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Multidrug Resistance Transporter-1 and Breast Cancer Resistance Protein protect against ovarian toxicity, and are essential in ovarian physiology

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Abstract

Ovarian protection from chemotoxicity is essential for reproductive health. Our objective is to determine the role of ATP-dependent, Multidrug Resistance Transporters (MDRs) in this protection. Previously we identified MDR-dependent cytoprotection from cyclophosphamide in mouse and human oocytes by use of MDR inhibitors. Here we use genetic deletions in MDR1a/b/BCRP of mice to test MDR function in ovarian somatic cells and find that *mdr1a/b/bcrp*^{−/−} mice had significantly increased sensitivity to cyclophosphamide. Further, estrus cyclicity and follicle distribution in *mdr1a/b/bcrp*^{−/−} mice also differed from age-matched wildtype ovaries. We found that MDR gene activity cycles through estrus and that MDR-1b cyclicity correlated with 17β-estradiol surges. We also examined the metabolite composition of the ovary and learned that the *mdr1a/b/bcrp*^{−/−} mice have increased accumulation of metabolites indicative of oxidative stress and inflammation. We conclude that MDRs are essential to ovarian protection from chemotoxicity and may have an important physiological role in the ovary.

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Keywords

ATP binding cassette (ABC); multidrug resistance transporter (MDR); P-glycoprotein; ovary; oocyte; chemotoxicity

1. Introduction

Cancer has in many cases become a survivable disease with an estimated 9.6 million women becoming survivors by 2024 ([1] [SEER.cancer.gov](https://seer.cancer.gov)). Breast cancer is the most common malignancy in women less than 45 years old, while young girls are more likely to suffer from central nervous system, hematologic, bone, and renal neoplasms. Over 80% of young female cancer patients will survive their disease due to more aggressive and advanced treatments ([1] [SEER.cancer.gov](https://seer.cancer.gov)). The rate of cancer cure is increasing, but many women undergo gonadotoxic therapies, which compromise both their reproductive function and long-term health. Women have the option to attempt preservation of their fertility through the established methods of *in vitro fertilization* (IVF) followed by embryo cryopreservation or by MII oocyte cryopreservation [2, 3]. Other approaches, including ovarian tissue cryopreservation followed by *in vitro* maturation (IVM) have been deemed experimental by the American Society of Reproductive Medicine [4], and ovarian suppression (e.g. administration of GnRH agonists) has insufficient evidence to be used for fertility preservation [5]. Unfortunately, none of these strategies alleviates the long-term health problems that parallel the loss of follicular function [6]. In fact, chemotherapy-induced toxicity is quickly becoming a leading cause of ovarian failure, which predisposes the patient to early onset of menopause-associated risks such as accelerated bone loss, increased cardiovascular risk, and sexual dysfunction, in addition to infertility [7]. The current thinking is that alkylating agents—such as busulfan, carboplatin, chlorambucil, cisplatin, cyclophosphamide, dacarbazine, ifosfamide, and thiotepa—deplete the woman's follicles [8] in a drug- and dose-dependent manner [9]. Research in mice has shown that cells of ovaries exposed to doxorubicin, an anthracycline, underwent apoptosis through acute injury of the vasculature and that apoptosis occurred in all follicular stages, but especially those in the secondary stage [10]. Soleimani et al., 2011 reported that doxorubicin-treated ovaries showed double-strand DNA-break-initiated apoptosis in primordial follicles, oocytes, and granulosa cells in a dose-dependent manner [11]. Their data also showed that stromal and vascular damage were implicated in ovarian failure post treatment with doxorubicin [11]. In a review by Morgan et al, (2012), 17 studies were selected to ascertain whether the mammalian oocyte or the granulosa cells were the main target of various chemotherapeutics including cisplatin, doxorubicin, and cyclophosphamide [12]. These studies could not distinguish whether germ or somatic cells are most sensitive to the drugs, and therefore most responsible for the observed ovarian damage [12].

Cyclophosphamide effects on the ovary are particularly worrisome given its widespread use to treat many malignancies in women, which include cancer of the breast [13, 14], endometrium [15], germ cell tumors [16], and gestational trophoblastic disease [17, 18]. Many autoimmune disorders are also treated with cyclophosphamide and have similar long-term health concerns. These include systemic lupus erythematosus [19, 20], rheumatoid

arthritis [21, 22], scleroderma [23], polyarteritis nodosa [24] and Wegner's granulomatosis [24], which disproportionately affects women [25, 26]. Unfortunately, even though cyclophosphamide is preferred in many cases for treatment, it is also associated with the highest risk compared to all other alkylating agents with approximately 6.3% of childhood cancer survivors developing acute ovarian failure and 8% of patients present with premature ovarian insufficiency [27]. A more recent study in young cancer survivors reported that approximately 28.6% of patients receiving cyclophosphamide treatments had diminished ovarian reserve and 10.2% had premature ovarian insufficiency [28].

