions of ABCG2 have been discovered, there are potentially more unrecognized functions of ABCG2.

1.1.3 Regulation of ABCG2

ABCG2 is regulated by gene amplification, epigenetic mechanisms, transcriptional and posttranslational mechanisms. ABCG2 gene amplification has been observed in drug resistant cell lines over expressing ABCG2 but not in the parental cells [35, 36]. However, ABCG2 gene amplification has not been observed in human tissues over-expressing ABCG2. Increased

ABCG2 expression has been correlated with the demethylation of the putative CpG island in the ABCG2 promoter in human multiple myeloma cell lines [37] and renal carcinoma cell lines [38]. Methyl binding proteins, which repress transcription by recruiting histone deacetylase 1, were bound to the methylated CpG island. However, the methylation status of the ABCG2 promoter did not change in hepatic carcinoma tissue compared to healthy donor liver tissue [39], suggesting that the epigenetic regulation of ABCG2 may be tissue dependent. There are several response elements in the 5' untranslated region of ABCG2 that have been shown to regulate the expression of ABCG2. These include an estrogen response element [40], a hypoxia response element [41], a peroxisome proliferator-activated receptor γ response element [42], a progesterone response element [43, 44], a dioxin response element [45], and an antioxidant response element [46]. ABCG2 is also regulated by post-translational mechanisms, such as by the microRNAs, miR-328 [47], miR-520h [48], miR-519c [49], and recently miR-212 [50], miR-200c [51], miR-487a [52], miR-142-3p [53], and miR-181a [54]. Wild-type ABCG2 is also subject to lysosomal degradation whereas improperly folded ABCG2 is degraded by the proteasome [55].

1.1.4 ABCG2 and Cancer

The clinical relevance of alterations in ABCG2 expression and function in cancer is somewhat controversial. Ross, et. al. first demonstrated increased ABCG2 expression in 33% of acute myeloid leukemia (AML) blast cells [56] and a correlation with prognosis and relapse [57]. However, others have found that ABCG2 expression does not correlate with AML [58, 59]. Similarly, in solid tumors some groups have found ABCG2 expression to be correlated and others have not. For example, ABCG2 expression was correlated to treatment response of breast

cancer patients treated with anthracyclines [60] but was not in breast cancer patients treated with doxorubicin-based chemotherapy [61]. The differences observed may be due to sample sizes and techniques used to measure ABCG2 expression (most have used RT-PCR, immunostaining, or flow cytometry). There is strong evidence that ABCG2 is upregulated in drug resistant cell lines [4, 6, 7]. Therefore, further work is required to understand the role of ABCG2 in cancer and how ABCG2 expression may affect treatment outcomes.

1.1.4.1 ABCG2 and Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer in American men and the second leading cause of cancer related deaths in men [62]. Prostate cancer is initially treated by radiation or prostatectomy but it often recurs. Androgen deprivation therapy is the next line of treatment, which induces apoptosis of androgen dependent cancer cells. Eventually this treatment becomes ineffective and the androgen dependent prostate cancer turns into androgen independent (castration resistant) prostate cancer. This form of prostate cancer is lethal and treatment with docetaxel and prednisolone increases overall median survival by 9 months when compared to prednisolone alone [63].

There are two reports of the single nucleotide polymorphism (SNP), C421A (Q141K) in the ABCG2 coding region that decreases the efflux function is correlated with survival in prostate cancer patients [64, 65]. One group observed that the presence of this SNP correlated with a shorter overall survival time than wild-type ABCG2. The authors hypothesized that the patients with the SNP retained more dihydrotestosterone (DHT) than the wild-type patients and the DHT increased tumor proliferation [64]. There is one report that identifies dihydrotestosterone as a potential ABCG2 substrate [66]. The other report observed that chemotherapy naïve androgen independent (castration resistant) prostate cancer patients with the

SNP and were treated with docetaxel had a longer survival time than wild-type patients. It was hypothesized that docetaxel could be a novel ABCG2 substrate [65]. Xie, et. al. also observed that ABCG2 was upregulated after CWR-R1 prostate cancer cells were chronically exposed to docetaxel. They also saw that other proteins involved in the multi-drug resistant phenotype were not upregulated in this docetaxel resistant CWR-R1 cell line [67]. Taken together, this data suggests that ABCG2 may play a role in prostate cancer progression and treatment outcome.

1.1.5 ABCG2 and Folates

Folate or vitamin B9 is essential for cell proliferation, purine and pyrimidine synthesis, methylation reactions, and polyamine biosynthesis. Folate is found naturally in foods such as beef liver and green leafy vegetables. In 1998, the United States required folic acid (synthetic folate) fortification of cereals, breads, and other grains after an association between folate deficiency and neural tube defects was observed. Humans cannot synthesize folate and must obtain it through diet and supplements. Dietary folate is typically polyglutamated and then hydrolyzed by prostate specific membrane antigen (PSMA) to monoglutamated folate in order to be transported into the cell. Once in the cell the monoglutamated folate is polyglutamated again by folylpolyglutamate synthetase (FPGS) and enters the one-carbon metabolism cycle. The metabolite 5-methyl-tetrahydrofolate donates the methyl group to homocysteine to form methionine and tetrahydrofolate. Methionine is converted to S-adenosylmethionine, which is required to donate the methyl group for methylation of DNA, RNA, histones, and other proteins. The donation of the methyl group results in S-adenosyl-homocysteine, which is a potent inhibitor of methyltransferases (reviewed in [68]).

Folate is considered to be a double edged sword in cancer [69]. In colorectal cancer, there are several epidemiological studies reporting a decreased risk of colorectal cancer in people with the highest intake of folate (reviewed in [70]). However, there is evidence that folate deficiency is correlated with decreased colorectal risk [71]. It is hypothesized that folate supplementation is protective before the occurrence of neoplastic lesions, but after the establishment of a premalignant lesion, folate deficiency may actually reduce the risk of cancer.

Folate deficiency has been studied in relation to the effect on ABCG2 expression and function. In the breast cancer cell line, MCF-7 and its subline that overexpresses ABCG2, MCF-7/MR, folate deprivation leads to a decrease in ABCG2 expression and function [72], which is due to ABCG2 confinement in the cytoplasm [73]. However, in colorectal cell lines, folate deprivation leads to an increase in ABCG2 expression and function [74-76]. This data suggests that folate deprivation can regulate ABCG2 expression but the induction or repression of expression is likely tissue specific.

1.1.6 Single Nucleotide Polymorphisms (SNPs) in ABCG2

Over 50 single nucleotide polymorphisms have been discovered in the ABCG2 gene [77-80]. Forty of these SNPs are located in the intronic regions and have not been thoroughly studied [78]. Other SNPs that have been observed are synonymous SNPs [81], meaning that the mutation in the nucleotide sequence does not change the amino acid. The SNPs that are the most interesting are non-synonymous, meaning that the amino acid is changed after the nucleotide mutation. Frequently observed non-synonymous polymorphisms of ABCG2 are G34A (V12M) and C421A (Q141K) [79]. Other non-synonymous SNPs include C376T (Q126stop), G1322A (S441N), and T1465C (F489C). These SNPs can be generally characterized by decreased

function of the ABCG2 protein to specific substrates, decreased expression compared to wild-type ABCG2, and may be differentially located [81-86]. However, the R482G/T SNP was discovered to be a gain of function mutation. This SNP has a higher affinity for anthracyclines and some fluorescent dyes (rhodamine 123 and lysotracker green) than the wild-type ABCG2 [87]. The only SNP in ABCG2 that has been associated with a human disease is the C421A (Q141K) polymorphism. It has been associated with causing approximately 10% of gout cases [20]. Therefore, it is the most widely studied SNP and is discussed in detail in the following section (1.1.7).

1.1.7 The Q141K variant ABCG2 protein

Imai, et. al. first reported the Q141K variant ABCG2 protein [88]. After screening 11 cancer cell lines, the group identified three variant cDNAs, one of which was a cysteine to adenine transversion at nucleotide position 421 that substitutes a lysine (K) for a glutamine (Q) at amino acid position 141. This polymorphism is located in the nucleotide binding domain and specifically in exon 5 [81] (**Figure 1-2)**.

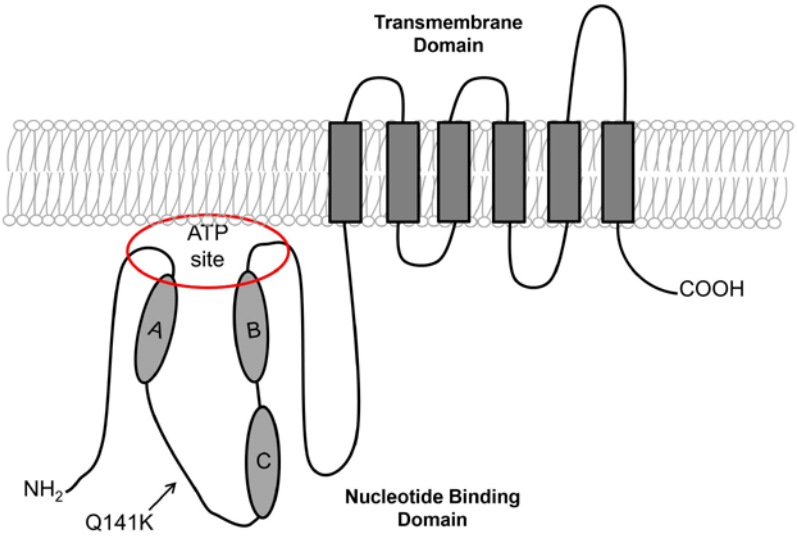

Figure 1-2. Location of Q141K variant.

The frequency of the Q141K variant in the human population varies by race. Several groups have analyzed the frequency of the Q141K variant allele. These results are summarized in **Table 1-1**. The variant allele is the most common in the Chinese and Japanese ethnic groups and it is the least common in African populations. The variability in the frequency of the Q141K variant has made it an important and highly studied polymorphism.

Table 1-1. Q141K variant allele frequencies in ethnic populations

Ethnicity	Allele Frequency (%)	Reference
African (north of Sahara)	0	[80]
African (sub-Saharan)	1	[89]
African American	0-5	[80, 89, 90]
American Indian	20	[90]
Ashkenazi Jew	5	[80]
Caucasian (American and European)	9-14	[80, 83, 89, 90]
Chinese	29-35	[80, 89, 91]
Japanese	20.5-35	[80, 88, 92-94]
Korean	28	[91]
Mexican	5	[80]
Mexican-American	19	[90]
Mexican-Indian	10	[80]
Middle Eastern	13	[80]
Pacific Islander	15	[80]
Southeast Asian	15	[80]
Vietnamese	31	[91]

1.1.7.1 RNA and Protein expression of the Q141K variant

When the Q141K variant was discovered, Imai, et. al. stably transfected wild-type ABCG2 and the Q141K variant into PA-317 cells. They found the Q141K variant protein expression was decreased compared to wild-type by western blot. However, by Northern blot they observed the mRNA expression was similar to wild-type ABCG2 [88]. Several other groups have shown that in transfected cell lines the Q141K variant protein is decreased compared to wild-type ABCG2 [82, 84-86, 95-98]. Some of these groups also looked at mRNA expression and found that the expression of mRNA in the Q141K variant was similar to the wild-type ABCG2 [84, 85, 95, 96]. Others have found that the protein expression between the Q141K variant and wild-type ABCG2 is similar [20, 81, 83, 97, 99, 100]. These groups also found that the mRNA in the Q141K variant were similar to the wild-type ABCG2 [81, 83]. ABCG2 protein

expression was measured in human intestinal samples but no relationship between the level of ABCG2 protein and the genotype of the sample was identified [80].

The Q141K variant and wild-type ABCG2 were exogenously expressed in a number of cell lines and with a variety of expression systems. This may explain the inconsistency in the level of protein expression observed in the Q141K variant. For example, several groups used the Flp-In-293 cell line, which utilizes Flp recombinase to integrate one copy of the Flp-In expression vector into a single Flp Recombination Target site, in order to study the effects of the Q141K variant *in vitro*. All of these groups observed decreased expression of the Q141K variant [84, 85, 95, 96]. Other groups generated stable cell lines and found similar expression between the variant and wild-type ABCG2 [81, 83, 99, 100], with the exception of Imai, et. al. who found decreased variant expression [88]. This group also used the PA-317 cell line where as the other groups used the polarized pig kidney epithelial cells, LLC-PK1 or human embryonic kidney cells, HEK293. Interestingly, the groups that performed transient transfections to induce expression of the Q141K variant or the wild-type ABCG2, found decreased Q141K protein expression [82, 97, 98]. Some groups have also used non-human model systems to study the Q141K variant. In Sf9 insect cells, there are conflicting reports in regards to the protein expression [86, 97] and in *Xenopus* oocytes the protein expression is comparable between the variant and wild-type [20, 98]. Overall, the system used to express exogenous Q141K variant and wild-type ABCG2 protein has a significant impact on the experimental plan. In order to determine the best model system for *in vitro* experiments, further analysis of human samples will need to be conducted.

1.1.7.2 Function of the Q141K variant

The presence of the Q141K variant decreases the function of the ABCG2 protein. It was established that ABCG2 effluxes drugs such as mitoxantrone, shortly after the discovery of ABCG2. Therefore, these drugs were used to study the function of variants of ABCG2. Many groups have demonstrated that the expression of the Q141K variant confers greater sensitivity to mitoxantrone compared to wild-type ABCG2, regardless of similar protein expression to wild-type [81, 83, 100] or decreased protein expression compared to wild-type [88, 95]. **Table 1-2** summarizes the effect of the Q141K variant on efflux of ABCG2 substrates. The model system chosen appears to have an effect on the function of the Q141K variant.

Table 1-2. Effect of the Q141K variant on ABCG2 function

Transfected Cell Line	Decreased function compared to wild-type	Increased function compared to wild-type	Similar function compared to wild-type	Reference
PA-317	Mitoxantrone, SN-38	Topotecan	-	[88]
LLC-PK1	Indocarbazole, Mitoxantrone, Topotecan	-	-	[83]
HEK293	Estrone-3-sulfate, Dehydroepiandrosterone sulfate, Methotrexate, *p*-aminohippuric acid	-	-	[82]
HEK239	Diflomotecan, Mitoxantrone, Topotecan, SN-38	-	Hoechst 33342	[81]
Sf9	Porphyrin	-	Mitoxantrone	[86]
HEK293	Imatinib	-	-	[99]
Flp-In-293	Camptothecin analogs	-	-	[85]
Flp-In-293	-	-	Pheophorbide A	[84]
Flp-In-293	SN-38	-	-	[96]
HEK293	Mitoxantrone	-	-	[100]
Xenopus Oocytes	Uric Acid	-	-	[20]
HEK293	-	-	Glyburide	[101]
Flp-In-293	Mitoxantrone	-	-	[95]
HEK293	Uric Acid	-	-	[98]
HEK293	PhIP	-	-	[64]

1.1.7.3 Localization, Trafficking, and Stability of the Q141K variant

The wild-type ABCG2 protein is localized to the plasma membrane in order to efficiently efflux its substrates from the cell. However, the location of the Q141K variant protein is different depending on the *in vitro* model system used. For example, groups that transfected the Q141K variant into the polarized cell line, LLC-PK1 found that the variant protein localized to the apical membrane, which is where the wild-type ABCG2 is localized [82, 83]. In addition other groups that have exogenously expressed ABCG2 observed by flow cytometry that the level of ABCG2 at the plasma membrane was similar in Q141K variant and wild-type ABCG2 [81, 101]. This suggests that the Q141K variant is properly trafficked. However, others that have induced the Q141K variant protein expression in HEK293 cells or the Flp-In-293 cells observed increased intracellular staining by immunofluorescence [81, 84, 85, 96-98]. One group observed the Q141K variant protein localizes to aggresomes (inclusion bodies where misfolded proteins can localize before being degraded) [95].

It has been hypothesized that the Q141K variant is differentially expressed and localized because it is improperly trafficked. One group tested this by treating Flp-In-293 cells expressing wild-type ABCG2 and the Q141K variant with MG132, a proteasome inhibitor and bafilomycin A_1, a lysosome inhibitor. They observed an increase in the Q141K variant ABCG2 protein after treatment with both inhibitors (wild-type ABCG2 protein expression remained the same after MG132 treatment and increased after bafilomycin A_1 treatment) [96]. They also observed an increase in cell membrane staining and increased resistance to SN-38 after MG132 treatment, suggesting that the Q141K variant was properly trafficked when proteasomal degradation was inhibited. Another group observed punctate intracellular staining in the Flp-In-293 cell line expressing the Q141K variant (this staining has not been observed by any other group using this

cell line). The group hypothesized that the Q141K variant was localizing to aggresome before being degraded [95]. After co-localization experiments, this group found that the Q141K variant was not co-localizing in the Golgi apparatus, but was co-localizing with γ-tubulin, a centrosome marker. Aggresomes are localized to the centrosome [102], therefore Basseville, et. al. predicted that the Q141K variant was aggregating before degradation. They also treated wild-type ABCG2 and the Q141K variant with MG132 and bafilomycin A_1 and found similar results to Furukawa, et. al. In addition, they treated the cell lines with 3-methyladenine, which prevents autophagosome formation. They found increased protein expression in the Q141K variant and no difference in the wild-type ABCG2 after the 3-methyladenine treatments. They concluded that the Q141K variant protein localizes to the aggresomes before degradation by the autophagy.

The location of the Q141K variant is in the nucleotide-binding domain and is in what is considered a mutational hotspot of the ABC transporter family. The Q141K variant is in a homologous region to the ΔF508 region in the cystic fibrosis cystic transmembrane conductance regulator gene [98]. The ΔF508 is the most common mutation that causes cystic fibrosis (reviewed in [103]). Woodward, et. al. observed that the phenylalanine adjacent to Q141K at amino acid position 142 in ABCG2 was homologous to the F508 of CFTR. There are known amino acids in CFTR that are known to affect the stability of the nucleotide-binding domain. This group mutated the homologous amino acids in ABCG2 to test the effect of the Q141K variant on the nucleotide-binding domain stability. They found that Q141K does decrease the stability of the nucleotide-binding domain but does not interfere with interdomain interactions or dimerization of ABCG2 [98].

1.2 RATIONALE AND AIMS

The importance of ABCG2 in prostate cancer progression and treatment outcome has not been widely studied. However, the potential role ABCG2 plays in other cancers and the known and potential ABCG2 substrates important for prostate growth and prostate cancer treatment (i.e. folate, dihydrotestosterone and docetaxel), have led to the following specific aims.

Aim 1: Investigate the role folate plays in the expression and regulation of ABCG2 in prostate cancer. Folate deprivation can lead to a reduction or induction of ABCG2 expression, which appears to be tissue specific [72, 74-76]. It has been shown in two studies that treatment of cells with an inhibitor of methyltransferases increases the expression of ABCG2 [37, 38]. This suggests that ABCG2 is regulated in part by promoter methylation. Other studies have demonstrated alterations in methyl-CpG binding proteins and histone modifications at the ABCG2 promoter [38, 104], though not in cells grown in a physiologic or deficient folate environment. Determining the mechanism by which ABCG2 transcription is regulated in prostate cancer will lead to a better understanding of the role ABCG2 plays in prostate cancer as well as potentially leading to personalized treatment and dietary recommendations for patients.

Specific Aim 2: Determine the effect of the Q141K variant on ABCG2 expression and function. ABCG2 is a known exporter of folate [13, 105] and a putative exporter of dihydrotestosterone [66] and docetaxel [65, 67]. Folate and androgens are important factors in prostate cancer growth. Docetaxel treatment is the standard of care for androgen independent prostate cancer patients. Therefore, modulation of ABCG2 expression and subsequently its function has the potential to have a dramatic impact on prostate cancer cell growth and treatment outcomes. The Q141K variant is correlated to survival times in prostate cancer patients [64, 65, 67]. Therefore it is crucial to understand how the Q141K variant affects the function of ABCG2.

2.0 THE ROLE OF FOLATE IN THE EXPRESSION AND REGULATION OF ABCG2

2.1 INTRODUCTION

Folate, a water soluble B vitamin, is a factor required in the one-carbon metabolism pathway. This pathway is responsible for synthesizing nucleotides and providing the methyl groups required for DNA, RNA, and histone methylation. When patients with adenomatous colorectal polyps were supplemented with folic acid, their global DNA methylation increased by 31% [106]. Others have observed global hypomethylation after several weeks of folate deprivation [107-109]. In addition to affecting the methylation of the genome, the level of folate may play a role in cancer risk. It is well documented that folic acid supplementation has dual roles in colon cancer risk (reviewed in [69]). There is also conflicting evidence regarding the effect of folic acid supplementation on prostate cancer diagnosis, progression and survival. For example, two studies concluded dietary folate had no effect on prostate cancer survival [110, 111]. Others have concluded that folic acid supplementation increases prostate cancer incidence [112, 113] and another showed a non-significant decrease in risk of advanced prostate cancer in men with high levels of folate [114]. Despite the conflicting results regarding folate and prostate cancer, it is known that the prostate has a high requirement for folate because of high polyamine biosynthesis and that decreasing folate levels severely decreases the proliferative capacity of prostate cancer

cell lines and primary cultures compared to colon cancer cell lines [115]. Because the data involving folate and prostate cancer is contradictory, further steps need to be made to help clarify the role of folate and prostate cancer development.

