Advances in Brief

The Multidrug Resistance Transporter ABCG2 (Breast Cancer Resistance Protein 1) Effluxes Hoechst 33342 and Is Overexpressed in Hematopoietic Stem Cells

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Abstract

The human ATP-binding cassette superfamily G (White) member 2 (*ABCG2*) gene and its murine homologue *breast cancer resistance protein 1* (*Bcrp1*) are recently described ATP-binding cassette transporters associated with drug resistance in tumor cell lines, including the MCF-7 cell line, selected for its resistance to mitoxantrone (MCF-7/MitoR). Infection of MCF-7 cells with the retroviral vector containing ABCG2 cDNA (G1-ABCG2) resulted in cells (MCF-7/ABCG2) that were resistant to mitoxantrone at levels similar to those observed in MCF-7/MitoR cells. Previous studies have shown that pluripotent hematopoietic stem cells overexpress the *multidrug-resistant transport* (*MDR1*) gene and efflux rhodamine, a substrate for the *MDR1* transporter. Other studies have identified a primitive hematopoietic stem cell population, or side population (SP) cells, which are identified by their efflux of the fluorescent dye, Hoechst 33342. In an attempt to identify the transport genes responsible for this phenotype, we examined the uptake of Hoechst 33342 into MCF-7, MCF-7/MitoR, and MCF-7 cells infected with a retroviral vector expressing the *ABCG2* gene (MCF-7/ABCG2). MCF-7/MitoR cells as well as MCF-7/ABCG2 cells demonstrated lower levels of Hoechst 33342 uptake compared with the parental MCF-7 cells. We also examined the level of the mouse Bcrp1 RNA in SP cells and non-SP cells isolated from mouse hematopoietic cells. Mouse SP cells expressed relatively high levels of Bcrp1 mRNA relative to non-SP cells. These results suggest that Hoechst 33342 is a substrate for the ABCG2 transporter and that ABCG2/Bcrp1 expression may serve as a marker for hematopoietic stem cells in hematopoietic cells.

Introduction

The emergence of drug-resistant tumor cells remains a major problem in cancer chemotherapy. The development of multidrug resistance is often characterized by reduced intracellular levels of many diverse classes of cytotoxic drugs and is frequently associated with overexpression of one or more members of the ABC[2] superfamily of membrane transporters (1). These transporters include P-glycoprotein, encoded by the *MDR1* gene (2), and the multidrug resistance-associated protein (*MRP*) gene (3). Another MDR gene has been described recently and referred to as the *BCRP* gene. The *BCRP* gene is an ABC half-transporter that is associated with resistance to mitoxantrone, anthracyclines, and camptothecins (4–6). *BCRP* is identical to *MXR* (5) and to the placental *ABCP1* gene (7). The Human Gene Nomenclature Committee has suggested recently that the *MXR/BCRP/ABCP1* gene be renamed *ABCG2* (8).

HSCs have been the object of considerable interest for decades because of their central role in the production of all hematopoietic cell lineages. Additionally, HSCs are a sensitive target of most chemotherapeutic agents (9). The isolation of HSCs from a number of species, including humans, has been facilitated by exploiting the phenomenon that these cells actively efflux fluorescent dyes such as rhodamine 123 (10). Previous studies have found that the *MDR1* gene product (P-glycoprotein) can efflux rhodamine 123 and is overexpressed in CD34+, rhodamine 123 dull HSC populations (10). Recently, another fluorescent dye, Hoechst 33342, has been used to identify a smaller stem cell population referred to as SP cells (11). The gene responsible for Hoechst 33342 transport has not yet been identified.

In this report, we describe the construction of a retroviral vector expressing the *ABCG2* gene (G1-ABCG2). MCF-7 cells infected with this retroviral vector (G1-ABCG2) expressed high levels of ABCG2 protein and developed resistance to mitoxantrone. We also found that MCF-7/MitoR cells as well as ABCG2-transduced MCF-7 cells (MCF-7/ABCG2) contained lower levels of Hoechst 33342 dye compared with wild-type MCF-7 cells. Furthermore, Hoechst 33342 dull hematopoietic

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[2] The abbreviations used are: ABC, ATP-binding cassette; MDR, multidrug resistance; BCRP, breast cancer resistance protein; ABCG2, ATP-binding cassette superfamily G (White) member 2; MXR, mitoxantrone resistance protein; ABCP, ABC protein; HSC, hematopoietic stem cell; MCF-7/MitoR, mitoxantrone-resistant MCF-7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; SP, side population; NSP, non-SP; RT-PCR, reverse transcription-PCR.