Our previous work showed that murine and human oocytes with decreased MDR-1 functionality had increased sensitivity to cyclophosphamide exposure [29] supporting the contention that cyclophosphamide was a substrate of at least the MDR-1 effluxer. Here we extend the analysis to focus on the somatic cell response to cyclophosphamide *in vivo*, with and without MDR functionality. An improved understanding of the inherent ovarian defensive mechanisms could assist in the development of pharmacologic drug development aimed at protecting the most vulnerable cell type.

Many neoplasms derive protection from chemotherapy with increased multidrug resistance (MDR) transporter function, though our understanding of the actual role of these gene products in normal tissue is quite limited. Such lack of understanding is particularly true in the ovary [30]. As a group, the super family of ATP-binding cassette (ABC) transporters are usually located in the plasma membrane and efflux molecules between cells and their environment. A subset of this family is also referred to as multidrug resistance transporters (MDRs) and these gene products are thought to provide protection to the cell by exporting toxins, metabolic products, and xenobiotics [31–33]. ABC effluxer expression seems to be highest in organs involved in the excretion of toxins such as the intestines, kidney, liver, and placenta [34] and especially in the syncytiotrophoblasts [35]. MDR-1 (also called P-glycoprotein) is the most well-studied of these transporters and it is a transmembrane protein with two domains each of six putative transmembrane segments and two intracellular nucleotide binding domains [36]. Other members of the MDR family have variations of this basic structure [37]. The newest identified member of the family is Breast Cancer Resistance Protein (BCRP), first described in the MCF-7 breast cancer line [38]. BCRP is actually a hemitransporter that functions as a dimer [39, 40]; it only has one nucleotide binding domain and six transmembrane segments.

The primary objective of this study was to test the importance of *mdr1a/mdr1b/bcrp* function in the mouse and their ability to protect somatic cells of the ovary from exposure to chemotherapy. The protocol included assessing viability of somatic cells after treatment with therapeutic (75mg/kg) and sterilizing (150 mg/kg) doses of cyclophosphamide given to wild type and *mdr1a/mdr1b/bcrp*^{-/-} knockout (triple knockout, (TKO)) mice. Surprisingly, we also discovered that the *mdr1a/mdr1b/bcrp* effluxers function in normal reproductive cyclicity of the ovary.

2. Material and Methods

2.1 Animals

An animal protocol was obtained from the Institutional Animal Care and Use Committee (IACUC) Protocol number 1407000080. Female mice 6–8 weeks of age were utilized for experimentation. The knock out mice, FVB.129P2 *Abcb1a*^{tm1Bor} *Abcb1b*^{tm1Bor} *Abcg2*^{tm1Ahs} N7 (*mdr1a/mdr1b/bcrp*^{-/-}) and wild type FVB/N were obtained from Taconic Biosciences (Hudson, NY). The mice were injected intraperitoneally with saline, with 75 mg/kg, or with 150 mg/kg of cyclophosphamide and then sacrificed at 24 and 48 hours following injection, with prompt bilateral ovariectomies after sacrifice. For estrous cycling and hormonal testing, FVB/N mice were compared to *mdr1a/mdr1b/bcrp*^{-/-} mice.

2.2 Viability Assay and Imaging

Ovaries were imaged with an Olympus FV-1000 MPE Multiphoton Microscope *ex vivo* after a 1-hour incubation at 37°C with the LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies Carlsbad, CA). Z-stacked sections totaling 6 mm were taken through each ovary; three spaced images per ovary were analyzed with 4 ovaries per condition to produce biological replicates. Nonviable cells in the ovary were quantified using a customized macro (Supplemental Data Figure 1) with an Otsu threshold in ImageJ to detect red fluorescent cells as a result of labeling with Ethidium homodimer-1. A dead cell:live area ratio was then calculated by dividing the number of red (dead) cells by the area of the ovary section. The mean of this ratio was then calculated along with SD in Prism Graphpad (La Jolla, CA).