ABCG2 expression is affected by folate deprivation in cancer cell lines. Ifergan, et. al. deprived the breast cancer cell line, MCF-7 of folate and observed a decrease in ABCG2 mRNA and protein expression that leads to increased accumulation of the ABCG2 substrates, Hoechst 33342, mitoxantrone, and folic acid and increased sensitivity to methotrexate. The MCF-7 cells grown in supraphysiological levels of folic acid (standard media) maintained the high expression of ABCG2 and still functioned properly [72]. However, in the colorectal cancer cell lines, Caco-2 and WiDr, Lemos, et. al. observed an increase in ABCG2 expression after deprivation of folic acid. They also observed an increase in resistance to mitoxantrone and gefitinib in the folic acid deprived cell lines compared to the cells grown in standard media [74-76]. The effect of folate deprivation has not been studied in other cancer cell lines.

The transcriptional regulation of ABCG2 has been investigated in very few cancer cell lines. To *et. al.* studied the promoter methylation in renal cell carcinoma cell lines. They found that the decreased promoter methylation was indicative of increased ABCG2 expression and histone 3 lysine 9 methylation (marker of chromatin opening). Conversely, increased promoter methylation silenced gene expression, and binding of methyl-CpG binding (MBD) proteins (marker of chromatin closing) [38]. A similar study was undertaken in human multiple myeloma cell lines. They used a mitoxantrone-resistant subline that overexpressed ABCG2. They similarly found that a decrease in promoter methylation led to an increase in ABCG2 expression at the mRNA and protein level. They also analyzed topotecan efflux as a measure of functionality and found that function also correlated with the promoter methylation status [37].

Taken together, these studies suggest that prostate cancer cell lines may respond in a similar manner but there is no published data on prostate cancer or the link to folate.

The goals of this chapter are to determine the effect folate deprivation has on ABCG2 expression in prostate cancer cell lines and to analyze the methylation at the ABCG2 promoter.

2.2 MATERIALS AND METHODS

2.2.1 CpG Island Prediction

To predict whether a CpG island was present in the ABCG2 promoter, the ABCG2 transcript sequence from NCBI (accession number NM_004827) was analyzed by The CpG Island Searcher program (http://www.uscnorris.com/cpgislands2/cpg.aspx). The following default parameters (lower limit values of 55% GC, 0.65 ObsCpG/ExpCpG, 500bp length, and 100bp gap between adjacent islands) were used for this analysis.

2.2.2 Cell Culture

LNCaP and PC3 cell lines were obtained from ATCC. The cells were maintained at 37°C and 5% CO_2. LNCaP cells were grown in RPMI, 10% FBS, 1000x L-glutamine, and 1000x penicillin/streptomycin. PC3 cells were grown in RPMI, 5% FBS, 1000x L-glutamine, and 1000x penicillin/streptomycin.

RPMI media contains approximately 2.3μM folic acid. The lab measures the folic acid concentration whenever a new lot of media arrives. The folate deprivation studies were

completed by diluting the 2.3μM folic acid RPMI to 100nM, 60nm, 25nM and/or 6.7nM with folate free RPMI. After passage of the cells, the diluted RPMI was added to the cells. Generally, the RPMI was changed every 3 to 4 days if the cells were grown for longer than 2 days. After trypsinization, the cells are harvested in the diluted RPMI to eliminate any contaminating folic acid.

2.2.3 Decitabine treatment

LNCaP and PC3 cell lines were treated with 3μg/mL decitabine (Sigma) for 9 days before cells were harvested. The media was replaced with new decitabine every 24 hours for the first 3 days and then every 72 hours until the cells are harvested for qPCR analysis.

2.2.4 RNA extraction

Cells were harvested by trypsinization after washing with PBS. Cells were spun at 1000 rpm for 5 minutes at room temperature. After removing the media, cells were washed again with PBS, transferred to a 1.5mL Eppendorf tube, and spun at 1000 rpm for 5 minutes at 4°C. After removing the PBS, the cells were completely resuspended by pipetting and vortexing in 1mL of Trizol (Invitrogen). This resuspension was incubated at room temperature for 3 minutes and 200μL of chloroform was added. The tubes were vigorously shaken for 15 seconds and then spun at 12,000x g for 15 minutes at 4°C. The aqueous phase (~500μL) was transferred to a new 1.5mL Eppendorf tube and 500μL of isopropanol and 2μL of 5μg/μL glycogen were added. This mixture was incubated at room temperature for 10 minutes and then spun at 12,000x g for 10 minutes at 4°C. A pellet should be visible after this centrifugation; then the liquid was removed

and 1mL 75% ethanol was added to wash the pellet. Next, the pellet was briefly vortexed and spun at 7,500x g for 5 minutes at 4°C. After centrifuging, all of the supernatant was removed and the pellet was air-dried for approximately 10-20 minutes depending on the size of the pellet. Twenty microliters of DEPC water was added and pipetted several times to fully resuspend the RNA. The RNA was incubated at 60°C for 10 minutes. The RNA was quantitated using a spectrophotometer and the $A_{260/280}$ ratio should be greater than 1.6 to ensure that the RNA is fully resuspended. To check for DNA contamination, 500ng of RNA was run on a 0.8% agarose gel and visualized by a UV transilluminator.

2.2.5 Reverse transcription

One microgram of RNA was mixed with 0.5µg Random Primer (Promega) and incubated at 70°C for 5 minutes in a thermocycler. Then, 200U MMLV Reverse Transcriptase, 5x MMLV buffer, 25U RNase Inhibitor (Roche Applied Science) and 500µM dNTPs was added and incubated at 37°C for 1 hour in a thermocycler. RNase-free, DNase-free water was added to the newly synthesized cDNA to bring the total volume up to 50µL. To check that the cDNA was properly synthesized, a PCR to amplify β-microglobulin was performed. Twenty-five microliter reactions contained 2.5µL 10x ThermoPol Reaction buffer (NEB), 1µL of 25µM B2MS primer (5'-AGCAGAGAATGGAAAGTCAAA), 1µL of 25µM B2MAS primer (5'-TGTTGATGTTGGGATAAGAGAA), 0.5µL of 10mM dNTPs, 0.1µL of Taq Polymerase (NEB), and 1µL of cDNA. Reactions were run on a thermocycler with the following conditions: Initial denaturation at 94ºC for 5 minutes, 40 cycles of 94ºC for 30 seconds, 57ºC for 30 seconds, and 72ºC for 45 seconds, and a final extension at 72ºC for 10 minutes. PCR products were visualized by a UV transilluminator on a 1.2% agarose gel.

2.2.6 Quantitative PCR

ABCG2 expression was measured in a 20µL PCR reaction containing iQ™ Supermix (BioRad), 500nM ABCG2 Forward (5'-CATTGCATCTTGGCTGTCAT), 500nM ABCG2 Reverse (5'-GTCCTGGGCAGAAGTTTTGT), 500nM ABCG2 probe ([FAM]-CCACGATATGGATTTACGGCTTTGC-[BHQ1]), and 2µL of cDNA. The cycling conditions were 95°C for 30 sec and 45 cycles of 95°C for 15 sec and 64°C for 1 min. GusB was used as a reference gene. Each 20µL PCR reaction contained 2X iQ™ Supermix (BioRad), 500nM GusB Forward (5'-CTCATTTGGAATTTTGCCGATT), 500nM GusB Reverse (5'-CCGAGTGAAGATCCCCTTTTT), 500nM GusB probe ([FAM]-TGAACAGTCACCGACGAGAGTGCT-[BHQ1]), and 2µL of cDNA. The cycling conditions were 95°C for 30 sec and 45 cycles of 95°C for 15 sec and 60°C for 1 min. The relative gene expression was calculated using the ΔΔCt method. Efficiency curves were optimized for each quantitative PCR. The ABCG2 efficiency was 100.9% (R^2=0.984). The GusB efficiency was 93.1% (R^2=0.993).

2.2.7 Microbiological Folate Assay

Intracellular folate concentrations were determined by the microbiological *Lactobacillus casei* assay in a 96-well plate. To extract folate from the cells, 600µL of 2% sodium ascorbate extraction buffer was added. The mixture was briefly sonicated (5 second pulses for 20 seconds) and an aliquot was removed for protein quantification. The remaining solution was boiled at 99ºC for 5 minutes, cooled on ice, briefly spun, and then the supernatant was treated with rat serum conjugase to deglutamate intracellular folates. This mixture was incubated at 37ºC for 2

hours, boiled for another 5 minutes, briefly spun, and the supernatant was transferred to a new tube.

Folic acid standards ranging from 0pg/µL to 200pg/µL and samples were run in replicates of six. Twenty microliters of each standard or sample was added to the *L. casei* master mix containing 150µL Casei media (9.4g Difco Folic acid Casei medium powder, 50mg L-Ascorbic Acid, and 100mL sterile ddH_2O), 8µL Working buffer (3.2g Sodium Ascorbate sodium salt, 1mL Potassium phosphate pH 6.1, 20mL sterile ddH_2O), 2.5µL *L. casei* bacteria, 17.6µL NaCl (9g/L), and 102µL water. Plates were incubated in the dark for 16 hours at 37ºC and then read at 600nm on a Molecular Devices SpectraMax M5 plate reader. The folate concentration was determined against the standard curve generated from the folic acid stock solution.

2.2.8 Bisulfite sequencing

To determine the effect of folate on the methylation of the ABCG2 promoter, LNCaP cells were grown in 2.3µM, 100nM, 60nM, and 6.7nM folic acid for 1 week. After cells were harvested, DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). The DNA concentration was measured by a spectrophotometer. One microgram of DNA was subjected to bisulfite conversion using the EZ DNA Methylation Kit™ (Zymo Research). It is important to complete all steps on ice or in a 4ºC centrifuge because the bisulfite-converted DNA is single stranded and unstable. During bisulfite conversion, the cytosines in the DNA sequence that were unmethylated are converted to uracil and the cytosines that were methylated are protected from the bisulfite conversion and remain intact.

The ABCG2 promoter was then amplified from the bisulfite converted DNA. The 25µL PCR reactions contained 2.5µL of 10x Titanium Taq buffer (Clontech), 0.4µL of 25µM ABCG2

1938 Forward primer (5'-GTTTGTGATTGGGTAATTTGTG), 0.4µL of 25µM ABCG2 2577 Reverse primer (5'-AACACTACCTCTTCCCTCCTAC), 0.5µL of 10mM dNTPs, 0.2µL Titanium Taq (Clontech), 19µL water, and 2µL bisulfite DNA. The cycling conditions were as follows: Initial denaturation at 95ºC for 1 minute, 40 cycles of 95ºC for 30 second and 68.5ºC for 1 minute, and a final extension at 68ºC for 10 minutes. PCR products were visualized by a UV transilluminator on a 1.2% agarose gel.

The PCR products were ligated into the p-GEM-T easy vector (Promega) and transformed into BIOLINE Peak™ Efficiency Competent Cells (Bioexpress) per manufacturer's instructions. The bacteria were plated onto Ampicillin-LB agar plates containing 100µL of 0.1M IPTG and 20µL of 50mg/mL X-gal (for blue/white colony selection) and incubated overnight at 37ºC. White colonies were picked and grown up in 2mL of Ampicillin-LB overnight at 37ºC while shaking. Ten clones each from cells grown in 2.3µM and 6.7nM and five clones each from cells grown in 100nM and 60nM were analyzed. DNA was extracted from bacterial cultures using the Wizard Plus SV Miniprep Kit (Promega). DNA was sequenced by MCLab and methylgrams were generated in Microsoft Office PowerPoint.

2.3 RESULTS

2.3.1 A CpG island in the ABCG2 promoter is predicted

A putative CpG island in the ABCG2 promoter region has been observed in the literature [116]. The ABCG2 sequence that this study used was from the NCBI transcript database (NM_004827). The CpG Island Searcher program predicted a CpG island 634 bases in length

with a GC content of 55% (**Figure 2-1**). This sequence was used as reference (i.e. to design primers and as the reference sequence for the methylgram) for the remainder of the study.

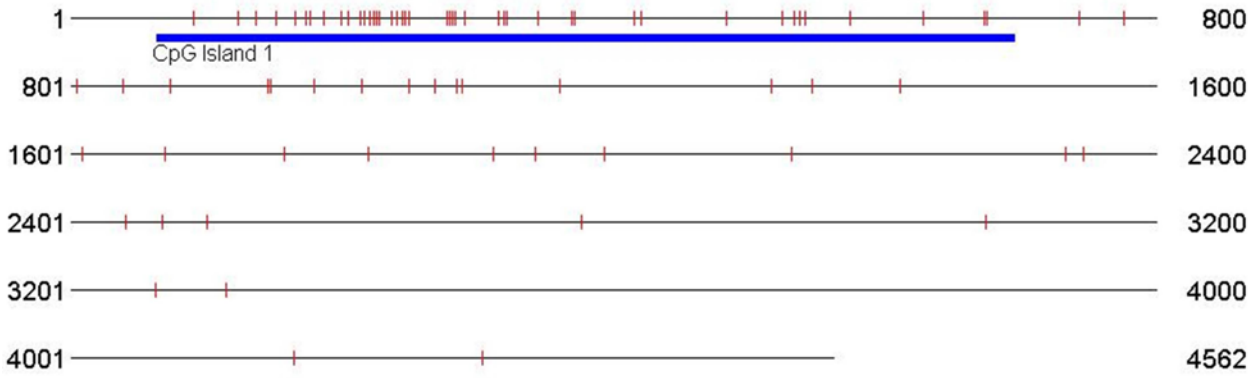

Figure 2-1. A CpG island is predicted in the ABCG2 promoter region. The program CpG Island Searcher was used to generate the image. The blue bar represents the CpG island and a red vertical line represents a CpG dinucleotide.

2.3.2 Decitabine treatment increases the expression of the ABCG2 gene

Methylation of the ABCG2 promoter has been shown to regulate gene expression in renal cell carcinoma cell lines [38] but has not been shown in prostate cancer cells. To test this, the prostate cancer cell lines, PC3 and LNCaP were treated with decitabine. Decitabine inhibits DNA methyltransferases and therefore is effective at hypomethylating DNA. The cell lines were treated with decitabine for 9 days and then RNA was extracted and reverse transcribed. I observed a 6-fold increase in LNCaP cells treated with decitabine ($p=0.0281$, $n=3$) and a 40-fold increase in decitabine-treated PC3 cells ($p=0.1565$, $n=6$) (**Figure 2-2**). The expression of ABCG2 in the PC3 cell line does not reach significance due to the variability between experiment replicates. However, even though the level of ABCG2 expression varied, decitabine treatment increased ABCG2 expression in all six experiments.

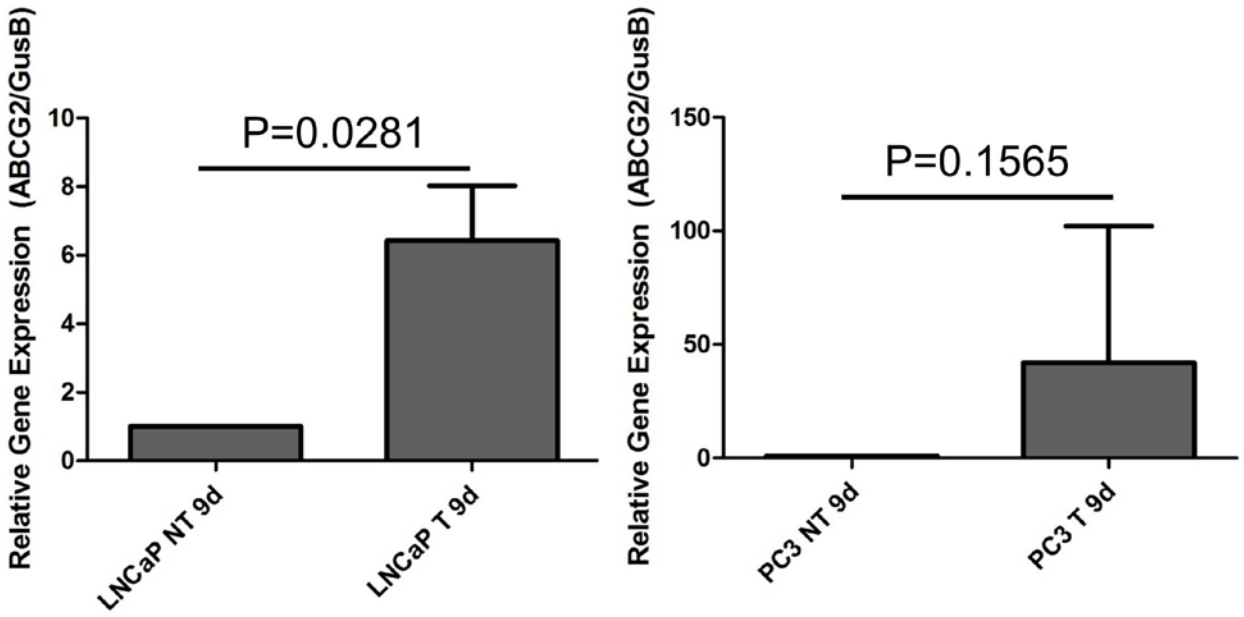

Figure 2-2. ABCG2 expression is increased after decitabine treatment. The expression of ABCG2 is significantly increased in decitabine treated LNCaP cells (p=0.0281, n=3). ABCG2 expression is increased in decitabine-treated PC3 cells but does not reach significance (p=0.1565, n=6). NT means not treated with decitabine and T means treated with decitabine. The cells were treated with decitabine for 9 days (9d). Relative gene expression was calculated by the ΔΔCt method.

2.3.3 ABCG2 mRNA expression increases after folate deprivation and may be caused by promoter hypomethylation

After observing an increase in ABCG2 mRNA after decitabine treatment, I hypothesized that growing the prostate cancer cells in decreased levels of folic acid would hypomethylate the ABCG2 promoter and lead to increased gene and protein expression. During one-carbon metabolism, folate donates the methyl group required to methylate DNA, RNA, and proteins. Therefore, if folate levels are decreased, then methylation should also decrease. I grew LNCaP and PC3 cells in 2.3μM (high), 25nM (physiologic), and 6.7nM (low) folic acid for 3 weeks. RNA and protein were harvested each week and the mRNA and protein expression was

measured. In LNCaP cells, the mRNA expression remained the same in all folic acid concentrations at one week, but at the two and three week time point the ABCG2 expression increased in the cells grown in 25nM and 6.7nM folic acid (**Figure 2-3**). In the PC3 cell line, the ABCG2 gene expression increased at all the time points in the cells grown in 25nM and 6.7nM (**Figure 2-3**).

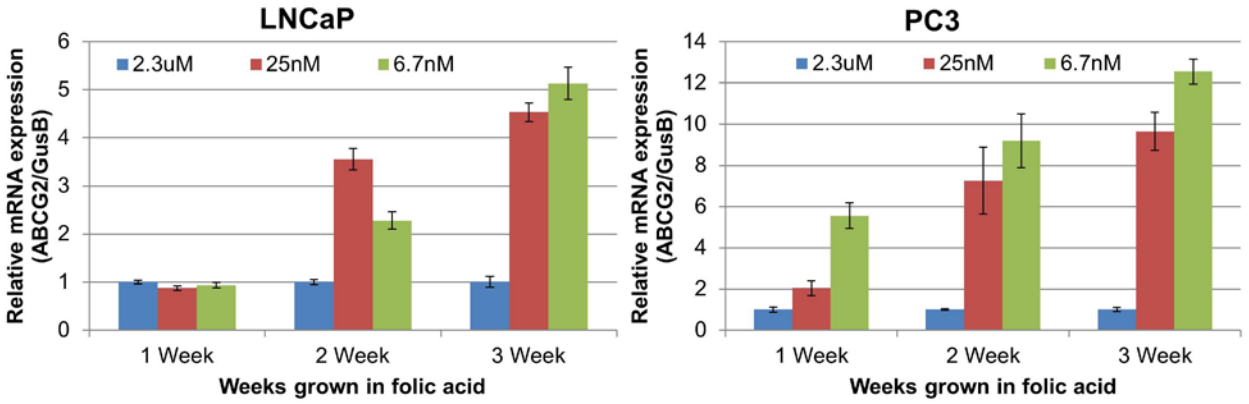

Figure 2-3. ABCG2 gene expression increases after folate deprivation. LNCaP and PC3 cells were grown in 2.3uM (high), 25nM (physiologic), and 6.7nM (low) folic acid for three weeks. ABCG2 gene expression increased in LNCaP cells grown in 25nM and 6.7nM after two weeks and remained increased at the three week time point. ABCG2 gene expression in PC3 cells grown in 25nM and 6.7nM folic acid increased after one week and remained increased at the two and three week time points. ABCG2 gene expression was measured by qPCR and analyzed using the ΔΔCt method. This is representative data from 3 experiments.

