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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.

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). 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). 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

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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 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 (75 mg/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 FVBN 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 h following injection, with prompt bilateral ovariectomies after sacrifice. For estrous cycling and hormonal testing, FVBN 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-h 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 Fig. S1 in the online version at DOI: 10.1016/j.reprotox.2017.02.002) 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 diaminobenzedine (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 μm thick sections were stained with hematoxylin and eosin. Using an EVOS® FL Auto