2.3 Immunohistochemistry

Five-micron paraffin sections were rehydrated into phosphate-buffered saline. Heat induced epitope retrieval was performed using sodium citrate buffer and the tissue was treated for possible endogenous peroxidase with a 3% aqueous peroxide solution. The tissue was then treated for endogenous biotin and avidin using an avidin biotin blocking kit (Vector, Burlingame, CA) and exposed to a PBS blocking buffer containing 15% normal rabbit serum and 1% bovine serum albumin. The prepared tissue sections were then incubated with a 1:200 solution of primary rabbit monoclonal to human P-glycoprotein (Abcam ab170904 Cambridge, MA) or with 1:400 BCRP/ABCG2 BXP-53 (Abcam 24115 Cambridge, MA) overnight at 4°C. After two washes in PBS, the Vector Peroxidase Goat IgG kit (Vector, Dallas, Texas) was used to label the primary antibody with biotinylated secondary antibody and diaminobenzidine (DAB) reaction product. Colorization of DAB was achieved using the Vector ImmPACT DAB peroxidase substrate kit and the sections were then counterstained with Harris hematoxylin (Thermo Fisher, Agawam, MA).

2.4 Follicle quantitation

Follicle counting and classification were performed using a method described by Flaws et al [41]. Briefly summarized here, serially sectioned, paraffin-embedded ovaries 5 micron thick sections were stained with hematoxylin and eosin. Using an EVOS® FL Auto Cell Imaging System at 40×, every tenth section was imaged and the follicles were categorized as: primordial, primary, preantral, antral, or corpora lutea based on the morphology of granulosa

cells, presence or absence of an antrum or luteinized cells of the corpus luteum as described [41]. This number was then multiplied by 10 and each follicle type of the FVBN mice was then plotted relative to *mdr1a/mdr1b/bcrp*^{-/-} mice whose ovaries were fixed, sectioned, counted, and plotted identically.

2.5 Serum Hormone Assays

Wild type and *mdr1a/b/bcrp*^{-/-} mice were sacrificed in diestrus or metestrus after confirming their cycle day with vaginal lavage and staining of the retrieved cells with methylene blue. Selected mice were exsanguinated, whole blood was allowed to clot at room temperature for 90 minutes, and the serum was separated after centrifugation at 2,000g for 15 minutes. The serum was sent to the University of Virginia School of Medicine Center for Research in Reproduction Ligand Assay and Analysis core [<https://med.virginia.edu/research-in-reproduction/ligand-assay-analysis-core/>] for quantitation of AMH, Inhibin B, FSH, Estradiol, and Progesterone.

2.6 TUNEL Assay

Five-micron paraffin sections of wild type and *mdr1a/b bcrp*^{-/-} knockout mouse ovaries were processed using the DeadEnd TUNEL Assay according to manufacturer's instructions (Promega, Madison, WI). The slides were also treated with VECTASHIELD® Mounting Medium for Fluorescence with DAPI. Using confocal microscopy with stitching in Zen Blue on the Zeiss LSM 800, entire ovarian sections were imaged and reconstructed. Densities of green fluorescence were analyzed in ImageJ and ratios of DAPI to green labeled cells were then compared. DNase treated sections were used as a positive control and as a standard for maximal labeling. Five ovarian sections from different areas of the ovary from three different mice for each condition were used for quantitation.

2.7 Dynamics of MDR1b and BCRP mRNA during estrous

To determine the levels of mRNAs encoding MDR1b and BCRP during the estrous cycle, primers were designed to unique regions of transcripts for MDR1b (F: 5'-CACAGCTTGTCAGCCAAT-3' and R: 5'-ACTTCTCGAAGATGGGCAA-3'), BCRP (F: 5'-CTAGGGGCCGAGGCTTATAC-3' and R: 5'-AGTGTGCTACAGACACCACA-3'), ERβ (F: 5'-AAACAGAGAGACCCTGAA-3' and R: 5'-CCTCTTGGCGCTTGGACTA-3') as well as to the transcript encoding β actin as a standard (F: 5'-TCTTGGGTATGGAATCCTGTGG-3' R: 5'-CAGCACTGTGTTGGCATAGAGG-3'). MDR1b was emphasized over MDR1a in these studies because it is more highly expressed in the ovary. Wild type mouse ovaries in various, defined phases of their estrous cycle were placed in Trizol (Thermo Fisher Scientific Waltham, MA) and stored at -80°C. After the RNA was extracted, cDNA was synthesized and used to perform RT qPCR. All values were normalized against the β-actin mRNA and represented as a fold-change relative to the amount of each RNA detected in the proestrus stage.