I next wanted to test if the increase in ABCG2 protein and mRNA was due to hypomethylation of the CpG island in the ABCG2 promoter region. Before I did this, I measured the intracellular folate levels in LNCaP and PC3 cells at different time points in to distinguish when the cells depleted their folate stores during folate deprivation. Knowing this information would determine how long to deprive the cells of folate before bisulfite sequencing the ABCG2 promoter. I initially grew LNCaP and PC3 cells in 2.3μM, 25nM, and 6.7nM folic acid for one

week. Intracellular folate stores were almost completely depleted after this length of time (**Figure 2-4a**). Then I grew the LNCaP and PC3 cells in 2.3μM, 25nM, and 6.7nM folic acid and harvested cells at 2, 3, 4, and 5 days. Again, the intracellular folate levels in the cells grown in 25nM and 6.7nM folic acid were extremely low (**Figure 2-4b**), suggesting that intracellular folate stores are depleted in less than 2 days. Finally, LNCaP and PC3 cells were grown in 2.3μM and 6.7nM folic acid for 24 hours and then harvested every four hours until 36 hours. A 48-hour time point was also included in this analysis (**Figure 2-4c**). At 24 hours, the intracellular folate stores are similar between the cells grown in 2.3μM and 6.7nM folic acid. By 36 hours, the cells grown in 2.3μM have started to replete their intracellular folate stores where as the intracellular folate stores in the cells grown in 6.7nM folic acid have plateaued. This finding suggests the cells may lose or deplete their intracellular folate stores after trypsinization and replating. When the cells are replated into media with low concentrations of folic acid, they are not able to increase their folate stores.

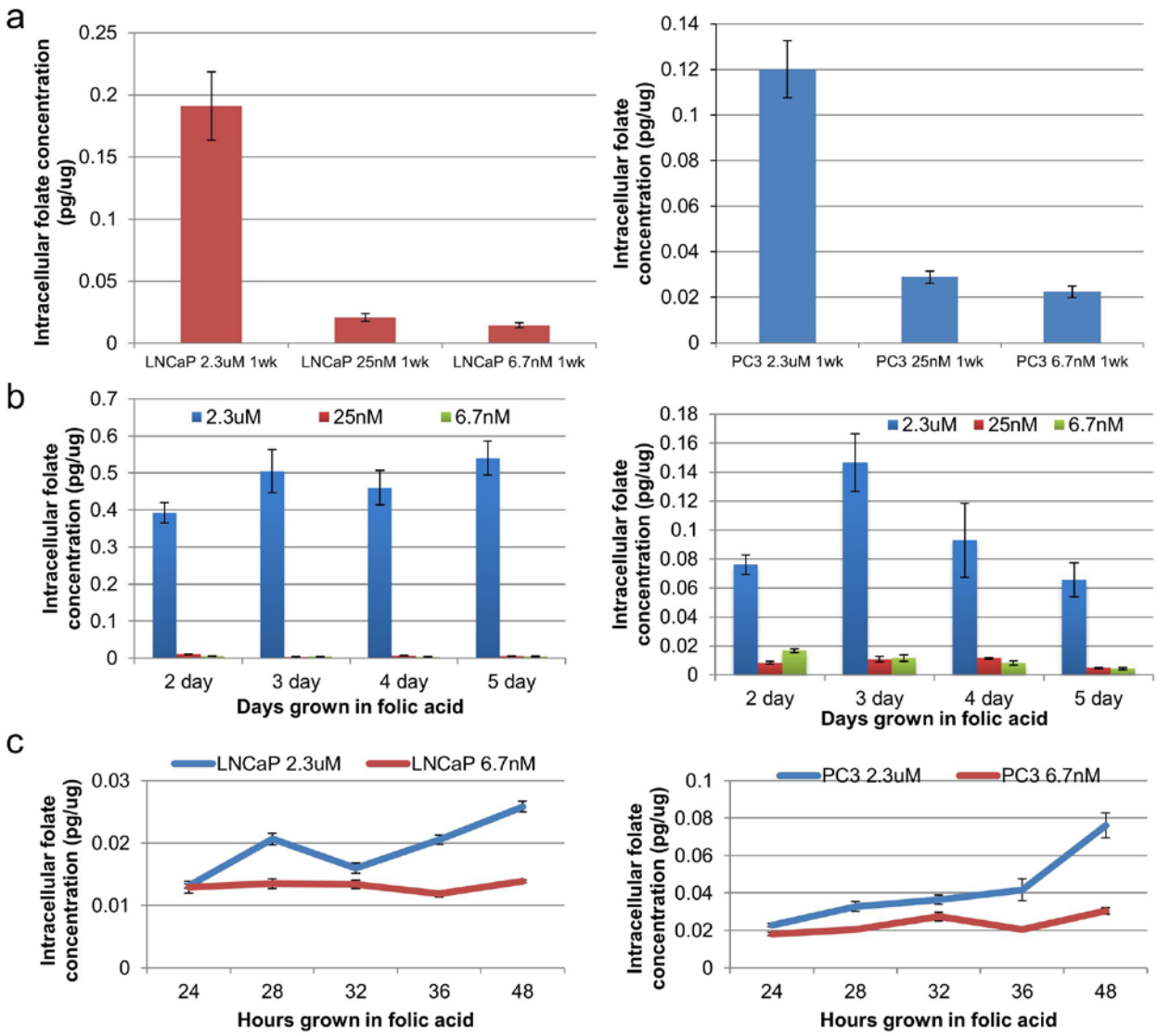

Figure 2-4. LNCaP and PC3 cells deplete intracellular folate stores by 36 hours. A. LNCaP and PC3 cells were grown in 2.3uM, 25nM, and 6.7nM folic acid for one week. Intracellular folate concentrations are decreased in the cells grown in 25nM and 6.7nM folic acid as measured by the microbiological folate assay. **B.** After growing LNCaP and PC3 cells in varying concentrations of folic acid for 2, 3, 4, and 5 days, the intracellular folate stores are still depleted in cells grown in 25nM and 6.7nM. **C.** LNCaP and PC3 cells were grown in 2.3uM and 6.7nM folic acid and harvested every 4 hours from 24-36 hours after being plated. Cells were also harvested at 48 hours. In both LNCaP and PC3, the intracellular folate stores begins to increase at 36 hours in the cells grown in 2.3uM, suggesting that after trypsinization and replating into 6.7nM folic acid, these cells will not replete their intracellular folate stores.

From these observations, it was decided that growing the cells in low levels of folic acid for one week would deplete intracellular folate and be sufficient time to determine whether there is an effect on the methylation of the CpG island in the ABCG2 promoter region. Initially, cells were grown in 2.3μM and 6.7nM folic acid and DNA was extracted and bisulfite converted. The ABCG2 promoter region was amplified by PCR and cloned. Ten clones from each folic acid treatment were sent for sequencing. After analysis of the sequence, a methylgram was generated to depict the methylated (black boxes) and unmethylated (white boxes) CpG dinucleotides. In **Figure 2-5**, the LNCaP cells grown in 6.7nM folic acid had a significantly hypomethylated ABCG2 promoter compared to cells grown in 2.3μM ($p<0.05$). I did a preliminary analysis on LNCaP cells grown in 100nM and 60nM folic acid and found that there was no significant difference in the ABCG2 promoter methylation in these treatment groups compared to the cells grown in 2.3μM. This data suggests that the depriving the cells of folic acid causes a hypomethylated promoter state, which is conducive to gene transcription.

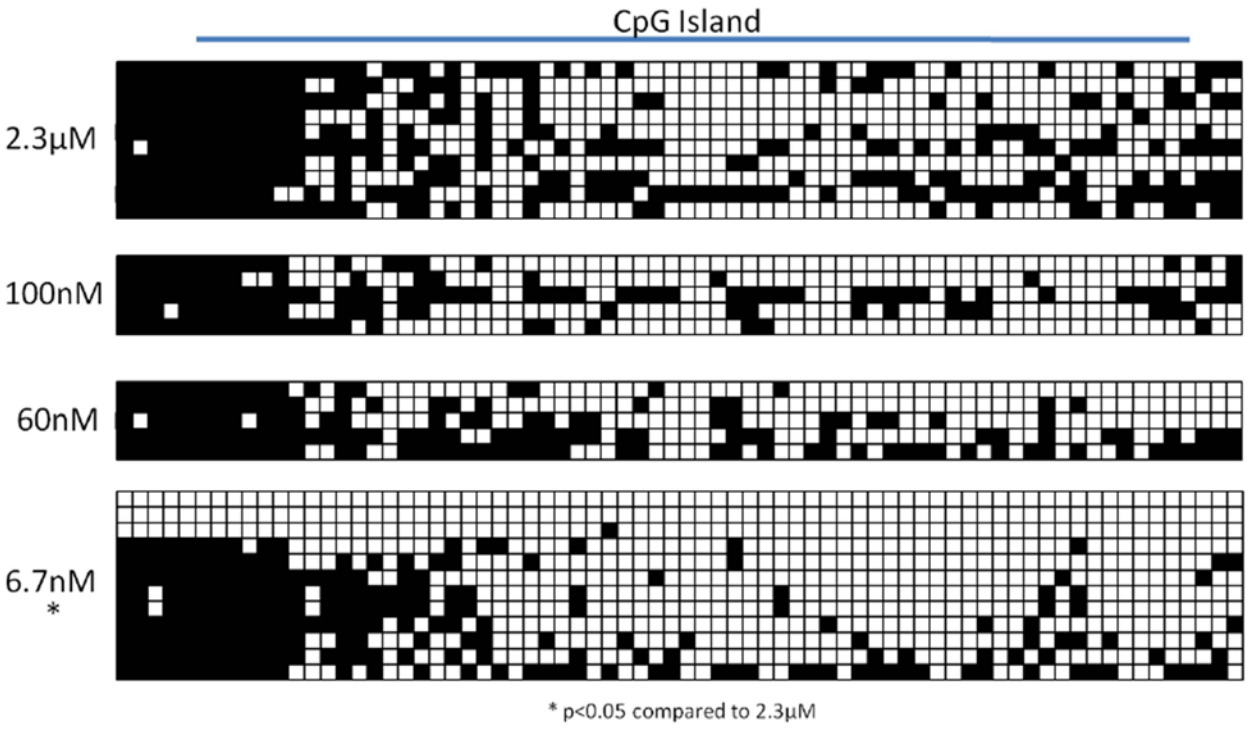

Figure 2-5. Folate deprivation causes the ABCG2 promoter to become hypomethylated. A methylgram of the ABCG2 promoter was generated after bisulfite conversion, PCR amplification, cloning, and sequencing of LNCaP cells grown in varying concentrations of folic acid. Each box represents one CpG dinucleotide in the ABCG2 promoter region. The black boxes are methylated CpGs and the white boxes are unmethylated CpGs. There was no significant difference in methylation in cells grown in 2.3µM, 100nM, and 60nM folic acid. However, the promoter of the cells grown in 6.7nM folic acid was significantly hypomethylated ($p<0.05$) when compared to cells grown in 2.3µM.

2.4 DISCUSSION

In the prostate cancer cell lines tested, I observed that ABCG2 expression is increased when treated with decitabine. Decitabine inhibits DNA methyltransferases that catalyze the addition of a methyl group to a cytosine in a CpG dinucleotide context. The increase in ABCG2 expression is likely due to transcriptional activation from a hypomethylated promoter state. The methylation

of the ABCG2 promoter was not analyzed in this experiment. However, several groups have observed global and promoter hypomethylation after decitabine treatment [38, 117-119] suggesting that this could also occur in the prostate cancer cell lines. The ABCG2 promoter also appears to be susceptible to promoter demethylation. There is evidence that promoters with a very high CpG content are less likely to be demethylated after decitabine treatment [120]. The ABCG2 promoter is 55% CpGs which is the cut-off for a CpG island qualification.

This data suggests that the mRNA expression of ABCG2 in prostate cancer cell lines is up-regulated after folate deprivation. The up-regulation is due in part to hypomethylation of the ABCG2 promoter in the cells grown in a low folate environment. Because folate is required for the methyl group donation for the cell to methylate DNA, RNA, and histones, it is logical that in low folate conditions, the amount of available methyl groups decreases, promoter hypomethylation occurs, and transcription factors are able to bind the promoter to initiate transcription. Indeed, increased S-adenosylhomocysteine (the resulting product after the methyl group is transferred from S-adenosylmethionine to the DNA) and decreased S-adenosylmethionine has been observed after folate deprivation [121, 122].

The observation that ABCG2 expression is increased after folate deprivation supports what Lemos, et. al. has observed multiple times in the colon cancer cell lines, Caco-2 and WiDr [74-76]. These studies and ours are in direct contrast to the finding by Ifergan, et. al. who observed a massive decrease in ABCG2 expression after folate deprivation in the breast cancer cell line overexpressing ABCG2, MCF-7/MR [72]. The authors postulate that ABCG2 is down-regulated as an adaptation to the folate deprivation. Because folate is required for cell growth, DNA repair, and methylation reactions, the cell is required to adapt in order to survive. By down-regulating ABCG2 in a folate deprived environment, the cell is able to retain more folates

and continue to survive. Ifergan also observed a decrease in ABCC1 (MRP1), another folate effluxer and an increase in folylpolyglutamate synthase (FPGS), which catalyzes the addition of glutamate to folate in order to retain and metabolize folates (reviewed in [123]). This cascade of gene up- and down-regulation allowed the MCF-7/MR cells to survive in a folate deprived environment. Lemos, et. al. did not observe a difference in ABCC1 expression, but observed an increase in RFC (reduced folate carrier, folate importer), FPGS, DHFR (dihydrofolate reductase, reduces dihydrofolate to tetrahydrofolate in one-carbon metabolism pathway), and TS (thymidylate synthase, generates dTMP). This data also presents a picture of the cell adapting. This study did not measure the expression of other genes involved in the one-carbon metabolism pathway, but I would hypothesize that folate importers, including RFC, the folate receptors and PCFT (proton-coupled folate transporter) would be up-regulated in a folate deprived environment in order to utilize any and all folate available.

The expression of ABCG2 in response to folate deprivation has not been thoroughly studied and as presented above, ABCG2 is either up- or down-regulated in cells deprived of folic acid when compared to cells maintained in standard media (2.3μM folic acid). The effect of the folic acid on ABCG2 expression is likely tissue specific or possibly cell line specific. The promoter methylation status in the MCF-7, Caco-2, and WiDr cell lines has not yet been characterized. It is possible that the ABCG2 promoter in the MCF-7 cell line is more methylated than in the colorectal cancer and prostate cancer cell lines. This may cause the MCF-7 cell line to be less susceptible to promoter demethylation, as observed in the LNCaP prostate cancer cell line. Further work will need to be conducted to understand the complexities of ABCG2 expression in different models after folate deprivation.

In conclusion, I observed for the first time that ABCG2 expression is increased in prostate cancer cell lines after decitabine treatment and folate deprivation. The increased expression appears to be in part due to demethylation of the ABCG2 promoter. ABCG2 is one of the genes involved in multidrug resistance phenotypes and is a target for pharmaceutical inhibition in order to increase intratumoral drug concentrations. By easily modifying dietary folate, a personalized diet could be given to patients in order to increase the efficacy of their chemotherapeutics.

3.0 EXPRESSION AND FUNCTION OF ABCG2 AND THE Q141K VARIANT IN PROSTATE CANCER

3.1 INTRODUCTION

The Q141K variant of ABCG2 is implicated in poor prognosis of adult AML treated with idarubicin-based chemotherapy [124] and longer progression free survival in advanced stage ovarian cancer patients treated with platinum and taxane-based chemotherapy [125]. I am interested in the role ABCG2 and the Q141K variant may play in prostate cancer. Prostate cancer is the most frequently diagnosed cancer and the second-leading cause of cancer related deaths in American men [62]. A retrospective study has shown that androgen independent prostate cancer patients with the Q141K variant have a shorter overall survival time than patients without the SNP (5.3 years vs. 7.4 years) [64]. The authors hypothesized that the decreased function of the variant ABCG2 protein allows increased intracellular concentrations of dihydrotestosterone that drives the proliferation of the cells. The authors tested to see if ABCG2 transported testosterone and found that neither the wild-type nor the Q141K variant was able to transport testosterone. However, Huss, et. al. has identified dihydrotestosterone as an ABCG2 substrate [66].

ABCG2 is also known to efflux folate, and tumor cells are known to divide directly in response to available folate. It was recently observed that the prostate has a high requirement for folate due to polyamine biosynthesis [115]. Our laboratory has recently found that an increased

fasting serum folate level in prostate cancer patients is correlated to increased Ki67 index, which is an indicator of cellular proliferation [126]. Therefore, we hypothesize that increased intracellular folate levels in the Q141K variant may also play a significant role in driving the proliferation of the tumor cells and thereby decrease patient survival.

In another study, the effectiveness of combining docetaxel, the first-line chemotherapeutic for castration resistant prostate cancer, with other drugs for the treatment of prostate cancer was examined. The authors found that patients heterozygous for the Q141K variant had a longer survival time than patients who were homozygous for wild-type ABCG2 [65]. This is likely because ABCG2 effluxes docetaxel and the patients expressing the Q141K variant were able to retain higher intratumoral concentrations of docetaxel than wild-type patients. Because ABCG2 functions as a homodimer, theoretically patients heterozygous for the allele encoding the variant protein will have the majority of their ABCG2 protein affected by any phenotype the variant imparts on the protein. Determining the effect the Q141K variant has on the efflux of molecules important for cell growth and death can provide a better understanding as to how to clinically manage patients, and whether treatment should differ based on the patient's ABCG2 genotype.

Identification of effective inhibitors of ABCG2 is currently a target for pharmaceutical development, therefore the goal of this chapter was to determine the potential dual roles of the Q141K variant in prostate cancer. In this chapter, I tested the hypothesis that folate, docetaxel and dihydrotestosterone are differentially effluxed by the wild-type ABCG2 and the Q141K variant using both patient samples and *in vitro* models.

3.2 MATERIALS AND METHODS

3.2.1 Microarray Analysis

To analyze the expression of ABCG2 in prostate cancer from published microarray data, the information was mined from online repository for gene expression information. Best, et. al. deposited their microarray data into GEO (Gene Expression Omnibus series number GSE2443) [127] and Liu, et. al. deposited their data into ArrayExpress (accession number E-TABM-26) [128]. This data was analyzed in GraphPad Prism.

3.2.2 ABCG2 Genotyping

Tissue from recurrent prostate cancer patients was procured from the University of Pittsburgh Health Sciences Tissue Bank under the University of Pittsburgh Institutional Review Board Protocol #970480. DNA was extracted from tissues with the QIAamp® DNA Micro Kit (Qiagen). The DNA region containing the site coding for the Q141K variant was amplified with the primers Q141K SNP Forward (5'-CCATCATTATGTCTCATTAAAATGC) and Q141K SNP Reverse (5'-CCTGAATGACCCTGATAATCCG). The PCR was performed in a 25µL reaction containing 10X Titanium buffer and 0.2µL of Titanium Taq (Clontech), 0.5µL of 25µM Forward and Reverse primer, and 0.5µL of 10mM dNTPs. The cycling conditions were: 94°C for 1 minute, 40 cycles of 94°C for 30 seconds, 55.6°C for 30 seconds, and 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. The PCR products were then digested with *MseI* (New England Biolabs) overnight at 37°C. Digested PCR products were visualized on a 2.5% TBE agarose gel. The CC genotype resulted in 2 bands (34bp and 124bp), the CA genotype resulted in

4 bands (34bp, 44bp, 80bp, and 124bp), and the AA genotype resulted in 3 bands (34bp, 44bp, and 80bp).

3.2.3 Microbiological Folate Assay

Intracellular folate concentrations were determined by the microbiological *Lactobacillus casei* assay as described in Section 2.2.7.

3.2.4 Immunofluorescence

Fresh frozen Gleason 7 prostate cancer tumors were embedded in O.C.T. compound (Tissue-Tek) and 8 micron sections were put on glass slides. Tissues were rehydrated with PBS before fixation with 2% paraformaldehyde for 15 minutes and permeabilization with 0.1% Triton X-100 for 15 minutes. Samples were washed 3 times with PBS and 5 times with 0.5% BSA/PBS. Samples were blocked in 2% BSA/PBS for 1 hour, washed 5 times with 0.5% BSA/PBS, incubated for 2 hours in the ABCG2 antibody (Millipore, clone BXP-21, 1:100 dilution), washed 5 times with 0.5% BSA, and incubated for 1 hour in the Alexa Fluor 488 secondary antibody (1:500 dilution). The slides were washed 5 times with 0.5% BSA/PBS, 5 times with PBS, then nuclei were stained with 0.75µg/mL tissues of Hoechst 33342 (Sigma) for 30 seconds and washed 2 times with PBS. Slides were coverslipped with Fluorescent Mounting Medium (Dako) and imaged on an Olympus FV1000 Confocal Microscope. Exposure times were kept consistent for all samples.

3.2.5 Cell Culture

The LNCaP and PC3 cell lines (obtained from ATCC) were maintained in standard RPMI 1640 medium supplemented with 10% or 5% FBS, respectively, L-glutamine, and penicillin/streptomycin. The HEK293 cell line (generous gift of Dr. Zhou Wang) was maintained in DMEM medium supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. Cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2.