SP stem cells isolated from mouse bone marrow cells expressed high levels of ABCG2 RNA. These studies suggest that the expression of the *ABCG2* transport gene may be a useful marker for pluripotent HSCs.

Materials and Methods

Cell Lines. MCF-7 human breast cancer cells (ATCC HTB-22), mitoxantrone-resistant MCF-7 cells (MCF-7/MitoR), and the amphotropic retroviral packaging cell line PA317 were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with penicillin (100 units/ml), streptomycin (100 units/ml), and 10% FBS. Cultures were maintained in a humidified incubator at 37°C in 5% CO_2-95% air.

Vector Construction, Retroviral Producer Clone Isolation, and Transduction. Wild-type ABCG2 cDNA was inserted into the Moloney murine retroviral backbone pG1 (Genetic Therapy, Inc., Gaithersburg, MD) digested with *Xho*I and *Hin*dIII to yield G1-ABCG2 (12, 13). This vector was introduced by calcium phosphate transfection into the amphotropic packaging line PA317, and 10 clones were isolated after exposure to 200 nM mitoxantrone. To titrate the retrovirus produced, each clone was grown to 80% confluency. The medium was changed, and 24 h later, the fresh medium was collected. NIH 3T3 cells were plated (2.5×10^4 cells) in 30-mm plates in 3 ml of medium (DMEM) containing 10% FBS and Polybrene at 4 μg/ml. Virus dilutions were made in the same medium, and 0.5 ml was added to each plate. After incubation for 48 h, the medium was removed, and fresh growth medium containing 200 nM mitoxantrone was added. After 2 weeks of incubation, the medium was removed, and the colonies were stained with 0.5% methylene blue dissolved in 50% (v/v) methanol. G1-ABCG2 producer clones designated PD2 (2.2×10^5 colony-forming units/ml) and PD3 (2.3×10^5 colony-forming units/ml) were isolated and used for subsequent experiments.

Cytotoxicity Assay. Cytotoxicity was assessed using colorimetric MTT assay (14). Cells (1500/well) were plated in 96-well plates and incubated overnight. Cells were then exposed to different concentrations of mitoxantrone and incubated for 7 days at 37°C. MTT (50 μl of 2 mg/ml) in 1× PBS was added to each well, and the cells were incubated for 4 h at 37°C. The plates were then centrifuged at $500 \times g$ for 10 min, and DMSO (120 μl) was added to each well and incubated 1 h on an orbital shaker. The $A_{570\,nm}$ was determined using an Ultra-microplate reader (ELx 808; Bio-Tek Instruments, Winooski, VT). Cytotoxicity was expressed as the percentage of the $A_{570\,nm}$ of treated cells relative to untreated cells.

Preparation of Cells for FACS and Fluorescence Microscopic Analysis. Cells were grown in 10-cm culture dishes and washed two times with PBS. Hoechst 33342 (4 μg/ml) was added directly to each culture dish, and the cells were incubated for 60 min at 37°C for FACS and fluorescence microscopic analysis. Murine bone marrow was extracted from the femurs and tibias of BALB/c mice, and a single-cell suspension was made by gentle passage of the bone marrow through an 18-gauge needle. The cells were pelleted by centrifugation, washed, resuspended at 10^6 cells/ml in 37°C DMEM containing 2% FBS, 1 mM HEPES, and 4 μg/ml Hoechst 33342 (Sigma Chemical Co., St. Louis, MO), and incubated for 90 min at 37°C. After Hoechst staining, cells were pelleted and maintained at 4°C before FACS analysis (Becton Dickinson & Co., Mountain View, CA).

Flow Cytometry and Fluorescence Microscopic Analysis. Analysis and cell sorting were performed using a dual-laser FACStar PLUS fluorescence-activated cell sorter (Becton Dickinson). The Hoechst 33342 dye was excited at 350 nm, and its fluorescence was measured at two wavelengths using a 450 BP 20 and a 675 EFLP optical filter (Omega Optical, Brattleboro, VT). Hoechst "blue" represents the 450 BP filter, the standard analysis wavelength for Hoechst 33342 analysis for DNA content. Hoechst "red" was detected at 675 nm. A live gate was defined on the flow cytometer using Hoechst red and blue axes to exclude dead cells (Hoechst red, very bright), red cells (no Hoechst stain), and debris. After collecting 10^5 events within the live gates, the SP was clearly defined. SP and NSP cells were sorted into sterile Eppendorf tubes containing 100% FBS. For fluorescence microscopy, cells were stained with Hoechst directly in dishes and photographed (excitation, 350 nm; emission, 450 nm).