2.8 Analysis of the estrous cycle in WT vs *mdr1a/b/bcrp*^{-/-} mice

Wild type and knockout mice underwent daily vaginal lavage with deionized water for 17 days. Cells in the washings were stained with 2.5% methylene blue, recorded, and analyzed for estrus cycle state. The times of each animal in each genotype were compared for proestrus, estrus, metestrus, and diestrus.

2.9 Metabolomic Profiling

Whole mouse ovaries taken from both wild type (3 animals) and *mdr1a/b/bcrp*^{-/-} (4 animals) mice during estrus were cleaned and had their bursae removed. The ovaries were then snap frozen in liquid nitrogen and stored at -80°C. Complete metabolome analyses were performed at Massachusetts Institute of Technology Metabolite Profiling Facility Whitehead Institute for Biomedical Research Cambridge, MA [<http://metabolomics.wi.mit.edu/>]. Each tissue (~10 mg) was homogenized on dry ice in 600 ul methanol using a disposable Pellet Pestle (Kimble-Chase). After addition of 300 ul water and 400 ul chloroform (HPLC grade), samples were vigorously shaken for 10 minutes at 4°C and then centrifuged at 15,000×g at 4°C. The two phase-separated layers (top: polar; bottom: organic) were collected separately, dried by Speedvac, and stored at -80°C. For polar metabolite analysis, samples were resuspended in 100 ul water, and 1 ul was injected for LC/MS analysis [42]. Non-polar metabolite analysis was performed on the same samples following resuspension in 50 ul of 65/30/5 acetonitrile:isopropanol:water (v/v/v), and 5 ul was injected for LC/MS analysis [43].

3.0 Results

3.1 MDR-1 and BCRP are expressed in somatic cells of the ovary

We used immunohistochemistry to localize MDR-1 (Figure 1) and BCRP (Figure 2) in the ovary. Both MDR-1 and BCRP appear to accumulate in oocytes, as expected from whole mount immunolabeling previously described [29] and in granulosa cells. Intense labeling was observed in the corpora lutea for MDR-1 while BCRP appeared to label non-luteinized granulosa cells. BCRP was also seen in capillaries of the ovary, as originally reported [44].

3.2 MDR-1 and BCRP are protective against cyclophosphamide in the ovary

We used the LIVE/DEAD® Viability/Cytotoxicity Kit with 2-photon microscopy to quantitate the immediate effect of typical and sterilizing doses of cyclophosphamide *in vivo* on cell viability in the ovaries of mice. Ovaries from wild type FVBN mice treated with saline show sporadic, low-level cell death in somatic tissues. The cells displaying the most cell death in the ovary of the cyclophosphamide-treated animals appear morphologically to be somatic cells, not germ cells (Figure 3) and histologically appear to be granulosa cells. Two – to – three fold increased cell death was seen at 24 hours in the TKOs relative to the WTs, and even at 48 hours, a nearly three-fold increase in cell death was seen in the higher dose of cyclophosphamide. Our results overall show that cell death and viability can be evaluated and quantified in a whole live ovary in real-time (Figure 4). These animals had similar exposure to cyclophosphamide at cumulative doses to which patients undergoing treatment with cyclophosphamide would be exposed [45]. In addition to the LIVE:DEAD

assay measuring cell viability in situ, we also performed a TUNEL assay to quantitate apoptosis in these same tissues. We found that there was more apoptosis in the knockout animal compared to wild type (Figure 5). This outcome supports the conclusion that MDRs are protective in the ovary against the harmful effects of cyclophosphamide, and both cell viability and apoptotic measurements converge on the same result. We also conclude that the vast majority of non-viable cells observed following cyclophosphamide exposure die via apoptosis.

3.3. MDR-1 and BCRP function in normal ovarian physiology

To determine how MDRs may function normally in ovarian physiology we tested several metrics of reproductive function. First, we assessed reproductive cyclicity in wildtype and *mdr1a/b/bcrp*^{-/-} females by 17 days of vaginal lavage and analysis of resultant cells. Although estrous cycling did not show statistically significant differences between the animal groups, on average the knockout females spent more time in estrus compared to wild type animals (Supplemental Figure 2). Further, histological examination of the ovaries revealed a remarkably different ovarian architecture between wild type and knockout animals. Ovaries of knockout mice had a higher percentage of preantral follicles (WT 24.84% vs KO 29.59% $p=0.0235$) and fewer corpora lutea (22.56% vs 7.45% $p=.0043$) (Figure 6). We then measured a profile of hormones in both animal groups. For the most part, the wildtype and *mdr1a/b/bcrp*^{-/-} animals showed similar average levels of AMH, Inhibin B, estradiol, and progesterone although FSH was significantly higher in the wild type ($p=.0099$) (Figure 7).