3.2.6 Lentiviral Transduction

The ABCG2 cDNA in the pCMV6-AC vector (Origene) was sub-cloned into the lentiviral vector, pLVX-puro (Clontech). To do this, *XhoI* was used to digest the pCMV6-AC vector 3' of the ABCG2 DNA (10µL 10x NEB buffer 4, 1µL 100x BSA, 7.6µL H_2O, 6.8µL *XhoI*, and 10µg of the ABCG2 plasmid at 37ºC for 3 hours). The pLVX-puro vector was digested at the 3' end of the multi-cloning site with *BamHI* (7.5µL 10x NEB buffer 3, 0.75µL 1000x BSA, 37.35µL H_2O, 5.1µL *BamHI*, and 10µg pLVX-puro plasmid at 37ºC for 3 hours). The digested vectors were blunt-ended (for pCMV6-AC-ABCG2: 100µL digested product, 12.5µL 1000µM primers, 3.4µL 1unit/µg DNA T4 pol, and 9.1µL H_2O and for pLVX-puro: 75µL digested product, 7.5µL 1000µM primers, 3.4µL 1unit/µg DNA T4 pol, and 14.1µL H_2O) at 12ºC for 15 minutes. Then 0.5M EDTA was added and the mixture was incubated at 75ºC for 20 minutes to inactivate the enzyme. The solutions were then purified using the Geneclean II kit (BIO 101 Systems). Ten microliters of Glassmilk was used according to manufacturer's instructions. The purified solutions were then digested with *EcoRI* (5µL 10x NEB EcoRI buffer, 31.6µL H_2O, 3.4µL *EcoRI*, and 10µL of the purified solution) for 3 hours at 37ºC. *EcoRI* digests the 5' end of both

the pLVX-puro and pCMV6-AC vector. After digestion, the entire 50µL was electrophoresed on a 0.8% TBE gel for 40 minutes at 100V. The bands were excised from the gel and purified using the Geneclean II kit (BIO 101 Systems). Ten microliters of glassmilk was used according to the manufacturer's instructions. One microliter was visualized on a 0.8% TBE gel for 40 minutes at 100V. The purified ABCG2 cDNA and purified pLVX-puro vector were ligated over night at 14ºC. Two ratios (1:1 and 2:1) of ABCG2:pLVX-puro were set up. For the 1:1 ratio the solution contained 2µL T4 ligase buffer, 5µL T4 ligase, 2µL purified ABCG2, 2µL purified pLVX-puro, and 9µL H_2O. For the 2:1 ratio, 4µL of the purified ABCG2 cDNA and 2µL purified pLVX-puro was used. After the ligation, the ligated vector was transformed into Peak Efficiency Chemically Competent bacteria (Genemate) according to manufacturer's protocol. The bacteria was plated on 400µg/mL ampicillin LB agar plates and grown overnight at 37ºC. Twenty colonies cultured overnight at 37ºC while shaking in 400µg/mL ampicillin LB. The DNA was extracted from the cultures using the Wizard Plus SV miniprep kit (Promega) per manufacturer's instructions. Colonies were screened for the ABCG2 cDNA insert by restriction digest. After a double digestion with *XbaI* and *XhoI*, the 2500bp ABCG2 cDNA insert was visualized on a 0.8% TBE gel. After a positive clone was identified, the miniprep was spiked into 50mL of 400µg/mL ampicillin LB and grown overnight at 37ºC with shaking. The DNA from the midiprep was isolated with the Wizard Plus Midiprep kit (Promega) per manufacturer's instructions. The entire ABCG2 cDNA insert was sequence verified with the following primers: pLVXpuro F (5'-CCATCCACGCTGTTTTGACC), pLVXpuro R (5'-GAAAAGCGCCTCCCCTACCC), pLVXpuro2636F (5'-TGAAGAGTGGCTTTCTACCTTGTCG), and pLVXpuro1777R (5'-TGGTCGTCAGGAAGAAGAGAACC).

The University of Pittsburgh Cancer Institute Lentiviral Core generated active viral particles that were transduced into the LNCaP, PC3, and HEK293 cell lines. A GFP lentiviral control vector (pLKO.1-puro-CMV-TurboGFP) was transduced into the three cell lines to act as a control. The transduced cells were selected with puromycin (2 μg/mL, 1 μg/mL, and 3μg/mL, respectively). After generation of the selected transduced cell lines, STR PCR technology was used to validate the cell lines [129]. All experiments used mixed clones to avoid effects attributable to specific integration of the transgene.

3.2.7 Site-Directed Mutagenesis

Site directed mutagenesis was used to generate the Q141K variant and the delABCG2 construct. The Quikchange XL site directed mutagenesis kit (Stratagene, Agilent Technologies) was used according to the manufacturer's instructions. Briefly, to generate the Q141K plasmid, the primers Q141K SDM Forward (5'- TGACGGTGAGAGAAAACTTAAAGTTCTCAGCAGCT) and Q141K SDM Reverse (5'- AGCTGCTGAGAACTTTAAGTTTTCTCTCACCGTCAG) were used. To generate the delABCG2 plasmid, the primers ABCG2 miRNA del SDM Forward (5'-gtatgatttatcctcacataaactcgagatcccgcgactctag) and ABCG2 miRNA del SDM Reverse (5'-CTAGAGTCGCGGGATCTCGAGTTTATGTGAGGATAAATCATAC) were used. The delQ141K plasmid was generated by using site-directed mutagenesis to mutate the delABCG2 plasmid. The plasmids were sequence-verified.

3.2.8 RNA isolation, Reverse Transcription and Quantitative PCR

RNA was extracted from cell lines using the Trizol reagent (Invitrogen) as described in Section 2.2.4. One microgram of RNA was reverse transcribed as described in Section 2.2.5. ABCG2 and GusB expression were measured in a 20µL PCR reaction as described in Section 2.2.6.

3.2.9 Western Blotting

Protein was extracted from cells using RIPA buffer. RIPA buffer contained 150mM NaCl, 1% Triton X-100, 50mM Tris, pH 8.0, 0.1% SDS, and 1mM EDTA-free protease inhibitors. Depending on the size of the pellet, 50-150µL of the buffer was used to resuspend the pellet. The mixture was briefly sonicated (5 second pulses for 15 seconds) and then incubated on ice for 30 minutes. After centrifugation at 16,000x g for 15 minutes at 4ºC, the supernatant containing the protein was transferred to a new tube.

The concentration of the protein was measured by a BCA protein assay (Pierce) or a Bradford protein assay (Amresco). BCA protein standards were made from a 2mg/mL BSA stock and standards ranged from 0mg/mL to 2mg/mL. Bradford standards ranged from 0mg/mL to 0.2mg/mL. Twenty microliters of each standard and sample were added to a 96 well plate in triplicate. The protein samples were diluted in a range from 1:50 to 1:200. For the BCA protein assay, 200µL of Reagent B was added to 9.8mL of Reagent A. One hundred eighty microliters of the Reagent AB mix was added to each protein standard and sample. The plate was incubated at 37ºC for 30 minutes and then read at 562nm on a Molecular Devices SpectraMax M2^{e} plate reader. For the Bradford protein assay, 180µL of the Bradford reagent was added to each protein standard and sample and immediately read on the plate reader at 595nm.

A 10% polyacrylamide gel was used for electrophoresis. The components of the resolving gel are 2.5mL of 4x lower gel buffer (dissolve 18.2g Tris Base in 60mL Milli-Q water, adjust pH to 8.8, add water to a total of 100mL, filter solution and add 0.4g SDS), 4.9mL Milli-Q water, 2.5mL 40% (37.5:1) acrylamide solution, 50µL 10% APS (prepared fresh), and 5µL TEMED. After the resolving gel has polymerized, the stacking gel is poured. The stacking gel contains 2.5mL of 4x upper gel buffer (dissolve 6.05g Tris Base in 40mL Milli-Q water, adjust pH to 6.8, add water to a total of 100mL, filter solution and add 0.4g SDS), 6.6mL water, 0.8mL 40% (37.5:1) acrylamide solution, 100µL 10% APS (prepared fresh), and 10µL TEMED. Fifteen to fifty micrograms of protein was mixed 1:1 with Laemmli buffer containing β-mercaptoethanol (1:20 dilution). It is important to note that when ABCG2 was the protein of interest, the samples were not boiled. For other proteins, the samples were boiled at 95ºC for 5 minutes. The Precision Protein Plus (BioRad) marker was used. Proteins were resolved at 150V for approximately 1.5 to 2 hours. The running buffer components were 1.52g Tris Base, 7.2g Glycine, and 0.5g SDS in 500mL Milli-Q water.

The proteins were transferred to a 0.45µm PVDF membrane. To do this, the PVDF membrane was incubated in methanol and then equilibrated in transfer buffer (3.03g Tris Base, 14.4g Glycine, 3.76mL 10% SDS, 200mL methanol, and 800mL Milli-Q water). The gel is also equilibrated in transfer buffer for 15 minutes. The proteins were transferred overnight in the cold room at 30V.

After the transfer, the PVDF membrane was incubated at 60ºC for 5 minutes with vacuum pressure. The membrane was incubated in methanol and rehydrated with water. The membrane was blocked with 5% milk/TBS for 2 to 4 hours. The membrane was rinsed briefly with TBS-T before the primary antibody was added. The ABCG2 antibody (Millipore) was used

at a dilution of 1:500 and incubated with the blot for 4 hours. The β-actin antibody (Sigma-Aldrich was used at a dilution of 1:10,000 and incubated with the blot for 1 hour. Then the membrane was washed 4 times, 5 minutes each with TBS-T. The secondary antibody was added and incubated for 1 hour. The membrane was washed 4 times, 5 minutes and Pierce ECL Western Blotting Substrate was added for one minute. Films were developed on an MXR developer.

3.2.10 miRNA expression

A modified protocol for stem-loop RT-qPCR for miRNA analysis from Current Protocols in Molecular Biology [130] and Chen, et.al [131] was used. Briefly, 100μM reverse transcription primers (primers from [132], miR-519c RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATCCTC, miR-520h RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCTA, and U74: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATTGT) were overlaid with 100μL mineral oil. To create a stem-loop, the primers were incubated at 95°C for 10 minutes, slowly reduced to 75°C (the temperature was decreased 0.4°C every minute), then incubated at 75°C for 1 hour, 68°C for 1 hour, 65°C for 1 hour, 62°C for 1 hour, and then held at 60°C for 9.5 hours. The primers were then aliquoted and frozen until needed. Total RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription reactions were performed as a 7.5μL reaction containing 50nM stem-loop RT primer, 1X MMLV RT buffer (Promega), 0.25mM of each dNTP, 3.33U/μL MMLV reverse transcriptase (Promega), 0.25U/μL RNase Inhibitor (Roche) and 1μg RNA. The reactions were incubated in an Eppendorf thermocycler for 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and held at 4°C. A reverse

transcriptase negative control was run for each primer and RNA sample. qPCR reactions were performed as 10µL reactions containing 1X iQ™ SYBR® Green Supermix (BioRad), 1.5µM specific miRNA forward primer (miR-519c: 5'-GGCGGGAAAGTGCATCTTTTT, miR-520h: 5'-GGCGACAAAGTGCTTCCCTT, and U74: 5'-CCTGTGGAGTTGATCCTAGTCTGGGTG), 0.7µM Universal Reverse primer (5'-GTGCAGGGTCCGAGGT), and 0.67µL of RT product. Products were amplified with the following conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on a BioRad iQ5 real time PCR thermocycler.

3.2.11 Flow Cytometry

The percentage of ABCG2 expressing cells was determined by flow cytometry. The protocol used was modified from Goodell, et. al. [133]. Briefly, $1x10^6$ cells/mL for each cell line was aliquoted and incubated for 10 minutes at 37°C and 5% CO_2 in pre-warmed RPMI with or without 1µM Ko143. The cells were incubated in 5µg/mL Hoechst 33342 for 1 hour at 37°C and 5% CO_2. Cells were washed once with ice cold PBS and resuspended in ice cold RPMI. To exclude non-viable cells from the analysis, 100µg/mL of propidium iodide was added to the cells. A Beckman Coulter (Cytomation) MoFlo High Speed Sorter was used to analyze and sort the ABCG2 expressing cells. Hoechst 33342 was excited at 355nm and a fluorescent profile was created for dual-wavelength analysis (450/50nm and 675/20nm).

3.2.12 MG132 treatment

The PC3-GFP, PC3-delABCG2, and PC3-delQ141K cell lines were treated with 20μM MG132 for 7 and 24 hours. The cells were lysed with RIPA buffer and processed according to the protocol in Section 3.2.9.

3.2.13 Docetaxel treatment

LNCaP cells were treated with 1nM docetaxel (Sigma) for 8 days and the ABCG2 mRNA expression was measured by qPCR as detailed in sections 2.2.4, 2.2.5, and 2.2.6.

3.2.14 Statistical Analysis

An unpaired t-test (Prism Graph Pad) was used to analyze the microarray data. SigmaPlot 12 was used for the remaining statistical analyses. Time to PSA recurrence in regards to patient ABCG2 genotype was analyzed by a Kaplan-Meier survival analysis. A t-test was used to analyze the serum folate concentration and genotype experiment. The miRNA data was analyzed by a t-test.

3.3 RESULTS

3.3.1 ABCG2 is down-regulated in primary prostate cancer and up-regulated as the disease progresses

To determine the expression of ABCG2 in normal human prostate tissue and prostate cancer tissue, I analyzed microarray data available on Oncomine. Lapointe, et. al. analyzed normal prostate and primary prostate carcinomas. The expression of ABCG2 is decreased in the prostate cancer compared to the normal prostate (**Figure 3-1a**, $p=3.51x10^{-9}$). Microarray data is also available for androgen independent vs. androgen dependent prostate cancer and prostate cancer categorized by Gleason score. This data was extracted from GEO [127] and ArrayExpress [128], respectively. There was a significant increase in ABCG2 expression in androgen independent prostate cancer ($p=0.0056$) compared to androgen dependent prostate cancer (**Figure 3-1b**). A significant up-regulation of ABCG2 expression was also observed in prostate cancer that was scored as 8 or higher compared to a lower grade prostate cancer ($p=0.0272$) (**Figure 3-1c**).

The mRNA expression of ABCG2 in prostate cancer cell lines and donor prostate tissue was examined by qPCR. The expression of ABCG2 in the prostate donor tissue was higher than the prostate cancer cell lines LNCaP, PC3, and DU143, the benign prostatic hyperplasia cell line, BPH1 (**Figure 3-1d**), and the normal prostate cell line, RWPE-1. This data supports the observations from Oncomine that normal prostate tissue expresses higher levels of ABCG2 than prostate cancer. However, there are some inconsistencies between the microarray data and the cell line data. For example, the normal prostate cell line, RWPE-1 expresses lower levels than the prostate cancer cell line DU145 (brain metastasis) and the androgen-independent cell line PC3 expresses less ABCG2 than the androgen-dependent cell line LNCaP. It is possible that the

culture conditions for the cell lines would affect the expression level of ABCG2 and the cells lines may not be representative of ABCG2 expression in human tissue.

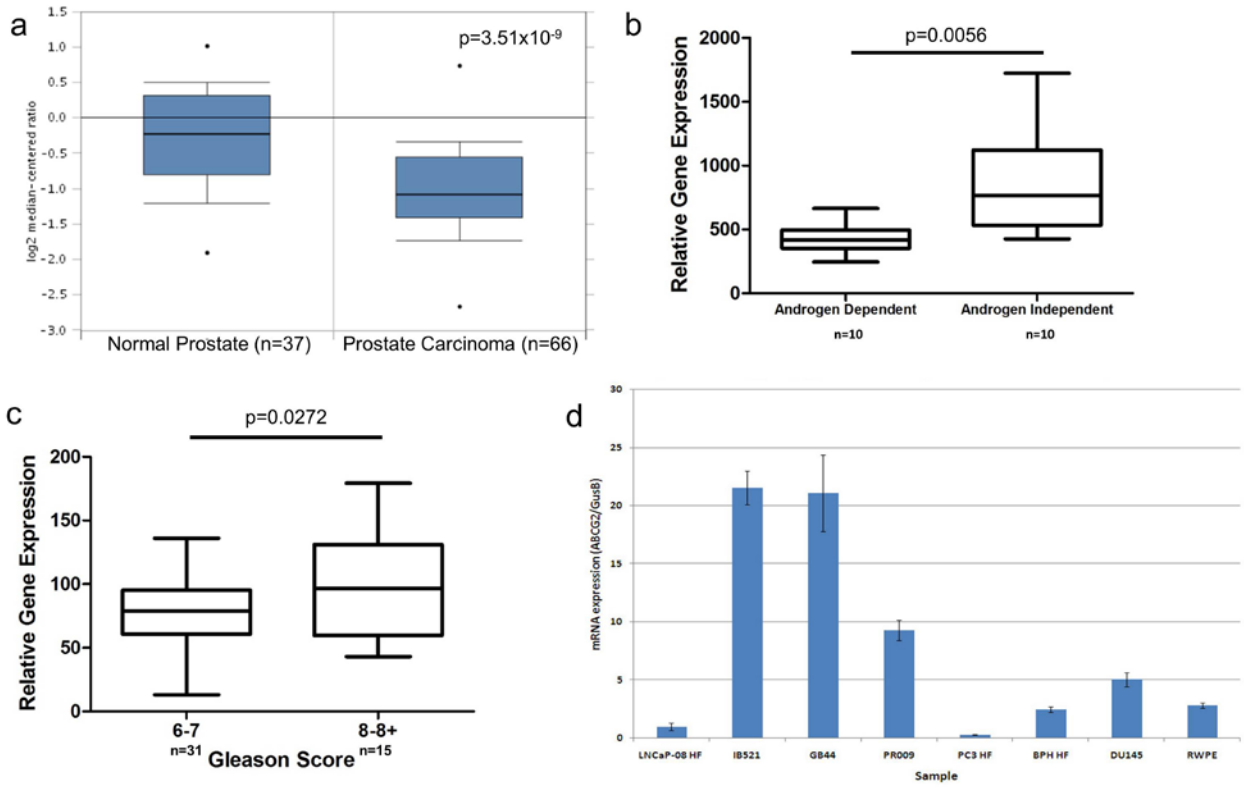

Figure 3-1. ABCG2 is down-regulated in primary prostate cancer and is up-regulated in later stage and higher grade prostate cancer. A. Data from Oncomine revealed a significant down-regulation of ABCG2 in primary prostate cancer tissue compared to normal prostate tissue ($p=3.51x10^{-9}$). **B.** Androgen independent prostate cancer patients from a phase II clinical trial of docetaxel and thalidomide were compared to androgen dependent patients who received a prostatectomy as their first-line therapy. A significant up-regulation of ABCG2 was observed in the androgen independent patients (p=0.0056). **C.** A Gleason score was assigned after radical prostatectomy and a significant up-regulation of ABCG2 was observed in the higher grade prostate cancers (p=0.0272). **D.** ABCG2 expression is increased in the prostate donor tissue compared to the prostate cancer cell lines and benign prostatic hyperplasia cell line. The normal prostate cell line, RWPE-1 expresses higher levels of ABCG2 than LNCaP and PC3, the same as BPH-1, and lower than DU145.

3.3.2 The Q141K variant decreases time to PSA recurrence in prostate cancer patients

To determine if there is a difference in recurrence or time to recurrence in prostate cancer patients expressing wild-type ABCG2 or the Q141K variant, lymphocytes from forty-one prostate cancer patients were genotyped at the ABCG2 C421A locus by PCR and *MseI* restriction digestion (the genotyping for this experiment was completed by Jessica L. Cummings). This cohort was selected from prostate cancer patients who had biochemical recurrence after undergoing a prostatectomy but receive no drug therapy. Seven patients were CA (heterozygous; encoding the QK protein) and 34 patients were CC (wild-type; encoding the QQ protein) (see **Figure 3-2** for a representative *MseI* digest gel). The rate of recurrence was not statistically different between the genotypes. A Kaplan-Meier survival curve for time to PSA recurrence was generated for the homozygous carriers of the wild-type ABCG2 protein versus carriers of the variant Q141K protein, substituting time to PSA recurrence post-prostatectomy for survival. One patient who recurred within one month of surgery was excluded from analysis. Patients with the Q141K variant had a significantly shorter time to PSA recurrence post-prostatectomy than patients expressing wild-type ABCG2 (mean time to PSA recurrence: 15.5 months vs. 51 months, P=0.01) (**Figure 3-3**).

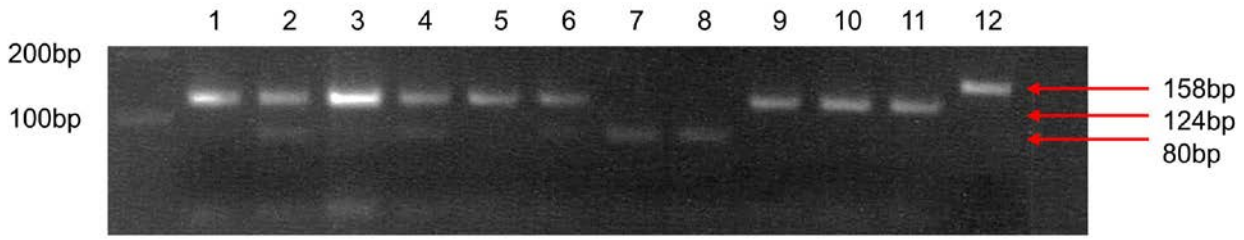

Figure 3-2. Representative banding pattern for the three genotypes at the 421 locus. The CC genotype produces 2 bands at 124bp and 34bp. The 34bp is too small to visualize on the agarose gel. Lanes 1, 3, 5, 9, 10, and 11 have the CC banding pattern. The CA genotype produces 4 bands at 124bp, 80 bp, 44bp, and 34bp. The 44bp band is also too small to visualize on this gel. Lanes 2, 4, and 6 have the CA banding pattern. The AA genotype produces 3 bands at 80bp, 44bp, and 34bp. Lanes 7 and 8 represent the AA genotype banding pattern. Lane 12 is the undigested PCR product (158bp). (This experiment was done by Kathryn M. Sobek.)