PCR Analysis. RNA was prepared according to the manufacturer's instructions (Qiagen, Inc., Santa Clarita, CA) from 10,000 SP and NSP cells. RT-PCR analyses were performed as described previously (15), using ABCG2 5′ end primer ^{1345}5′-GTC AGC TGT GGA GCT GTT CGT AG^{1367} and 3′ end primer ^{1829}5′-CAC AAG TGC TGT TGT CCG TTA CA^{1807}.

Nested ABCG2 PCR used primers located within the first amplified PCR fragment spanning nucleotides 1551–1726 (ABCG2 nested 5′ end primer, ^{1551}5′-TTA CCC TTA TAA TGG TGG CTT ATA CGG^{1577}; 3′ end primer, ^{1762}5′-CAA AGC TGT GAA GCC ATA TCG AG^{1739}). The PCR conditions for the nested ABCG2 reaction were 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension of 7 min at 72°C.

The PCR for actin was performed as a control using 5′ end primer ^{305}5′-TGT TAC CAA CTG GGA CGA CA^{324} and 3′ end primer ^{858}5′-AAG GAA GGC TGG AAA AAG AGC^{837}. Actin PCR used the same conditions as the initial ABCG2 PCR reaction.

Western Blot Analysis. Whole cell lysates were prepared and subjected to Western blot analysis as described previously (16). Blots were probed with polyclonal anti-ABCG2 (dilution 1:2000; kindly provided by Dr. Susan Bates, National Cancer Institute, Bethesda, MD). A secondary horseradish peroxidase-conjugated antirabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was applied for 1 h (dilution 1:1000), followed by enhanced chemiluminescence detection (Pierce, Rockford, IL) and exposure on Kodak X-OMAT AR autoradiography film to visualize immunoreactive bands.

Immunofluorescent Staining. To determine the subcellular localization of ABCG2, cells were plated at a density of 50,000 cells/35-mm dish on coverslips, grown for 48–72 h to 70% confluence, and then rinsed in PBS, followed by fixation for 5 min in PBS containing 3.8% paraformaldehyde. SP and NSP mouse stem cells were collected by cytospin and fixed. The fixed cells were permeabilized by incubation for 5 min in cold methanol (−20°C). Coverslips were incubated for 45 min at 37°C with the polyclonal anti-ABCG2 antibody (dilution 1:800). Unbound antibodies were removed by rinsing in PBS, followed by incubation for 45 min at 37°C with FITC-conju-

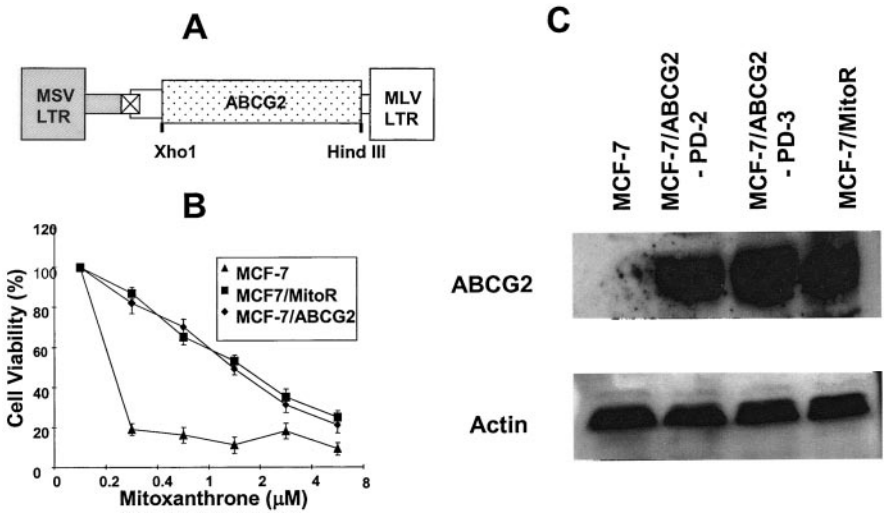

Fig. 1 *A,* MCF-7 cells were transduced with a retroviral vector containing the ABCG2 cDNA (G1-ABCG2), and transduced cells (MCF-7/ABCG2) were isolated as described in "Materials and Methods." *MSV,* Murine Sarcoma Virus; *MLV,* murine leukemia virus; *LTR,* long terminal repeat. *B,* sensitivity to mitoxantrone of wild-type MCF-7, MCF-7/ABCG2, and MCF-7/MitoR cells were determined by MTT assay as described in "Materials and Methods." *Bars,* SD. *C,* Western blot analysis was performed as described in "Materials and Methods" using anti-ABCG2 and anti-actin antibodies.

gated antirabbit antibody as a secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Fluorescence images were collected using a wide-field fluorescence microscope.