3.4 MDR-1 and BCRP expression are dynamic in the estrous cycle

We learned previously that different stages of oocyte meiotic maturation had different levels of MDR1 [29]. These changes influenced the level of protection to chemotherapeutics within the oocyte. Here we tested *mdr1b* and *bcrp* mRNA levels throughout the estrous cycle to determine if certain periods of reproduction might elevate such activity. We found that levels of MDR1b transcripts cycled with a greater than 2-fold change during estrus in wild type mice. The period of increased accumulation of MDR1b mirrors the surge in 17 β estradiol during proestrus (Figure 8) which may reflect transcriptional regulation by estrogen receptors, whereas the BCRP mRNA steady-state level was actually decreased during this same time. The result is surprising since both MDRs have a conserved estrogen receptor binding site. While estradiol was not directly measured here we did use a surrogate for estradiol by tracking the expression of estrogen receptor beta, which is responsive to estradiol, in relationship to the expression of MDR1b and BCRP. Perhaps post-transcriptional regulation of these two mRNAs includes selective turnover of the transcripts and may reflect changing demands of the effluxers during transitions in the estrus cycle. Were we to identify normal biological substrates of these effluxers, it may contribute as to how the ovary normally fluctuates in estrus and what cellular demands may be involved in response to dynamic hormonal activities. In the least, this result shows dynamic regulation of the MDRs and encourages molecular evaluation of the molecular mechanism in these changes during estrus cycling.

3.5 Loss of MDR-1 and BCRP leads to accumulated metabolites in the ovary, indicative of oxidative stress and inflammation

Here we learned that MDR-1 and BCRP function in protection from cyclophosphamide damage. We reasoned, though, that these effluxers must have endogenous substrates for function as well, given the alterations in follicle counts, and the differences in the estrous cycling. We therefore examined what differences in metabolites might be present in the ovary when lacking these MDRs with the premise that certain metabolite substrates of MDR-1 and BCRP may accumulate within the cell from lack of effluxer activity when the genes are knocked out. While we know from our research that the oocyte alone has at least 25 different detectable mRNAs encoding MDRs [46, 47], we reasoned that the somatic cells of the ovary must also have a large repertoire that likely overlap in substrate functionality. We thus did not anticipate many significant differences in the metabolic profiles of the MDR + vs MDR – ovaries. We tested for over 500 polar and non-polar metabolite candidates and found several significant differences in metabolite concentrations in the KO relative to the wild type (Figure 9). The metabolic profiles of wild type FVBN ovaries and FVB.129P2 *Abcb1a^{tm1Bor} Abcb1b^{tm1Bor} Abcg2^{tm1Ahs}* N7 (*mdr1a/mdr1b/bcrp*–/–) in estrus showed statistically significant over-accumulation of orotate (CV=0.033 R=.982), dihydroorotate (CV=.001R=0.998) kynurenine (CV=.029 R=.979), and 3-ureidopropionate (CV=.035 R=.993). The premise that the knockout animals may be more susceptible to ROS is further supported by overexpression of kynurenine, which has been shown to scavenge hydrogen peroxide and superoxide [48]. The metabolite 3-ureidopropionate has been linked to neurotoxicity and generation of reactive oxygen species [49]. Fructose 1,6 biphosphate, GTP (CV=.118 R=.978), and GSH (CV=.129 R=.999) are under-accumulating in the knockout ovaries. Reduced glutathione, also known as GSH, is a major thiol bound to proteins and its markedly low levels in the TKOs may indicate a near complete oxidation. The non-polar metabolic analysis also shows that ceramides were under-accumulating in the knockouts versus wild type (the fold change for ceramides (TKO/WT)=0.881, p=0.015). Cholesterol esters were also over-accumulated in the knockout versus wild type (the fold change for cholesterol esters (TKO/WT)=1.978, p=0.065). Overall, a thematic difference seen in the ovaries from *mdr1a/b/bcrp*–/– knockout animals show a metabolic profile of increased oxidative stress and inflammation (Figure 9).