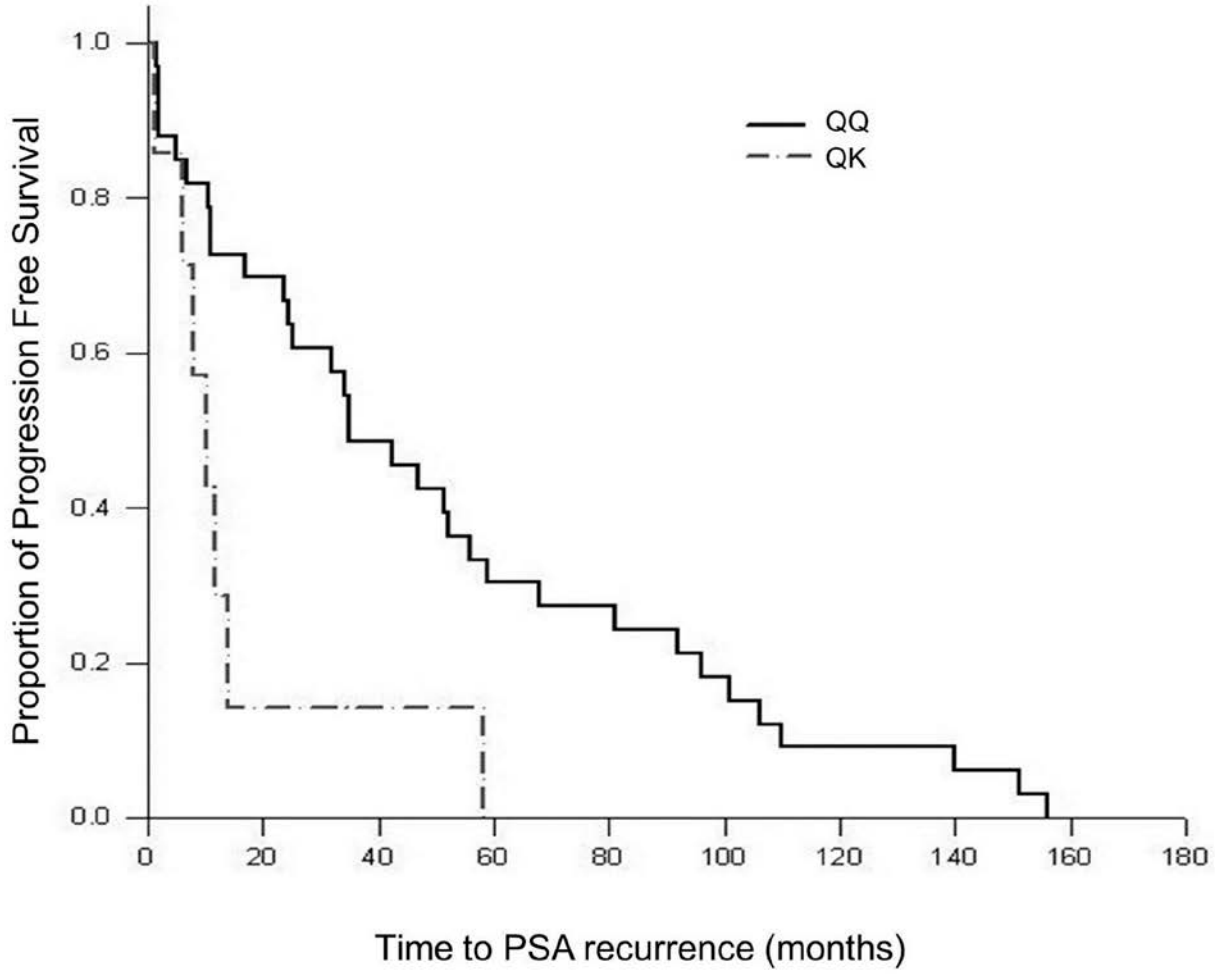

Figure 3-3. Q141K variant decreases time to PSA recurrence. Prostate cancer patients that had recurred after prostatectomy and received no drug treatment were evaluated for time to PSA. The genotype at the 421 locus that encodes for the Q141K variant was determined by PCR and restriction digest. Patients with the CA genotype (QK variant, n=7) had a significantly shorter time to PSA recurrence than patients with the CC genotype (QQ wild-type, n=33) (mean time to PSA recurrence:15.5 months vs. 51 months, P=0.01).

3.3.3 Patients with the Q141K variant have reduced systemic levels of folate

I hypothesized that the ABCG2 genotype at the 421 locus is correlated with serum folate levels because ABCG2 effluxes folates and the Q141K variant decreases the function of ABCG2. To

test this, the microbiological folate assay was used to measure the fasting serum folate levels of Gleason 7 prostate cancer patients (the folate assays were completed by Jessica L. Cummings and Jeffrey J. Tomaszewski). The genotype at the 421 locus was determined by PCR and *MseI* digestion as described in section 3.3.2. I observed a significantly lower serum folate level in the patients with the Q141K variant than patients with the wild-type ABCG2 (**Figure 3-4**, P=0.029). This finding suggests that the Q141K variant-expressing patients may be retaining more folate within the liver, which is the storage tissue for folate. This observation also indicates an important role for ABCG2 in systemic folate regulation.

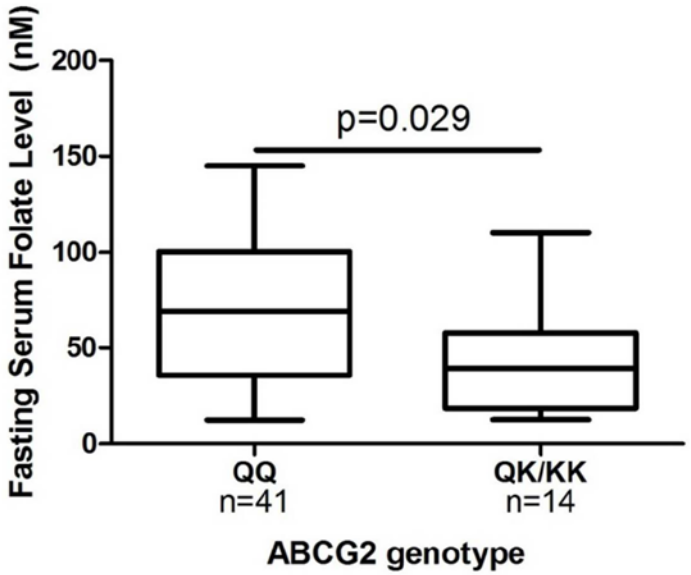

Figure 3-4. The Q141K variant is correlated to low systemic folate levels in prostate cancer patients. Folate from Gleason 7 prostate cancer patients' serum was measured using a microbiological assay. Patients with the variant ABCG2 (either QK or KK) have significantly lower serum folate concentrations compared to patients expressing wild-type ABCG2 (P=0.029).

3.3.4 Localization of ABCG2 in patient prostate cancer tissue

It has been demonstrated that wild-type ABCG2 and the Q141K variant localize differently *in vitro* (reviewed in Chapter 1). I wanted to determine the effect of the Q141K variant on localization in prostate cancer patients. In our cohort of fifty-five Gleason 7 prostate cancer patients, 14 patients (25%) were homozygous or heterozygous for the Q141K ABCG2 variant (KK or QK). Of these 14 patients, 2 were homozygous for the ABCG2 variant (KK). Immunofluorescence and confocal microscopy were utilized to analyze the localization in 2 patients for each genotype (QQ, QK, and KK). All genotypes had a mixture of cytoplasmic and cell membrane localization (**Figure 3-5**). There were no observable differences in localization or staining frequency and intensity in the wild-type homozygotes (QQ) and heterozygotes (QK). However, it was observed that the ABCG2 staining intensity and frequency in the two homozygous variant (KK) patient samples were noticeably lower than in the wild-type (QQ) or heterozygous variant (QK) at the same exposure time. Surprisingly, we also detected nuclear localization of ABCG2 for all genotypes (**Figure 3-5**, arrows). ABCG2 nuclear localization has been observed in glioblastoma multiforme cells [134] but has never been reported in normal prostate or prostate cancer cells.

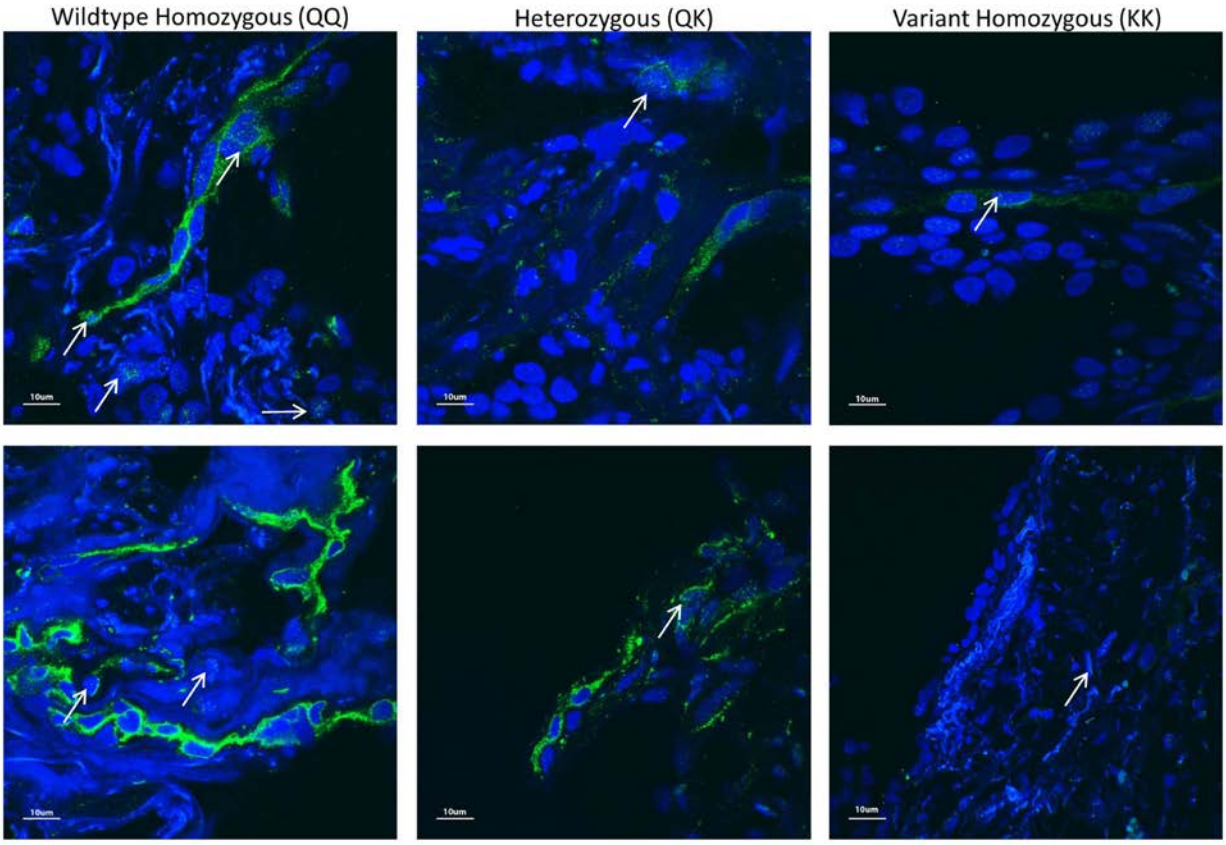

Figure 3-5. Localization of ABCG2 in prostate cancer patient tissue. Prostate cancer tissue was stained with the ABCG2 antibody, BXP-21and an Alexa Fluor 488 secondary antibody and the nuclei were stained with Hoechst 33342. Tissue from all three genotypes (QQ, QK, and KK) showed ABCG2 (green) staining at the cell membrane, intracellular, and nuclear. Arrows represent nuclei that contain ABCG2 staining.

3.3.5 Exogenous ABCG2 protein is not expressed in the prostate cancer cell lines despite expression of ABCG2 mRNA

The endogenous expression of ABCG2 is virtually undetectable by western blot in the prostate cancer cell lines, LNCaP and PC3. To examine the function of wild-type ABCG2 and the Q141K variant in an *in vitro* prostate cancer model, the prostate cancer cell lines LNCaP and PC3 were

initially transduced with the lentivirus expressing ABCG2 (pLVX-ABCG2) and a control (pLVX-GFP). After puromycin selection, the mixed clone cell lines were named LNCaP-ABCG2, LNCaP-GFP, PC3-ABCG2 and PC3-GFP. ABCG2 protein levels were determined by western blot (**Figure 3-6a**). Despite selection, expression of ABCG2 in the transduced prostate cancer cell lines was undetectable (the HEK293 cell line transduced with ABCG2 was used as a control). In addition to not expressing the ABCG2 protein, the LNCaP-ABCG2 cell line grew poorly. Therefore, further analysis on this cell line could not be conducted.

ABCG2 mRNA expression was measured in the PC3-ABCG2 and PC3-GFP cell line to confirm the transcription of the ABCG2 lentiviral vector. Expression of ABCG2 mRNA in the PC3-ABCG2 cell line was approximately 280 times greater than in the PC3-GFP cell line (**Figure 3-6b**). This confirmed that the ABCG2 cDNA in the lentiviral vector was being transcribed but that a post-transcriptional or post-translational mechanism was repressing the ABCG2 protein.

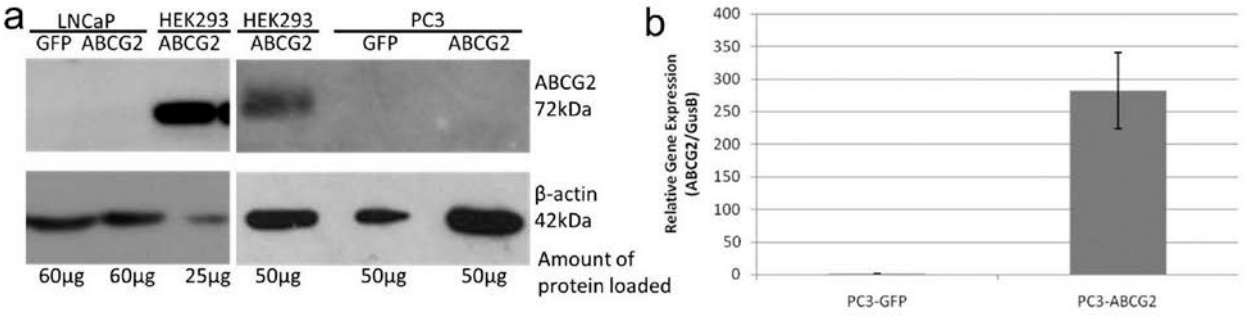

Figure 3-6. Exogenous ABCG2 expression is undetectable in transduced LNCaP and PC3 cells despite mRNA expression. A. ABCG2 protein expression was undetectable in the LNCaP and PC3 cell line transduced with ABCG2. The HEK293 cell line transduced with ABCG2 was used as a positive control. **B.** Because the LNCaP-ABCG2 cell line grew poorly, the mRNA expression of ABCG2 could not be measured. In the PC3-ABCG2 cell line, the expression of ABCG2 was ~280 greater than in the PC3-GFP cell line.

3.3.6 ABCG2 may be repressed by miR-519c and/or miR-520h in the prostate cancer cell lines

The ABCG2 cDNA from Origene used for this study contains the 5'-UTR and part of the 3'-UTR. There are two miRNA (miR) recognition sites in the 3'-UTR of this cDNA that are known to repress ABCG2 protein expression. ABCG2 has been shown to be repressed by miR-519c in the MCF-7 breast cancer cell line [132] and by miR-520h in the PANC-1 pancreatic cancer cell line [135]. We used the stem loop RT-qPCR method to measure expression of these two miRNAs in the PC3-ABCG2 and PC3-GFP lines, as well as 293-ABCG2. The expression of miR-519c was significantly increased in the PC3-ABCG2 cell line relative to the 293-ABCG2 and PC3-GFP cell lines ($p=0.016$) (**Figure 3-7a**). The expression of miR-520h was similar in the PC3-ABCG2 and PC3-GFP cells (**Figure 3-7b**). To test whether miR-519c was repressing the translation of the ABCG2 mRNA in the lentiviral transduced cell lines, I used site-directed mutagenesis to delete the miRNA recognition site from the 3'-UTR region of the pLVX-ABCG2 plasmid. The miR-519c and miR520h recognition sites overlap and both sites were deleted from the pLVX-ABCG2 plasmid. After we confirmed that the plasmid did not have the miRNA recognition sites by sequencing, we renamed it pLVX-delABCG2 and transduced the plasmid into LNCaP and PC3 parental cells. After puromycin selection, the mixed clone cell lines were named LNCaP-delABCG2 and PC3-delABCG2. ABCG2 protein expression was detected by western blot but at levels much lower than in the 293-ABCG2 cell line (**Figure 3-7c**). This data demonstrates for the first time that ABCG2 in prostate cancer cell lines can be regulated by miR-519c and/or -520h.

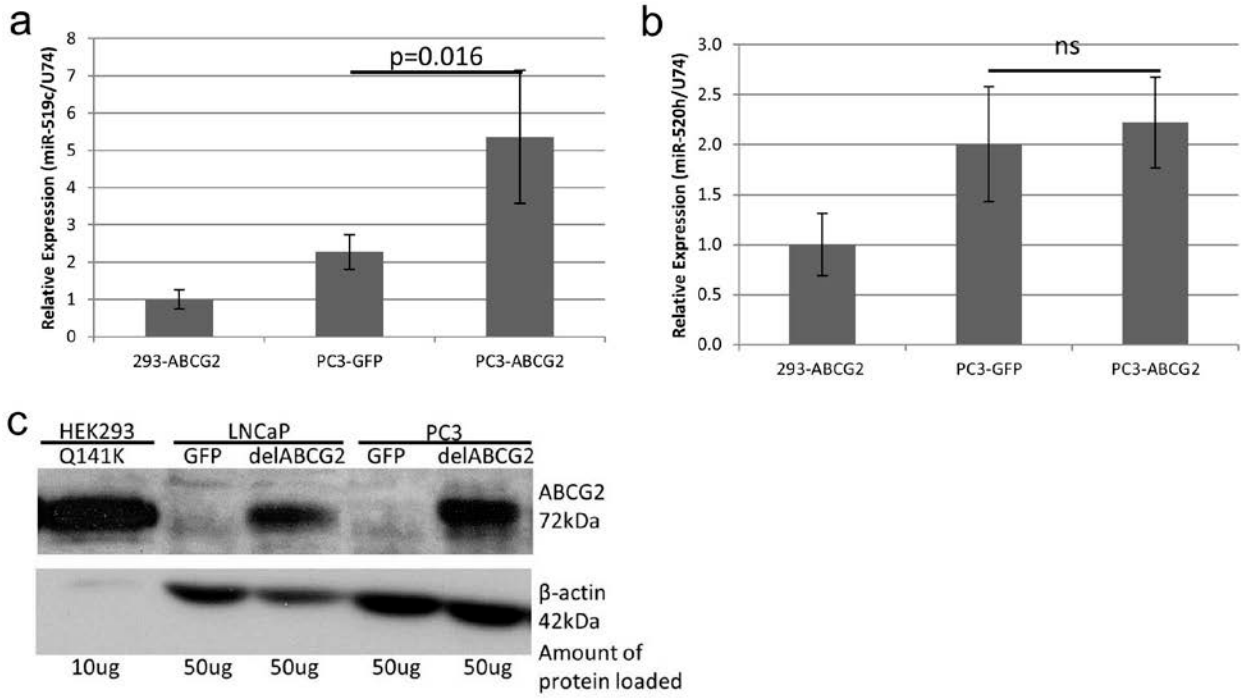

Figure 3-7. ABCG2 expression is increased after deletion of miR-519c and miR-520h recognition sites in the 3'-UTR. A. MicroRNAs were measured by qPCR. Expression of miR-519c was significantly increased (P=0.016) in the ABCG2-transduced PC3 cell line compared to the control PC3-GFP cell line. **B.** Expression of miR-520h was not significantly increased in the PC3-ABCG2 cell line compared to PC3-GFP (P=0.571). The ΔΔCt method was used to quantitate mRNA and miRNA expression **(**n=2). **C.** After deletion of the miRNA recognition sequence in the 3'-UTR of the ABCG2 cDNA, expression of ABCG2 protein was detectable in LNCaP and PC3 cell lines after lentiviral transduction with the deleted ABCG2 plasmid.

3.3.7 ABCG2 effluxes Hoechst 33342 in the transduced prostate cancer cell lines

Flow cytometry and the ABCG2 specific substrate Hoechst 33342 were used to determine if the exogenous ABCG2 protein expressed in the prostate cancer cell lines was functional. The ABCG2 expressing (Hoechst 33342 effluxing) cells were also sorted in order to enrich them and perform the folate efflux experiments and the PSA induction experiment. In the LNCaP-delABCG2 cell line 50.62% of cells were able to efflux Hoechst 33342 compared to 2.88% in

LNCaP-GFP. When Ko143 was added to the cells, the effluxing cells in LNCaP-ABCG2 and LNCaP-GFP was 13.24% and 4.36%, respectively (**Figure 3-8a**). The percentage of cells effluxing Hoechst 33342 in the PC3-delABCG2 cell line was 39.58% compared to 2.18% in the PC3-GFP cell line (**Figure 3-8b**). When the ABCG2 specific inhibitor Ko143 was added to the cells, the percentage of effluxing cells in the PC3-delABCG2 cell line decreased to 3.33%. These data demonstrate that the exogenous ABCG2 protein is functional in the PC3 and LNCaP cell lines.

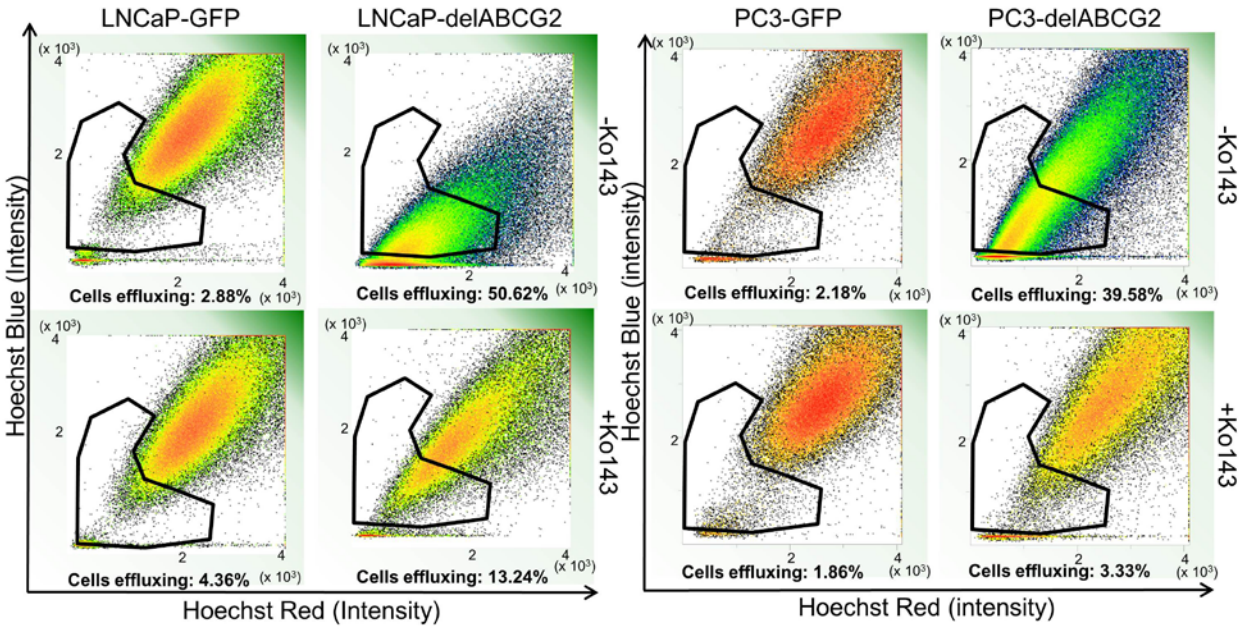

Figure 3-8. Flow cytometry analysis of LNCaP-delABCG2 and PC3-delABCG2. The functionality of the exogenous ABCG2 protein expressed in the transduced prostate cancer cell line was determined by flow cytometry. The ABCG2 specific dye Hoechst 33342 was used to determine the percentage of cells effluxing the dye. The ABCG2 specific inhibitor Ko143 was added to show that when ABCG2 is inhibited, more cells retain Hoechst 33342 and have a higher Hoechst Blue and Hoechst Red intensity. **A.** LNCaP-delABCG2 had more ABCG2 expressing cells than LNCaP-GFP (50.62% vs. 2.88%, respectively). When the ABCG2 specific inhibitor Ko143 was added, the percentage of cells able to efflux Hoechst 33342 decreased to 13.24%. **B.** PC3-delABCG2 had more ABCG2 expressing cells than PC3-GFP (39.58% vs. 2.18%, respectively). When the ABCG2 specific inhibitor Ko143 was added, the percentage of cells able to efflux Hoechst 33342 decreased to 3.33%.

After culturing the PC3-delABCG2 and LNCaP-delABCG2 cell line for several weeks, ABCG2 protein expression was undetectable in LNCaP-delABCG2 and decreased in PC3-delABCG2 by western blot (**Figure 3-9**). Therefore, further analysis was not conducted on these cell lines.

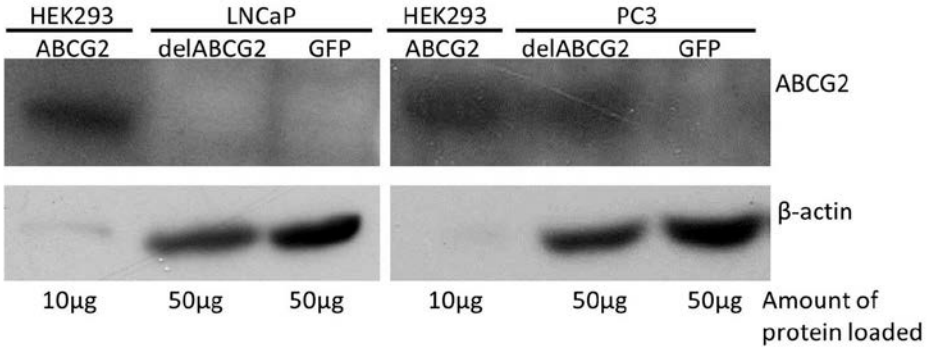

Figure 3-9. ABCG2 expression is decreased in the prostate cancer cell lines. Expression of ABCG2 protein decreased in the selected LNCaP-delABCG2 and PC3-delABCG2 cell lines after several weeks of culturing.

3.3.8 The exogenous Q141K variant is not expressed in the prostate cancer cell lines

The functional assays used to test the ability of ABCG2 to efflux folate and DHT did not reveal interpretable differences between ABCG2 expressing and non-expressing cells. However, a difference was detected in the Hoechst 33342 flow cytometry assay. It was decided to use this assay to test the general hypothesis that wild-type ABCG2 and the Q141K variant differentially efflux in prostate cancer cells. First, the Q141K variant was generated. Site-directed mutagenesis was used to mutate the cytosine at the 421 locus to an adenine in the pLVX-delABCG2 plasmid. The new plasmid was sequence verified and named pLVX-delQ141K. A lentiviral transduction with the pLVX-delQ141K plasmid was performed on the LNCaP and PC3 cell lines. After

transduction, the cells were selected and maintained in puromycin. I maintained them in puromycin to encourage the expression of the Q141K variant. The resulting cell lines were named LNCaP-delQ141K and PC3-delQ141K. ABCG2 protein was undetectable in the LNCaP-delQ141K cell line, but was expressed in the PC3-delQ141K cell line (**Figure 3-10a**). The ABCG2 mRNA expression was measured in the LNCaP and LNCaP-delQ141K cell line to determine if the plasmid was being expressed. The LNCaP-delQ141K cell line expressed over 1000-fold more ABCG2 mRNA than the parental LNCaP cell line (**Figure 3-10b**).

After generation of the PC3-delQ141K cell line, the PC3-delABCG2 cell line was thawed and maintained in media containing puromycin. Before the flow cytometry experiment, the ABCG2 protein level was measured in the PC3-GFP, PC3-delABCG2, and PC3-delQ141K cell lines. Unfortunately, the protein expression in the PC3-delQ141K cell line was undetectable (**Figure 3-10c**). The time frame between the two westerns was two weeks and the PC3 cells repressed the expression of the Q141K variant protein. The ABCG2 mRNA expression was determined in the PC3 and PC3-delQ141K cell line by qPCR. The PC3-delQ141K cell line expressed 124.5 times more ABCG2 mRNA than the parental PC3 (**Figure 3-10d**) suggesting that the plasmid is being expressed. There is evidence in the literature that the Q141K variant protein can be degraded by the proteasome. The PC3-GFP, PC3-delABCG2, and PC3-delQ141K cell lines were treated with MG132 for 7 hours and then protein expression was detected by western blot. ABCG2 protein expression was still undetectable in the PC3-delQ141K cell line after MG132 treatment (**Figure 3-10e**).

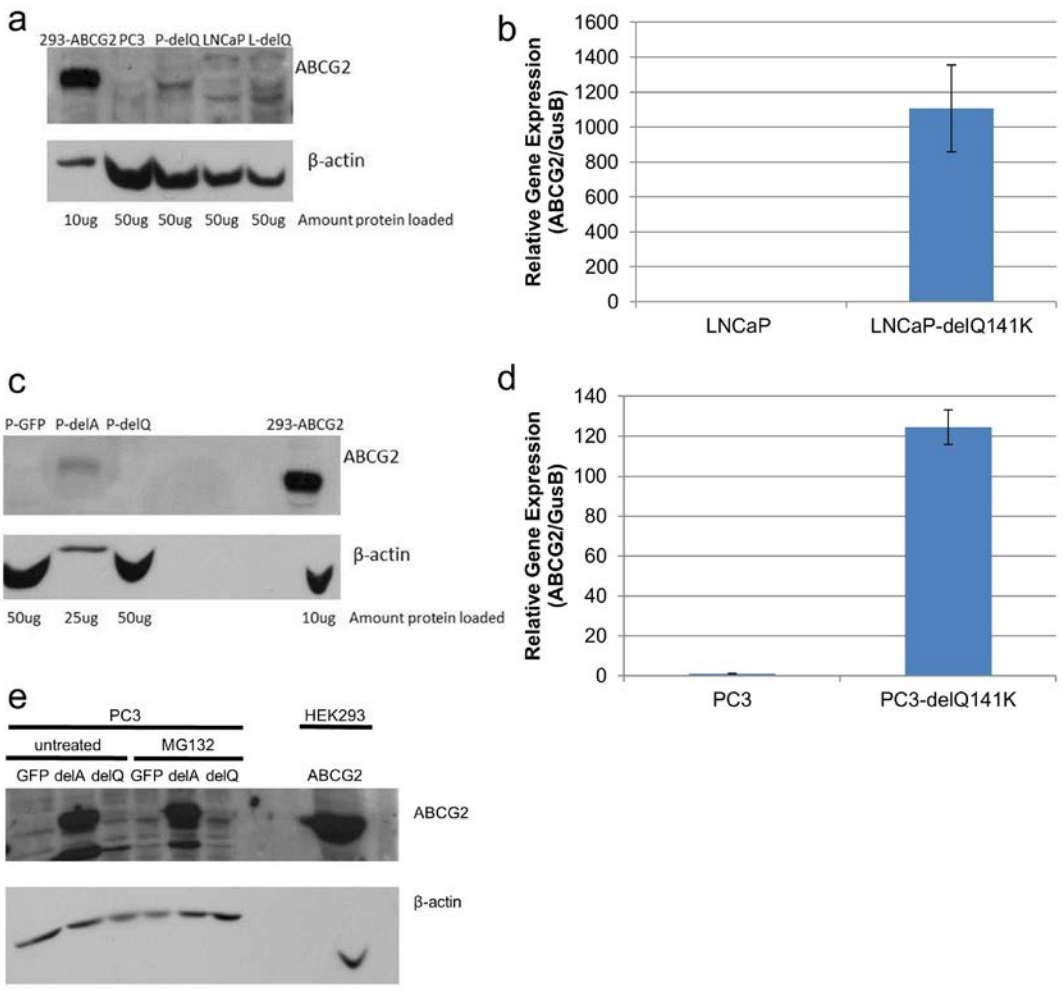

Figure 3-10. The Q141K variant is not expressed in the prostate cancer cell lines. A. The pLVX-delQ141K plasmid was transduced into the LNCaP and PC3 cell lines. Protein was undetectable by western blot in the LNCaP-delQ141K (abbreviated L-delQ) cell line but was present in the PC3-delQ141K (abbreviated P-delQ) cell line. The 293-ABCG2 cell line was used as a positive control. **B.** The ABCG2 mRNA expression was measured in the LNCaP and LNCaP-delQ141K by qPCR. ABCG2 expression was increased in the LNCaP-delQ141K cell line over 1000-fold over the parental LNCaP cell line. **C.** Before the PC3 cell lines were analyzed for their ability to efflux Hoechst 33342, the protein expression was measured. The PC3-delQ141K cell line lost protein expression despite being maintained in the selective agent, puromycin. **D.** The PC3-delQ141K cell line had 124.5 times more ABCG2 mRNA expression than parental PC3 cells after analysis by qPCR. **E.** After treatment with MG132 for seven hours, the PC3-delQ141K (abbreviated delQ) cell line does not express the ABCG2 protein. The ABCG2 protein in PC3-delABCG2 (abbreviated delA) cell line is unaffected by the MG132 treatment.

3.3.9 Docetaxel treatment induces ABCG2 expression

To determine if ABCG2 plays a role in docetaxel efflux, the expression of ABCG2 was measured after growing the cells in 1nM docetaxel for 8 days. I observed a 5-fold induction of ABCG2 mRNA expression (**Figure 3-11**). This data suggests that the LNCaP cells are increasing the expression of ABCG2 in response to docetaxel treatment.

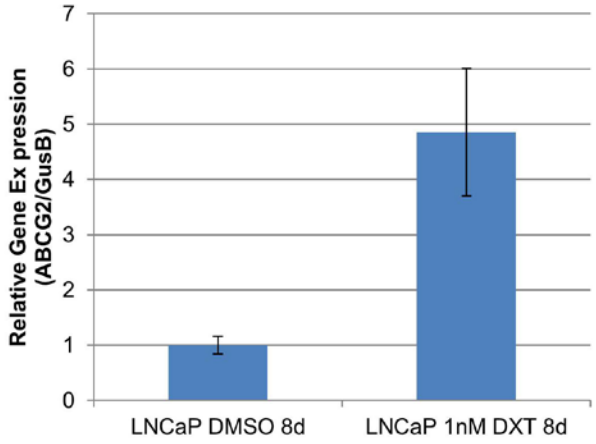

Figure 3-11. ABCG2 is induced after treatment with docetaxel. LNCaP cells were treated with 1nM docetaxel for 8 days and the ABCG2 mRNA expression was measured by qPCR. ABCG2 expression was induced ~5 fold in the docetaxel treated LNCaP cells. Relative gene expression was calculated using the ΔΔCt method.

3.4 DISCUSSION

ABCG2 expression is decreased in prostate cancer when compared to normal prostate tissue. This is consistent with microarray data available for other cancers, such as ductal breast carcinoma in situ [136] and colon and rectal adenomas [137]. This could be an indication that it is advantageous for cancer cells to down-regulate ABCG2. There may be unknown growth

factors that are ABCG2 substrates that the tumor cells want to retain. Microarray analysis also revealed an up-regulation of ABCG2 in androgen independent prostate cancer compared to androgen dependent prostate cancer. The androgen independent prostate cancer patients in this study had been treated with a variety of protocols, including zoladex, flutamide, lupron, casodex, orchiectomy, and radiation [127]. Casodex or bicalutamide is a known ABCG2 substrate and it is likely that ABCG2 was upregulated in the two patients who received bicalutamide treatment. The other drugs used to treat these patients may also be unknown ABCG2 substrates. ABCG2 expression was also increased in prostate cancers with a higher Gleason score. A higher Gleason score is an indicator of a poorly differentiated and more aggressive tumor and usually a worse prognosis. There are a few reports that ABCG2 expression is positively correlated to cellular proliferation [138, 139]. This could explain why there is an increase in ABCG2 expression in higher Gleason scores. However, the mechanism by which ABCG2 increases cellular proliferation is unclear.

Gardner, et. al. compared the time from initial prostate cancer diagnosis to death in androgen independent prostate cancer patients treated under a variety of protocols. They reported a shorter overall survival time in prostate cancer patients expressing the ABCG2 Q141K variant (5.3 years) compared to patients expressing wild-type ABCG2 (7.4 years) [64]. It was determined that unlike time-to-death, which can include all causes of death, time to PSA recurrence would be a specific indicator of prostate cancer disease progression. The cohort of men chosen had not been treated with any drugs that may confound the results (in the Gardner, et. al study the men had been treated with numerous drugs including thalidomide, docetaxel, and suramin). The observation that patients with the Q141K variant have a significantly shorter time to PSA recurrence supports the hypothesis put forward by Gardner, et al. [64], which is patients

carrying the ABCG2 Q141K variant have a shorter time to disease-specific recurrence. It is known that ABCG2 effluxes folic acid [13, 105], and it is well established that the proliferation rate of cancer cells in culture is dependent on the availability of folate (most cell culture media contains supraphysiological levels of folic acid e.g. ~2.3μM folic acid in RPMI and average physiological serum folate levels in humans is 25nM). Our laboratory has previously demonstrated that patients with Gleason 7 prostate tumors with high circulating serum folate levels have tumors that proliferate on average six times faster than those patients with low serum folate levels [126]. Therefore, a person expressing the variant ABCG2 protein could have an increased tissue/intracellular folate level compared to a person expressing the wild-type ABCG2 protein. In a cancer patient, this could potentially allow cancer cells (that have already lost control of growth) to proliferate faster than cells effluxing these molecules. As a result of the increased proliferation, the time to PSA recurrence could be shortened in prostate cancer patients carrying the Q141K variant. Further investigation is needed in order to determine whether the level of intracellular folates in patient tissues could lead to increased proliferation rates.

I also observed that prostate cancer patients either homozygous or heterozygous for the Q141K variant ABCG2 protein had significantly lower systemic (fasting) folate levels compared to patients expressing the wild-type ABCG2. The liver is an important organ for folate storage and metabolism [140] and ABCG2 is expressed in the liver canalicular membrane [28]. Therefore, folates would be excreted through the liver by ABCG2 and expression of the Q141K variant could prevent the excretion of folate. This correlation provides new evidence that ABCG2 can have a significant effect on regulating systemic levels of folate. However, the levels of systemic folate levels may not be indicative of disease progression because this information does not directly tell us the level of a particular tissue's intracellular folate level. In addition to

folate affecting the proliferation of prostate cancer cells, ABCG2 has been shown to efflux dihydrotestosterone [66], which will have an effect on the growth of prostate cells. Further work analyzing the expression of wild-type ABCG2 and the Q141K variant in a specific organ (e.g. prostate) will need to be conducted in order to determine the likelihood that retained molecules (e.g. folate and dihydrotestosterone) are contributing to cell proliferation and disease progression. Overall, this finding suggests that ABCG2 may be a major player in folate regulation and its role in disease progression will require additional studies.

There are a few reports that the Q141K variant does not have an effect on ABCG2 cellular localization [82, 83, 101]. Others have observed increased intracellular ABCG2 staining in Q141K variant transduced/transfected cells compared to cells transduced/transfected with wild-type ABCG2 [84, 85, 95, 96, 98, 141] . It was also recently been reported that the Q141K variant protein may be retained at the aggresome, a perinuclear structure where misfolded proteins can accumulate [95]. These studies were observing the localization of ABCG2 in transfected cell line models. This study observed that the localization of ABCG2 in prostate cancer patients is cytoplasmic and intracellular for all genotypes, even though the staining intensity is noticeably lower in the homozygous variant patients. ABCG2 was also detected in the nucleus. This is the first report of ABCG2 co-localizing with the nuclei of prostate cancer patients. Bhatia, et. al. observed nuclear ABCG2 localization in multiforme glioblastoma cells [134]. They hypothesized that ABCG2 localizes to the nuclear membrane in order to efflux cytotoxic drugs from the nucleus into the cytoplasm. There are other possible roles of ABCG2 localizing to the nucleus. For example, when plasma membrane proteins are processed from the Golgi to the cell membrane by exocytosis, the protein is essentially ‘inside-out.’ In the secretory vesicle, the direction of ABCG2 would allow for influx of substrates from the cytoplasm to the

interior of the secretory vesicle. As the secretory vesicle merges with the cell membrane, the direction of ABCG2 allows the efflux of substrates from the cytoplasm to the extracellular space. Therefore, if ABCG2 is localizing to the nucleus (or other organelles), when the secretory vesicle merged with the nuclear membrane, ABCG2 would be in the influx direction. ABCG2 may actually be importing substrates into the nucleus. Further investigation is required in order to test this hypothesis and determine the significance of ABCG2 nuclear localization.

We found after transducing the prostate cancer cell lines LNCaP and PC3 with the ABCG2 lentiviral vector that the ABCG2 protein is not expressed, even though the mRNA was being transcribed in the PC3 cell line. The expression of one miRNAs that is known to repress the expression of ABCG2 in breast cancer cells [132] was increased in the PC3-ABCG2 cell line. After deletion of the miRNA recognition sites and expression in the prostate cancer cell lines, ABCG2 protein was detected. This is the first report that miR-519c and/or miR-520h are able to repress ABCG2 expression in prostate cells.

There were larger percentages of Hoechst 33342 effluxing cells in the LNCaP-delABCG2 and PC3-delABCG2 cell lines compared to the LNCaP-GFP and PC3-GFP cell lines. Because Hoechst 33342 is an ABCG2 specific substrate, it is assumed that cells that are effluxing Hoechst 33342 also express high levels of ABCG2. To further prove that ABCG2 is effluxing the Hoechst 33342 dye, the ABCG2 specific inhibitor Ko143, was added to the ABCG2 expressing cell lines and the percentage of Hoechst 33342 effluxing cells was similar to the GFP cell lines. This data indicated that the LNCaP-delABCG2 and PC3-delABCG2 cell lines were expressing functional ABCG2.