4. Discussion

Accessibility to oncofertility centers that offer oocyte and embryo cryopreservation is extremely limited outside of Western Europe and the Americas. Even in countries with such access, the delay in oncologic care and perhaps the lack of counseling regarding fertility preservation options result in many girls and women having irreparable damage to their ovary. Recently, Salih et al reported that Dexra (dexarazoxane), an inhibitor of topoisomerase 2, protects the ovary from doxorubicin by preventing DNA damage [50]. However, currently no medications are approved by the Federal Drug Administration to provide undisputed protection to the ovary from gonadotoxic agents routinely used in chemotherapy. Therefore, our understanding of the endogenous protective mechanisms such as multidrug resistance transporters and their regulation are critical to the development of

therapeutics that could protect the ovary from both the necessary chemotherapies and reproductive toxicants in general.

Our results demonstrate that MDRs are abundantly expressed in the ovary and thus likely have many functions normally in ovarian physiology. Metabolomic profiles during estrus of ovaries lacking only three of the many MDRs present show significant shifts towards oxidative stress and inflammation in the cells. One explanation of this result is that the lack of these three MDRs causes over-accumulation of metabolites that also happen to be byproducts of oxidative stress. The knockout ovaries also lack several metabolites implicated in the remediation of oxidative stress. Perhaps this indicates a condition in which either they face increased consumption or decreased expression. This is of importance since oxidative stress in gametogenesis has been reported to alter follicular maturation and quality [51]. Most of the metabolites that were over-accumulated are associated with pyrimidine synthesis, which has been shown to be associated with ROS and herald apoptosis, at least in plants [52]. Dihydroorotate dehydrogenase (DHODH) for example, is involved in *de novo* pyrimidine biosynthesis which involves the ubiquinone-mediated oxidation of dihydroorotate to orotate, which are found to be over-accumulated in the profile of these ovaries. DHODH is implicated in the formation of reactive oxygen species (ROS) and involved in apoptosis [53] so perhaps its substrate over-accumulation suggests that the loss of ABC effluxers leads to increased ROS. Protein thiols, including GSH, play an important role in determining the redox status of cells. The decreased accumulation of glutathione (GSH) further suggests that glutathione is possibly being consumed or under-produced, and either scenario leads to increased susceptibility to ROS and free radical damage. Low glutathione levels causes accelerated ovarian aging [54]. The non-polar metabolic profile changes associated with MDR-1 and BCRP loss may also indicate a shift towards inflammation. These increased oxidative stresses and inflammation may cause premature senescence in the ovary with overt aberrations in reproductive cycling and follicle regulation. Thus, although not definitively learning that these metabolites are natural substrates of the MDRs knocked out here, they do provide a link to the MDRs and to the altered reproductive physiology seen in these mice.

We definitively show that ABC effluxers work to protect the ovary from the most gonadotoxic chemotherapeutic—cyclophosphamide. Loss of MDR in the whole ovary leads to more somatic cell death very rapidly as we show damage as early as 24 hours following cyclophosphamide administration of 75 mg/kg and 150 mg/kg. At 48 hours, no difference in cell death was noted in the knockouts given 75 mg/kg but this is likely because of variability of cell death associated with the lower dosages, and/or with the most sensitive, and affected cells already cleared from the tissue. These results are suggestive that somatic cells are more sensitive to chemotoxicity with the loss of MDRs and/or have more retention of toxins at baseline and even more so with the loss of MDRs compared to oocytes, which were not TUNEL positive nor fluorescently labeled as non-viable with ethidium homodimer-1. Martin et al 2016 reported that incubation with etoposide *in vitro* is even more toxic to cumulus oophorus complex enclosed oocytes compared to denuded oocytes [55]. This further supports the possibility of increased toxin retention, as we have appreciated anecdotally that comparative efflux assays between oocytes and granulosa cells are technically not feasible because the indicator dye is retained in the granulosa cells to saturating levels. Therefore,

how ABC effluxers are perhaps differentially expressed and regulated in each cell type will be important to inform us on how they can be manipulated.