In addition, I observed that after several weeks in culture, the expression of ABCG2 decreased in the LNCaP-delABCG2 and PC3-delABCG2 cell lines, suggesting that another

mechanism represses the ABCG2 protein in prostate cancer cells. It is not surprising that tumor cells would adapt to find another way to repress ABCG2 because it effluxes folate, a nutrient required for cell proliferation and dihydrotestosterone [66], a potent androgen that stimulates the androgen receptor. In addition, the prostate requires a higher level of folate than most tissues because of polyamine biosynthesis and decreased folate levels decreases proliferation in prostate cancer cell lines and primary cultures compared to colon cancer cell lines [115]. It is interesting that the prostate cancer cell lines appear to repress ABCG2 whenever possible. This might indicate that ABCG2 is effluxing a substrate or substrates, other than folates and DHT that are crucial for the prostate cancer cell lines to survive. Further investigation is needed to determine why the prostate cancer cell lines repress expression of the ABCG2 protein.

After discovering that the flow cytometry/Hoechst 33342 assay was sensitive enough to determine differences between the ABCG2 expressing and non-expressing cells, the Q141K variant was expressed in the prostate cancer cell lines. Unfortunately, the LNCaP cell line never expressed the Q141K variant and the PC3 cell line lost expression of the Q141K variant after several weeks in culture despite maintaining selection with puromycin. I determined that ABCG2 was not induced after MG132 treatment in the PC3 cell line expressing the Q141K variant. There have been other reports that the Q141K variant can be degraded by lysosomes or autophagy [95, 96]. Additional experiments will need to be conducted in order to determine the mechanism by which the prostate cancer cell lines are repressing the Q141K variant.

In this chapter, I observed that the Q141K variant has physiological effects in prostate cancer patients. The presence of the Q141K variant decreases the time to PSA recurrence in prostate cancer patients. This data suggests that ABCG2 may be involved in the progression of prostate cancer. Because the Q141K variant is known to be less functional than the wild-type

ABCG2, a retained substrate could be contributed to a faster disease recurrence. I also observed a decrease in fasting serum folate levels in patients with the Q141K variant, suggesting that the liver (the main folate storage and metabolizing organ) in a person expressing the Q141K variant may be retaining more folates than a person expressing wild-type ABCG2. This provides evidence that ABCG2 may be important in regulating systemic folate levels. ABCG2 expression can be repressed by miR-519c and/or miR-520h in prostate cancer cell lines. However, after deletion of the miRNA recognition sites and several weeks of culturing, ABCG2 expression is repressed, indicating another mechanism may also repress ABCG2 expression. Taken together, the Q141K variant likely plays an important physiological role, but the further works needs to be conducted in the prostate cancer cell lines to provide a reliable *in vitro* model to study our hypotheses.

4.0 EFFECT OF THE Q141K VARIANT ON ABCG2 EXPRESSION AND FUNCTION

4.1 INTRODUCTION

The effect of the Q141K variant on ABCG2 function was indeterminable in the prostate cancer cell lines due to unknown repressive mechanisms. I wanted to determine the functional differences between wild-type ABCG2 and the Q141K variant in another model system. *In vitro* studies investigating the differences between wild-type ABCG2 and the Q141K variant have been undertaken in PA-317 cells [88], LLC-PK1 cells [82, 83], HEK293 [82, 97-101, 141], Sf9 insect cells [86, 97], Flp-In-293 cells [84, 85, 95, 96], *Xenopus* oocytes [20, 98], and CHO cells [98]. Exogenous wild-type ABCG2 and the Q141K variant were either transiently or stably expressed. The expression, localization, and function of wild-type ABCG2 and the Q141K variant appear to be dependent on the cell line and expression system. For example, the expression of wild-type ABCG2 and the Q141K variant is equal in *Xenopus* oocytes but the Q141K variant expression is lower than wild-type in CHO cells transiently transfected [98]. The authors demonstrated that expression of the Q141K variant in the CHO cells could be increased by growing the CHO cells at a lower temperature (similar to the oocytes), which can enable folding and stabilization of the protein.

In this chapter, I used the HEK293 cell line and the lentiviral transduction system to stably express the wild-type ABCG2 and Q141K variant proteins. I used this model system to determine the effect of the Q141K variant folic acid efflux and to test the hypothesis that docetaxel is an ABCG2 substrate.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture

The HEK293 cell line (generous gift of Dr. Zhou Wang) was maintained in DMEM medium supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. Cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2.

4.2.2 Lentiviral transduction and site directed mutagenesis

HEK293 cells were transduced with the pLVX-ABCG2 plasmid generated in Section 3.2.6 and the pLVX-Q141K plasmid generated in Section 3.2.7. The GFP control vector (pLKO.1-puro-CMV-TurboGFP) was also transduced into the HEK293 cell line. After transduction, the cells were selected with 3μg/mL of puromycin. All experiments used mixed clones to avoid effects attributable to specific integration of the transgene.

4.2.3 Western Blotting

Protein was extracted from cells, processed and analyzed as described in Section 3.2.9.

4.2.4 RNA isolation, Reverse Transcription and Quantitative PCR

RNA was extracted from cell lines using the Trizol reagent (Invitrogen) as described in Section 2.2.4. One microgram of RNA was reverse transcribed as described in Section 2.2.5. ABCG2 and GusB expression were measured in a 20µL PCR reaction as described in Section 2.2.6.

4.2.5 Transient transfection

The HEK293 cell line was transiently transfected with 1µg pLVX-ABCG2 or pLVX-ABCG2Q141K with PolyJet™ DNA In Vitro Transfection Reagent (SignaGen Laboratories) according to manufacturer's instructions. Cells were harvested and analyzed by western blot 48 hours post transfection.

4.2.6 Immunofluorescence

Cells were grown on chamber slides for two days prior to staining. Cells were washed with PBS before fixation with 2% paraformaldehyde for 15 minutes and permeabilization with 0.1% Triton X-100 for 15 minutes. Samples were washed 3 times with PBS and 5 times with 0.5% BSA/PBS. Samples were blocked in 2% BSA/PBS for 1 hour, washed 5 times with 0.5% BSA/PBS, incubated for 2 hours in the ABCG2 antibody (Millipore, clone BXP-21, 1:100 dilution), washed 5 times with 0.5% BSA, and incubated for 1 hour in the Alexa Fluor 488 secondary antibody (1:500 dilution). The slides were washed 5 times with 0.5% BSA/PBS, 5 times with PBS, then nuclei were stained with 0.5µg/mL tissues of Hoechst 33342 (Sigma) for 30 seconds and washed 2 times with PBS. Slides were coverslipped with Fluorescent Mounting Medium (Dako) and

imaged on an Olympus FV1000 Confocal Microscope. Exposure times were kept consistent for all samples.

4.2.7 Hoechst 33342 Retention and Uptake

To test whether the exogenous ABCG2 proteins were functional, a Hoechst 33342 retention and uptake assay were performed. For the retention assay, 25,000 cells/well were plated in a black fluorescence 96-well plate and allowed to grow overnight. Phenol-red free RPMI with or without 1µM Ko143 were added to the wells and incubated at 37°C and 5% CO_2 for 10 minutes. Phenol-red free RPMI and 5µg/mL Hoechst 33342 (Sigma) were added to the wells and incubated at 37°C and 5% CO_2 for 20 minutes. Cells were then washed twice with phenol-red free RPMI. After a one hour incubation at 37°C, the dye was excited at 350nm and the emission was read at 461nm on a Molecular Devices SpectraMax M2^e plate reader. Samples were analyzed in quadruplicate and readings were also taken from untreated cells.

For the uptake assay, 25,000 cells/well were plated in a black fluorescence 96-well plate and allowed to grow overnight. Cells were incubated in phenol-red free RPMI with or without 1µM Ko143 for 15 minutes at 37°C and 5% CO_2. Next, 1µg/mL Hoechst 33342 was added to the cells and then immediately read every 5 minutes for 1 hour as described above on the plate reader. Cells without treatment were read as a blank measurement and samples were analyzed in quadruplicate. GraphPad Prism 5.0 was used to generate a one-phase association non-linear fit of the data.

4.2.8 Tritiated Folic Acid Transport Assay

The transport assay protocol was modified from Hamid, et. al. [142]. Cells were grown to approximately 80% confluency, counted by hemocytometer, and aliquoted in triplicate or quadruplicate at $1x10^6$ cells per tube. Cells were incubated in incubation solution pH 5.5 (10mM HEPES, 10mM Tris, 80mM mannitol, 100mM MES) for 30 minutes before 25nM ^{3}H-folic acid (Moravek Biochemicals) was added. Cells were incubated for 30 minutes in ^{3}H-folic acid at 37°C and 5% CO_2. The reaction was stopped with ice-cold stop solution pH 7.4 (280mM Mannitol, 20mM Tris, 20mM HEPES) and cells were rinsed four times with cold PBS. After the final rinse, cells were added to scintillation fluid and retained tritiated folic acid was measured on a Wallac 1409 DSA Liquid Scintillation Counter (Wallac, Perkin-Elmer).

4.2.9 Docetaxel Cytotoxicity Assay

Cytotoxicity assays were performed to determine the IC50 values of docetaxel in 293-GFP, 293-ABCG2, and 293-Q141K. The MTT assay was used to measure the respirating cells after growth in docetaxel. Cells were plated at a concentration of 5,000 cells/well in a 96-well dish 24 hours prior to the addition of methotrexate or docetaxel. Docetaxel ranging from 0-100nM was added to the plated cells and grown at 37°C and 5% CO_2. Cells were grown for 96 hours. At the time of analysis, fresh media and 10µL 5mg/mL MTT reagent A (BioExpress) were added to each well. After a four hour incubation at 37°C and 5% CO_2, the media and reagent A were removed and 100µL of DMSO was added to each well to dissolve the formazan product. Plates were read on a SpectraMax M2^e plate reader at a wavelength of 540nm (Molecular Devices). Samples were done in replicates of six.

4.2.10 Statistical Analysis

A one-way ANOVA with a Bonferroni's post-test was used to analyze the cell proliferation data (GraphPad Prism). SigmaPlot 12 was used for the following statistical analyses. The Hoechst accumulation and uptake experiments were analyzed by t-test and Mann-Whitney Rank Sum test. The tritiated folic acid and methotrexate transport, docetaxel cytotoxicity, and intracellular folate levels were analyzed by one-way ANOVAs for repeated measures, and adjusted for pair-wise multiple-comparisons using the Holm-Sidak method.

4.3 RESULTS

4.3.1 Exogenously expressed wild-type and Q141K variant ABCG2 protein in HEK293 cells is similarly expressed and differentially localized

To test the hypothesis that the Q141K variant differentially effluxes folic acid and docetaxel compared to wild-type ABCG2, the HEK293 cell line (endogenous ABCG2 protein is undetectable) was transduced with the lentiviral vectors for wild-type ABCG2 (pLVX-ABCG2), the Q141K variant, (pLVX-Q141K) and a control (pLVX-GFP). After puromycin selection, the mixed clone cell lines were named 293-ABCG2, 293-Q141K, and 293-GFP, respectively. ABCG2 mRNA levels were measured by real-time quantitative PCR (**Figure 4-1a**). A significant difference between the mRNA expression in the wild-type and Q141K variant was not detected. ABCG2 protein levels were determined by western blot (**Figure 4-1b**), and

accordingly, cells stably transduced with the wild-type ABCG2 and Q141K variant expressed similar levels of ABCG2 protein.

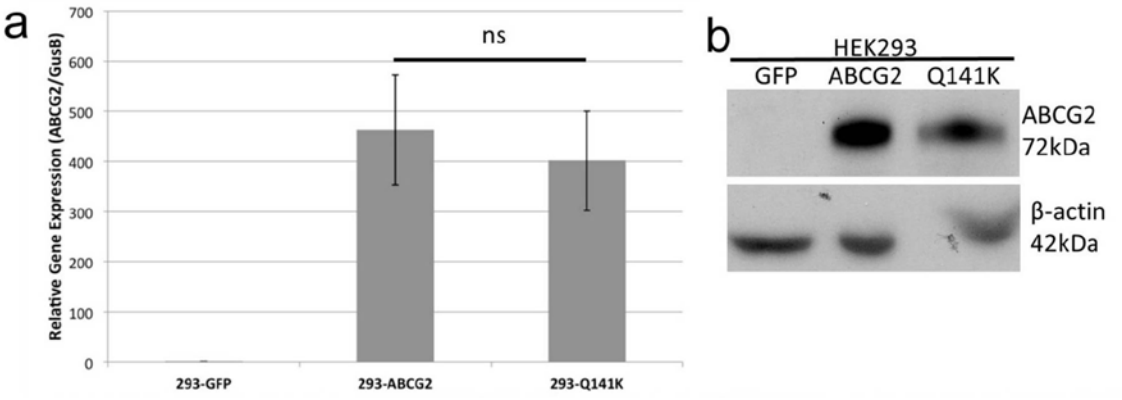

Figure 4-1. 293-ABCG2 and 293-Q141K express similar levels of ABCG2 mRNA and protein. A. ABCG2 mRNA expression was measured in 293-GFP, 293-ABCG2, and 293-Q141K. There is no significant difference in ABCG2 mRNA expression between the wild-type and variant cell lines. **B.** A western blot was performed to determine the expression of ABCG2 protein. Protein in 293-GFP was undetectable whereas ABCG2 expression in 293-ABCG2 and 293-Q141K was similar.

However, others have reported lower expression of the Q141K variant protein compared to wild-type ABCG2 by western blot [82, 84-86, 88, 95, 96, 98], but used different expression systems. One group did a stable transfection, three groups did transient transfections, and four groups used the Flp-In-293 cell line. It was decided to determine if a transient transfection would result in decreased Q141K variant protein expression. HEK293 cells were transiently transfected with the pLVX-ABCG2 and pLVX-Q141K plasmids, and then protein, RNA, and DNA were harvested after 48 hours. By western blot, a significant reduction in Q141K variant protein was observed compared to the wild-type protein (**Figure 4-2a)**, even though the mRNA expression were similar (**Figure 4-2b)** and DNA copy number was higher (**Figure 4-2c**). The wild-type ABCG2 mRNA in the transient transfection was 11-12 times higher than in the stable lentiviral

transductions. The ABCG2 protein expression in the 293-ABCG2 transient transfection was also increased compared to the ABCG2 expression in the stable transduction. In summary, this data suggests that the Q141K variant is synthesizing less ABCG2 protein than wild-type ABCG2, even though the level of RNA is the same in the variant and wild-type. This indicates that the cell does not want to synthesize and process the Q141K variant.

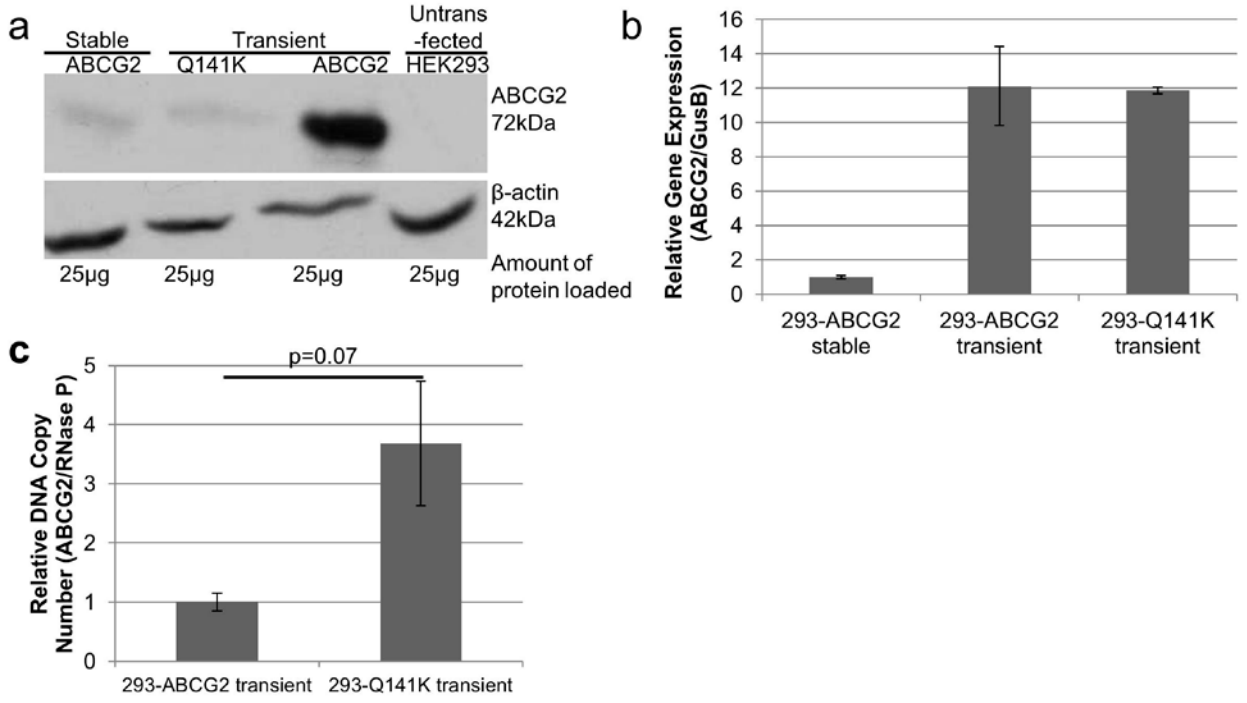

Figure 4-2. A transient transfection results in decreased Q141K variant protein expression. HEK293 cells were stably transduced with ABCG2 or transiently transfected with wild-type ABCG2 or the Q141K variant ABCG2. **A.** The level of wild-type ABCG2 protein expression in the transiently transfected HEK293 cells is greater than expression in the transiently transfected Q141K variant or the stably transduced wild-type ABCG2 cell line. **B.** The ABCG2 mRNA expression in the transiently transfected cell lines was determined by qPCR. The mRNA expression in the wild-type and Q141K variant is similar. GusB was used as a control for mRNA loading. **C.** The copy number of the DNA vector was determined by qPCR. More DNA from the Q141K variant was detected compared to the wild-type. RNase P was used to control for DNA loading. The ΔΔCt was used to determine expression.

It has been reported that the localization of the wild-type ABCG2 is predominantly at the plasma membrane and the Q141K variant is intracellular [84, 85, 96, 98, 141] and in the Flp-In-293 model system, the variant may localize specifically in aggresomes [95]. Immunofluorescence and confocal microscopy were used to determine where the ABCG2 protein was localizing in our model system. The wild-type ABCG2 was predominantly at the plasma membrane, whereas the Q141K variant protein was intracellular compartment as well as on the plasma membrane (**Figure 4-3**).

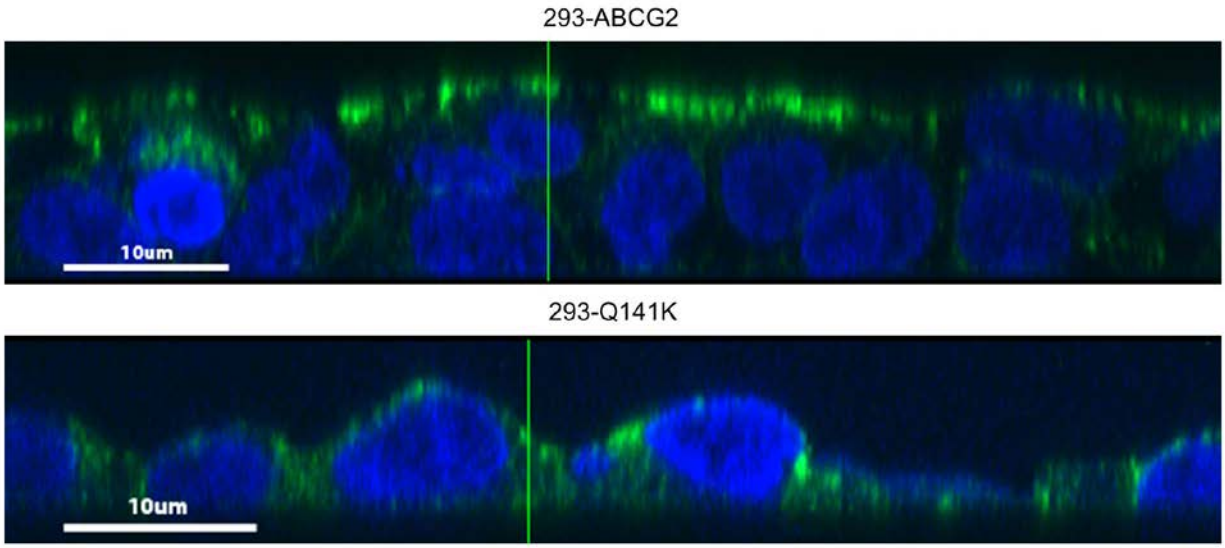

Figure 4-3. ABCG2 is localized intracellularly in the Q141K variant. The 293-ABCG2 and 293-Q141K cell lines were stained with ABCG2 (green) and Hoechst (blue) and imaged by confocal microscopy. The localization of ABCG2 is predominately at the cell membrane in the 293-ABCG2 cell line and both in the cytoplasm and cell membrane in the 293-Q141K cell line.

The functionality of the exogenous wild-type and variant protein was determined by measuring the accumulation and uptake of the ABCG2-specific substrate, Hoechst 33342. To measure the accumulation and thereby the efflux, the fluorescence of the retained dye was read on a plate reader in 293-GFP, 293-ABCG2, and 293-Q141K. After one hour, the 293-ABCG2

cell line retained 3 times less Hoechst 33342 than 293-GFP ($P<0.001$) (**Figure 4-4a**). The 293-Q141K cell line retained 1.3 times more dye than 293-ABCG2 (approaching significance, $P=0.115$), suggesting that the ABCG2 Q141K variant cell line does not efflux the dye as efficiently as the wild-type cell line. Addition of the ABCG2 specific inhibitor, Ko143 increased the level of accumulated Hoechst 33342 in both the wild-type and Q141K variant.

The uptake of the Hoechst 33342 dye in the cell lines was measured every five minutes for one hour. After an hour, the 293-ABCG2 cell line retained 3.2 times less dye than 293-GFP ($P<0.001$) and 293-Q141K retained 2 times less dye than 293-GFP ($P=0.029$) (**Figure 4-4b**). Again, when Ko143 was added, the amount of Hoechst 33342 retained increased in both the wild-type ABCG2 and the Q141K variant. Together these data demonstrate that the exogenous wild-type and variant ABCG2 proteins are capable of effluxing one of its substrates, Hoechst 33342. Importantly, this data shows that cells expressing the variant ABCG2 protein cannot efflux its substrate as well as the cells expressing similar levels of the wild-type protein and that ABCG2 function can be abolished by the addition of the ABCG2 specific inhibitor, Ko143.

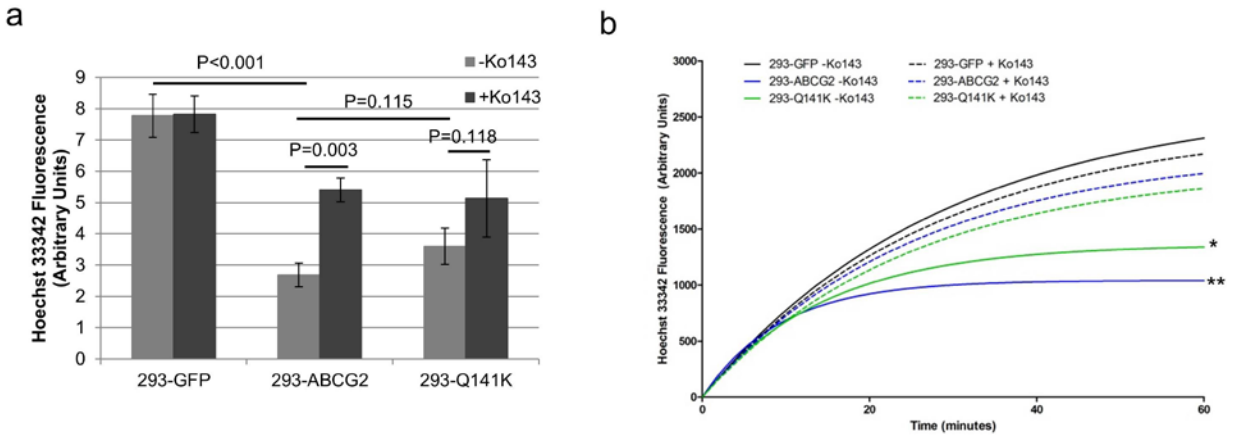

Figure 4-4. Hoechst 33342 retention and uptake in 293-ABCG2 cells is impaired compared to 293-GFP. A. A Hoechst 33342 retention assay was performed on each cell line to determine that the transduced protein was functioning properly. 293-ABCG2 retained 3 times less Hoechst 33342 compared to 293-GFP (P<0.001). 293-Q141K retained 1.3 times more Hoechst dye than 293-ABCG2 (P=0.115). When the ABCG2 specific inhibitor Ko143 was added, the amount of Hoechst 33342 accumulated in 293-ABCG2 and 293-Q141K increased 2-fold (P=0.003) and 1.4-fold (P=0.118), respectively. **B.** Hoechst 33342 uptake was also evaluated in 293-GFP, 293-ABCG2, and 293-Q141K. After 60 minutes, wild-type 293-ABCG2 (blue line) retained 3.2 times less Hoechst dye than the control 293-GFP (**, P<0.001) (black line) The variant 293-Q141K (green line) retained 2 times less Hoechst dye than the control 293-GFP (*, P=0.029). When the ABCG2 specific inhibitor Ko143 was added, the wild-type ABCG2 retained 1.8 times more Hoechst (P<0.001) (blue dash) and the Q141K variant (green dash) retained 1.4 times more Hoechst (P=0.071) when compared to the untreated cell lines.

4.3.2 The Q141K variant of ABCG2 retains more folic acid than wild-type ABCG2

To determine whether the Q141K variant affects the efflux of folic acid, tritiated folic acid transport assays were performed in the 293-ABCG2, 293-Q141K, and 293-GFP cells. The cell lines were incubated with tritiated folic acid with and without the ABCG2 specific inhibitor

Ko143 for 30 minutes. The cells were washed four times before counts were determined on a scintillation counter. Values were normalized against the results obtained for the 293-GFP cells that were assayed in the same experiment. As expected the 293-ABCG2 cells retained 33.7% less folic acid than the 293-GFP cells (**Figure 4-5**, $P<0.001$) and the addition of Ko143 increased the amount of retained tritiated folic acid by 21% ($P<0.001$). This demonstrates that ABCG2 is able to efflux folic acid. The 293-Q141K cell line retained 18% less tritiated folic acid compared to the 293-GFP control cells ($P<0.001$) and the addition of the inhibitor increased the retained folic acid by 14% ($P=0.012$). Importantly, a statistically significant difference in efflux between the wild-type ABCG2 and Q141K variant ($P=0.002$) was observed. This data demonstrates for the first time that the Q141K variant decreases folic acid efflux.

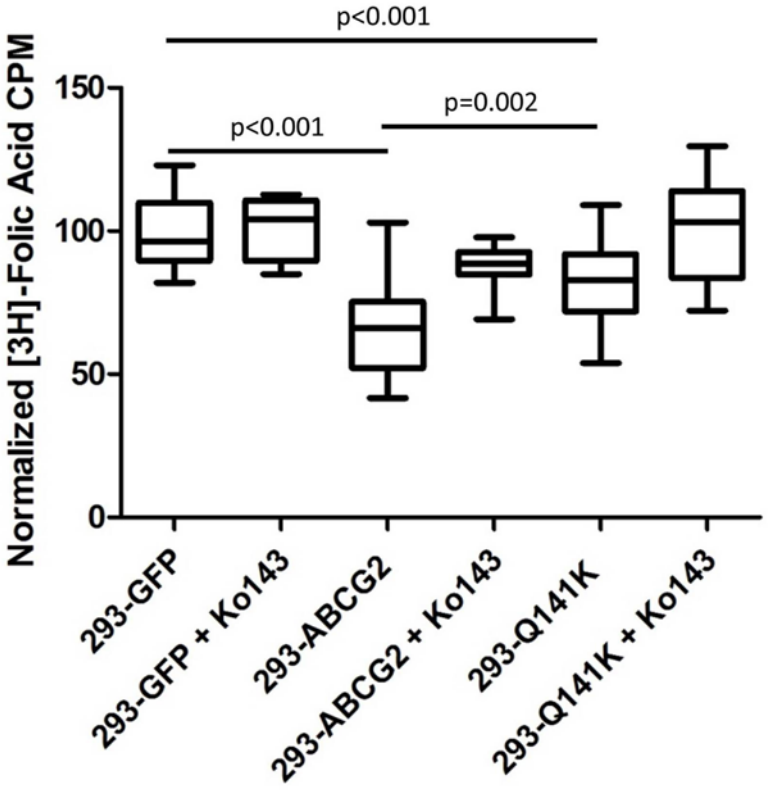

Figure 4-5. The Q141K variant cells retain more folic acid than the wild-type ABCG2 cells. Radiolabeled folic acid retention was measured in the cell lines 293-GFP, 293-ABCG2, and 293-Q141K. 293-ABCG2 retained 33.7% less folic acid than 293-GFP (P<0.001). 293-Q141K retained 17.8% less folic acid than 293-GFP (P<0.001). 293-Q141K also retained significantly more folic acid than 293-ABCG2 (P=0.002). There were 3 or 4 replicates for each cell line (experiment n=6). When the ABCG2 specific inhibitor Ko143 was added, the 293-ABCG2 and 293-Q141K cell lines retained more folic acid (21% and 14%, respectively).

4.3.3 Expression of wild-type ABCG2 confers docetaxel resistance and the Q141K variant leads to partial resistance

A 5 fold up-regulation of ABCG2 mRNA was observed in the prostate cancer cell line, LNCaP, after an 8 day treatment with 1nM docetaxel (Chapter 3). To test whether ABCG2 effluxes

docetaxel and to determine if expression of the Q141K variant would affect the efflux of docetaxel, a 96-hour cytotoxicity assay was performed. As shown in **Figure 4-6**, the cells expressing wild-type ABCG2 showed resistance to docetaxel compared to the 293-GFP control cells (IC_{50}=9.96nM vs. 4.50nM, P=0.001). Confirmation that the increased resistance was due to expression of ABCG2 was shown as the ABCG2-specific inhibitor Ko143 abrogated the growth advantage in docetaxel (IC_{50}=9.96nM vs. 3.69nM; P=0.007). Relative to the control 293-GFP cells, the Q141K variant was significantly more resistant to docetaxel (IC_{50}=7.51nM, P=0.011), but was less resistant than the wild-type 293-ABCG2 cells (not significant). Taken together, the wild-type ABCG2 can efflux docetaxel leading to a resistant phenotype and the Q141K variant appears to confer partial resistance to docetaxel.

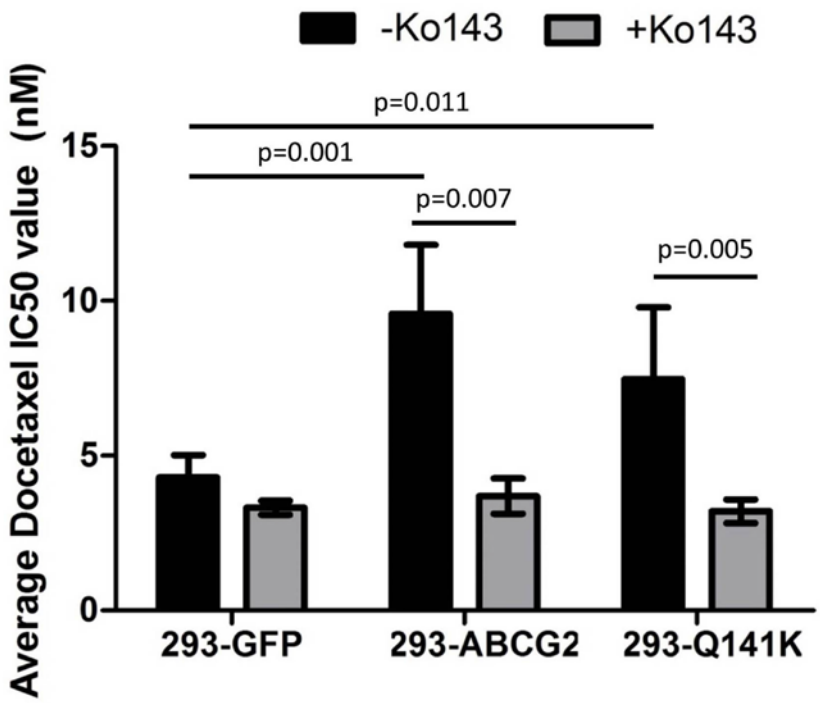

Figure 4-6. ABCG2 increases resistance to docetaxel. An MTT assay was used to determine the IC50 values of 293-GFP, 293-ABCG2, and 293-Q141K treated with docetaxel. 293-ABCG2 was 2.2 times more resistant to docetaxel than 293-GFP (P=0.001). The wild-type ABCG2 was 1.3 times more resistant than the Q141K variant, but this difference was not significant. However, the Q141K variant was 1.7 times more resistant than the control (P=0.011). By adding Ko143, the resistance to docetaxel observed in the wild-type and variant cell lines was abolished (P=0.007 and P=0.005, respectively).

4.4 DISCUSSION

The Q141K variant expressed similar levels of mRNA and protein compared to the wild-type ABCG2 in the HEK293 cell line, consistent with reports from other groups [83, 100, 141, 143]. Others have found that the decreased functionality of the Q141K variant is due to decreased amounts of protein detectable by western blot [82, 84-86, 88, 95, 96, 98]. The discrepancy could

be explained by the difference in the model or gene delivery systems. To test this, transient transfections were performed. I also observed a decrease in the Q141K variant protein relative to our stable transductions even though there was increased mRNA in the transient transfections compared to the stable transduction. This suggests that the model system can greatly affect the expression of the ABCG2 protein and mRNA. Exogenously expressing a protein through a transfection or transduction system is known to generate massive levels of the mRNA and protein. It is known that the cell has difficulty properly trafficking the Q141K variant protein in the Flp-In-293 cell line [95, 96], which is utilized by many of the groups observing decreased levels of the Q141K variant protein. If a cell is generating a considerable level of Q141K variant protein that is unable to be trafficked to the cell membrane, then the cell will degrade this protein. Indeed, these reports have provided evidence that the Q141K variant is targeted for proteasomal degradation [96] or degraded by an autophagic pathway after accumulation in an aggresome [95]. However, a small amount of the Q141K variant protein is still trafficked to the cell membrane. After staining the ABCG2 protein in the 293-ABCG2 and 293-Q141K cell lines, I observed more intracellular ABCG2 staining in the Q141K variant compare to the wild-type. In addition, the overall ABCG2 staining was similar in both cell lines, which corresponds to the similar levels of protein detected by western blot. In this lentiviral transduction system in HEK293 cells, there may not be as much protein produced and the cell is able to efficiently traffic more of the variant protein and/or does not degrade the Q141K variant protein.

In addition to decreased protein expression and improperly trafficked protein, it has been hypothesized that disruption at the nucleotide-binding domain could cause the decreased function observed in the Q141K variant. After structural analyses of homologous ABC transporters, it has been suggested that the Q141 position contributes to a critical domain-domain

interface between the cytosolic nucleotide-binding domain and the transmembrane domain. Recently, Woodward et. al. demonstrated that the ABCG2 Q141K variant leads to instability in the nucleotide binding domain thus causing decreased steady-state protein levels and functionality. Stabilization can be restored by decreasing the temperature at which the cells are grown, or treating with small molecule inhibitors that potentially bind directly to the protein to stabilize it [98]. Similarly, disruption of this interface in P-glycoprotein and CFTR (two other proteins in the ABC superfamily) also results in altered protein trafficking and function [144, 145].

This is the first report to show differential efflux of folic acid between the wild-type and Q141K variant ABCG2. This difference is likely due to the differences in ABCG2 protein localization detected in the 293-ABCG2 and 293-Q141K cell lines. There are a few reports that the Q141K variant does not have an effect on ABCG2 cellular localization [82, 83, 101]. We have shown though, as well as others [84, 85, 96, 98, 141], increased intracellular ABCG2 staining in Q141K variant transduced/transfected cells compared to cells transduced/transfected with wild-type ABCG2. This finding suggests that cells expressing the Q141K variant are retaining more folates, which could lead to increased proliferation.

This study revealed a difference in resistance to docetaxel between the 293-GFP, 293-ABCG2, and 293-Q141K cell lines. Cells expressing wild-type ABCG2 have an IC_{50} value 2.2 times greater than control cells. This difference is completely diminished when the potent and specific ABCG2 inhibitor Ko143 was added to the ABCG2 expressing cells, indicating a role for ABCG2 in resistance to docetaxel. Xie et. al. has reported an up-regulation in ABCG2 protein in docetaxel resistant CWR-R1 prostate cancer cells [67]. An increase in ABCG2 mRNA in LNCaP prostate cancer cells treated with docetaxel for 7 and 8 days has also been observed (Chapter 3).

In addition, the IC_{50} value trends lower in the Q141K variant compared to wild-type ABCG2, which suggests that the presence of the Q141K variant may increase the sensitivity of the cell to docetaxel. Hahn et. al. observed an increased survival time in prostate cancer patients expressing the Q141K variant being treated with docetaxel compared to patients expressing wild-type ABCG2 [65]. Taken together, docetaxel may be a novel ABCG2 substrate and the Q141K variant affects the clinical response to this drug.

In conclusion, using the HEK293 cell line to exogenously express the wild-type ABCG2 and the Q141K variant enabled me to study the differences in folic acid efflux between the two cell lines. If this *in vitro* data is translated to the patient, it suggests that a patient expressing the Q141K variant would retain more folates, which could have an effect on one-carbon metabolism. Further *in vitro* and clinical data needs to be collected to establish the link between the Q141K variant causing increased intracellular folates levels, which in turn increases cellular proliferation. This study also presented further evidence that docetaxel is an ABCG2 substrate and the clinical outcome of docetaxel treatment could be predicted based on the patient's ABCG2 genotype.

5.0 SUMMARY AND FUTURE DIRECTIONS

This dissertation sought to understand the role folate has in the regulation and expression of ABCG2, and how the Q141K variant effects the expression and function of ABCG2 in prostate cancer. The ultimate goal is to understand the interactions between folate and ABCG2 and the differences between the Q141K variant and wild-type ABCG2 in order to personalize a dietary and treatment plan for a patient based on their gene expression and mutation profile.

In Chapter 2, the expression of ABCG2 was increased in response to a folate-deprived environment, which was partially due to demethylation of the CpG island in the ABCG2 promoter. Because folate is required for methylation reactions, decreasing the amount of folate available to the cell will change the expression of the entire genome. It is important to understand the role folate plays in the regulation of ABCG2 because it is a dietary substance and is easily modifiable. The demethylation of the ABCG2 promoter occurred within one week of the cells being deprived of folic acid. This time frame suggests that an active, rather than passive, demethylation process is occurring. Currently, there are no known DNA demethylases and the mechanism by which active demethylation occurs is unknown. Understanding the demethylation mechanism is important in understanding the dysregulation of genes as well as how genes are demethylated during development.

A natural extension of this work is to translate the *in vitro* ABCG2 methylation findings to patient samples. If decreased methylation of the ABCG2 promoter is correlated to increased

expression of ABCG2 in prostate cancer patients, then the expression of ABCG2 could be modulated during cancer treatment. In this case, by inhibiting ABCG2 the efficacy of chemotherapeutics that may be substrates for ABCG2 would be increased. In addition, clinicians could recommend increasing or decreasing folate intake in order to alter the expression of ABCG2.

In Chapter 3, the goal was to study the expression and function of wild-type ABCG2 and the Q141K variant in prostate cancer. There is clinical evidence that the Q141K variant is correlated with decreased survival in prostate cancer patients. However, when patients are treated with docetaxel, the survival is increased in patients with the Q141K variant, suggesting that ABCG2 may be involved in docetaxel resistance. After evaluating the time to PSA recurrence after prostatectomy in a cohort of prostate cancer patients, a significant decrease in time to recurrence was observed in patients with the Q141K variant. To propose a mechanism by which the Q141K variant may decrease time to recurrence, ABCG2 was exogenously expressed in two prostate cancer cell lines. However, even after deleting recognition sites for miRNAs known to repress ABCG2, the prostate cancer cell lines stopped expressing or never expressed the wild-type ABCG2 or the Q141K variant. It is important to understand why the prostate cancer cell lines do not express ABCG2. There is another miRNA (miR-328) that is known to repress ABCG2 expression and perhaps there are other unknown miRNAs. In addition, other repressive mechanisms, such as DNA methylation and histone acetylation may be contributing to the repression of ABCG2. Endogenous ABCG2 could also be upregulated by low-dose chronic drug exposure. By treating the prostate cancer cell lines with an ABCG2 substrate, such as doxorubicin, over a long period of time, ABCG2 can be upregulated to efflux the drug and allow the cell to live. This technique may allow the function of wild-type ABCG2 to be assessed in

prostate cancer, but it would not enable us to do experiments to examine the differences between the wild-type an Q141K variant because a prostate cancer cell line expressing the Q141K variant has not been identified.

A decrease in serum folate levels was observed in Gleason 7 prostate cancer patients with the Q141K variant compared to patients with the wild-type ABCG2. This observation reveals a potential role for ABCG2 in regulating systemic folate levels. It suggests that patients with the Q141K variant are retaining more folate in the liver, the organ where over 50% of the body's folate is stored and metabolized. Because more folate is theoretically being retained in the liver of a Q141K patient, it is not released into the bloodstream and therefore serum folate levels are decreased. The next goal in this project is to if there are differences in the tissue (e.g. liver, prostate) folate concentrations between wild-type and Q141K variant patients.

In Chapter 4, exogenous wild-type ABCG2 and Q141K variant protein were expressed in the HEK293 cell line to determine whether there are functional differences in folate and docetaxel efflux. For the first time, differential efflux of folic acid was observed between the Q141K variant and the wild-type ABCG2. Additional evidence supporting the hypothesis that docetaxel is an ABCG2 substrate was obtained. However, additional experiments need to be completed. Using radio-labeled docetaxel and measuring the amount of retained docetaxel would provide direct evidence that ABCG2 effluxes docetaxel. In addition, radio-labeled dihydrotestosterone and measuring the level of retained DHT would provide additional support that DHT is an ABCG2 substrate. By proving that docetaxel and DHT are ABCG2 substrates, ABCG2 and ABCG2 genotype at the 421 locus could potentially be used as a biomarker to predict response to docetaxel treatment and prognosis.

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