The regulation of ABC effluxers is likely dependent in part on ovarian hormones. The literature in cancer cell lines suggests that the primary substrates of BCRP are sulfonated estrogens [56] and that their activity and expression are regulated by estrone (E1), estradiol (E2), and estriol (E3) [57–59] given the presence of an estrogen response element. However, conflicting reports complicate our understanding of which sex steroids regulate which MDRs. A recent paper by Arias et al 2014 showed that ethinyl estradiol (EE) and genisten, a phytoestrogen, upregulated MDR-1 expression but did not change BCRP expression in the colorectal cancer cell line Caco-2 [60]. Another paper by Dankers et al 2012 demonstrated that BCRP is actually inhibited by estradiol, testosterone, progesterone, and androstenedione in baculovirus-transduced HEK293 cells [44]. Yet, another paper by Mutoh et al 2006 [61] showed that both MDR-1 and BCRP in MCF7 cancer cells were downregulated by estradiol, estrone, and DES, but this down regulation was mitigated by tamoxifen. Overall these results stem from various cancer cell lines and not native tissues within the animal. However, we have shown that MDR1b responds to surges in 17β estradiol suggesting that the most prevalent hormone produced by the ovary upregulates the steady state levels of this transporter. It is possible that *MDR1b* and *BCRP* cycle in the mammalian ovary so that during ovulation there is heightened efflux of toxins during ovulation or perhaps improved export of sex steroid hormones necessary for the coordination of the reproductive tract, sperm, and oocytes. The cyclic nature of ABC effluxers could potentially be exploited to induce their expression during times of toxic exposure.

The mammalian ovary appears to depend on normal ABC effluxer functionality for normal follicular recruitment and development. Our findings show that the ovaries of the TKO mice have a higher percentage of preantral follicles (WT 24.84% vs KO 29.59% $p=0.0235$) and fewer corpora lutea (WT 22.56% vs KO 7.45% $p=.0043$), which may mirror the ovarian phenotype described in superoxide dismutase-1 knockout mice [51]. These data suggest that perhaps there is aberrant follicular maturation and decreased ovulation in combination with accelerated atresia in the ovaries of these knockout mice. This conclusion is further supported by our data which suggest that the *mdr1a/b/bcrp*^{−/−} animals spend more days on average in estrus. Although these differences were not significantly different from wild type controls, it possibly foreshadows premature ovarian insufficiency as seen in other mouse models such as *Taf4b* hypomorphs [62]. We also noticed hormone profiles indicative of ovarian reserve—such as AMH, Inhibin B, and estradiol—were lower, but not statistically different. Ovarian insufficiency may not be as pronounced here because these animals were young in age and were older animals tested, we may have seen additional defects in the physiology. Testing the age-dependent reproductive changes in these animals compared to wild-type may reveal additional reproductive differences.

Perhaps the significantly increased FSH levels are a harbinger of what is to come. Although we do not know the basis for this significantly elevated gonadotropin, we note that this TKO was selected because of the ABC family members targeted. It is, however, a constitutive TKO, not a conditional, or tissue specific TKO. Therefore, we have to broadly consider the many HPO feedback loops that figure into the hormone levels. An MDR deficiency in the

hypothalamus, or the anterior pituitary, etc, may cause an increase in FSH levels for reasons not directly related to oocyte function. We also do not see normal cycling in these tissues, and as a consequence, feedback pathways yielding FSH may be altered.

Our results explore both the native physiological role of MDRs and also their crucial function in chemoprotection. We show that loss of MDR leads to increased indication of oxidative stress and inflammation even without the presence of a toxic insult. These findings highlight the essential function of these transporters in the ovary. We have also successfully documented that MDRs contribute to normal ovarian physiology. Our results indicate that MDRs are important in follicular recruitment and development. Poor follicular development in the MDR knockouts may be indicative of ovarian insufficiency without the presence of multidrug resistance transporters. Our work also suggests that MDR-1 is responsive to estradiol which further supports the notion that ABC effluxers are regulated by the ovary and therefore have a critical and endogenous function in the female gonad.

Our future work will test how MDR expression in the ovary changes with aging with the hypothesis that decreased MDR activity over time can cause changes in metabolites that directly, or indirectly, affect the quality of the ovarian function. An improved characterization of normal transporters and their estrogen regulation may be of great value for exploitation of these ABC effluxers for drug development. The creation of an accessible and affordable ovarian protective drug is crucial for preventing ovarian insufficiency as our clinical diagnostics have greatly improved early detection and curative treatment. A medication that can be prescribed by an oncologist may lead to universal treatment as opposed to traditional oncofertility, which is cost prohibitive and requires specialized care and significant time and resources.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MDR-1 is present throughout the mouse ovary

MDR-1 is highly expressed in immature follicles compared to more developed follicles. MDR-1 appears to be abundant in the corpus luteum, but also in granulosa cells, and the oocyte. Each follicular stage is seen in the analysis primordial follicle (B, H, M) primary follicle (C, N) pre-antral follicle (D, I, O), antral follicle (E, J, P), and corpus luteum stage follicle (F, K, Q). Stain is significantly less for WT Control and the TKO samples, compared to the WT immunolabeled sections. Scale bar in A 1mm, same for G and L. The scale bar in F (as for all other panels except A, G and L) equals 100 μ m.

Figure 2. BCRP is diffusely expressed throughout the ovary in the non-luteinized granulosa cells of the follicles

Mature follicles appear to express more BCRP compared to immature follicles. Each follicular stage is labeled as follows: primordial follicle (C, H, L), primary follicle (B, C, H, I, L), pre-antral follicle (B, D, J, O), antral follicle (J), and corpus luteum stage follicle (E, G, M). Stain is significantly less for Negative control and TKO samples, compared to the WT immunolabeled sections. Note capillary label of BCRP in especially D. Scale bars: 1000 μm for A, F, and N, and 100 μm for all other panels.

Figure 3. Ovarian somatic cell sensitivity to cyclophosphamide

Whole non-fixed ovaries from wild type animals are imaged with a multiphoton microscope using the LIVE/DEAD® Cell Viability Assay. (A) Ovary from a wild type animal treated with saline (diluent for cyclophosphamide). (B) The ovary on the right is from a wild type animal treated intraperitoneally with 75 mg/kg of cyclophosphamide 24 hours before sacrifice. The green signal in the cells represents viable cells with intact esterase activity, while the red fluorescence is Ethidium Homodimer-1 (EH-1), and only appears when the cell membrane has been compromised. Many of the most sensitive (EH-1 positive) cells have a morphology consistent with granulosa cells whereas the arrows in B point to oocytes. Scale bar equals 1 mm.

Figure 4. Quantitation of cell death – viability ratios within ovaries

FVB.129P2-*Abcb1a*^{tm1Bor} *Abcb1b*^{tm1Bor} *Abcg2*^{tm1Ahs} N7 (*mdr1a/mdr1b/bcrp*^{-/-}) animals have more immediate cell death from cyclophosphamide exposure compared to wild type controls. This is evident at 24 hours (Top) in both 75 mg/kg and 150 mg/kg treatments. The sensitivity to cyclophosphamide persists through 48 hours post-treatment (Bottom), with the greatest difference at the 150 mg/kg treatment.

Figure 5. TUNEL Assay of ovaries exposed to cyclophosphamide suggest a significant cause of cell death is by apoptosis

Although only the 150 kg/mg treatment of cyclophosphamide at 48 hours was statistically significant, apoptosis appears to be a contributing mechanism of cell death in the ovarian somatic cells.

Figure 6. Ovarian follicle distribution is different when lacking MDRs
 FVB.129P2-*Abcb1a*^{tm1Bor} *Abcb1b*^{tm1Bor} *Abcg2*^{tm1Ahs} N7 (*mdr1a/mdr1b/bcrp*^{-/-}) animals have abnormal follicular development. Although no difference is seen in the number of follicles in an ovary (A), the distribution of those follicles is different when lacking the MDRs studied here (B). C–F show representative histological sections used to make follicle counts.

Figure 7. Hormone profiles show that FSH levels are distinct in FVB.129P2-*Abcb1a*^{tm1Bor}*Abcb1b*^{tm1Bor}*Abcg2*^{tm1Ahs} N7 (*mdr1a/mdr1b/bcrp*^{-/-}) animals

The TKO animals tended to have lower hormone profiles but did not show statistical significance except for FSH ($p=0.0099$). FSH, follicle stimulating hormone; AMH, anti-Mullerian hormone; P4, progesterone.

Figure 8. MDR-1 mRNA levels peak during the estrus phase of the cycle which may correlate with gonadotropins surges and peak estradiol levels
MDR-1 expression is significantly higher ($p=0.0298$) in estrus phase compared to proestrus directly after the 17 β estradiol surge.

Figure 9. Metabolic profile of FVB.129P2-*Abcb1a*^{tm1Bor} *Abcb1b*^{tm1Bor} *Abcg2*^{tm1Ahs} N7 (*mdr1a/mdr1b/bcrp*—) animals show indication of oxidative stress

A graphic representation of over-accumulation of orotate (CV=0.033 R=.982), dihydroorotate (CV=.001R=0.998) kynurenine (CV=.029 R=.979), and 3-ureidopropionate (CV=.035 R=.993). Fructose 1,6 bisphosphate, GTP (CV=.118 R=.978), and GSH (CV=.129 R=.999) are under-accumulating in the knockout ovaries.