Results

Retroviral Transfection of *ABCG2* Gene into MCF-7 Cells. To study the function of the *ABCG2* gene product, wild-type MCF-7 cells were transduced with a retroviral vector expressing ABCG2 (G1-ABCG2; Fig. 1*A*). After incubation with G1-ABCG2 retroviral supernatants, wild-type MCF-7 cells were incubated in the presence of 200 nM mitoxantrone for 2 weeks, and the surviving cells were pooled (MCF-7/ABCG2). For comparison, wild-type MCF-7 cells not exposed to retroviral supernatant were grown in parallel in 200 nM mitoxantrone, and after 2 weeks no surviving cells were present.

As shown in Fig. 1*B*, MCF-7/ABCG2 cells were markedly resistant to mitoxantrone compared with parental MCF-7 cells. The level of mitoxantrone resistance of G1-ABCG2-transduced MCF-7 cells was comparable with that of MCF-7/MitoR cells, which were described previously (17), and were obtained by serial passage of MCF-7 cells in increasing concentrations of mitoxantrone for multiple passages.

Western blot analysis was performed to evaluate ABCG2 protein levels in two clones of retrovirally transduced MCF-7 cells (MCF-7/ABCG2-PD2 and -PD3). As shown in Fig. 1*C*, two clones of G1-ABCG2- transduced MCF-7 cells demonstrated high levels of ABCG2 protein comparable with that observed in MCF-7/MitoR. In contrast, wild-type MCF-7 cells have undetectable ABCG2 protein levels.

Hoechst 33342 Accumulation in MCF-7 Cells. As noted earlier, incubation of murine bone marrow cells with Hoechst 33342 identifies several distinct cell populations including SP cells, which demonstrate dull staining with Hoechst 33342 (11). Subsequent studies have demonstrated that SP cells obtained from nonirradiated mice can protect recipient mice from lethal irradiation and that SP cells are capable of regenerating both lymphoid and myeloid cell lineages in lethally irradiated mice (11). Although the *MDR1* multidrug resistance drug transport gene is overexpressed in CD34+, rhodamine 123 dull HSCs (18), other studies have suggested that other transporters may be implicated in the transport of Hoechst 33342 dye from SP cells (19).

To examine the possible role of *ABCG2* gene expression in cellular accumulation of Hoechst 33342, wild-type and MCF-7/MitoR cells were incubated with Hoechst 33342, and cellular uptake of dye was evaluated by FACS analysis. As shown in Fig. 2, Hoechst 33342 uptake is markedly reduced in MCF-7/MitoR cells, which overexpress *ABCG2* gene compared with wild-type MCF-7 cells. Fig. 2*B* shows Hoechst 33342 uptake was also markedly reduced in two clones of PA317 packaging cells (PD2 + PD3) transduced with the G1-ABCG2 retroviral expression vector compared with the control nontransduced PA317 cells. Hoechst 33342 uptake was also assessed in wild-type MCF-7 breast cancer cells as well as MCF-7 cells infected with retroviral supernatants obtained from ABCG2 packaging cell lines. As shown in Fig. 2*A*, Hoechst 33342 uptake is greater in MCF-7 cells compared with ABCG2-transduced MCF-7 cells (MCF-7/ABCG2). As shown in Fig. 2*B*, the FACS profile of ABCG2-transduced cells reveals an increase in the proportion of cells that exclude Hoechst 33342 (R1). Whereas 1.8% of the proportion of parental MCH-7 cells preferentially excluded Hoechst 33342, this increased to 8.0% after incubation of a retroviral vector expressing ABCG2 (MCF-7/ABCG2). Fluorescence microscopy (Fig. 3) also revealed that Hoechst 33342 fluorescence was greater in wild-type MCF-7 cells compared with MCF-7/MitoR cells or ABCG2-transduced MCF-7 cells (MCF-7/ABCG2). Similar results were observed in G1-ABCG2 retrovirus-producing cell lines compared with the parental PA317 packaging cell line (Fig. 3). These results strongly support the role of ABCG2 transporter in the intracellular accumulation of Hoechst 33342.

Isolation of SP Cells and Detection of ABCG2 Transporter. Because the studies presented above indicate a role for the ABCG2 transporter in intracellular Hoechst 33342 accumulation, we next examined ABCG2 expression in murine bone marrow hematopoietic cells that were incubated with Hoechst

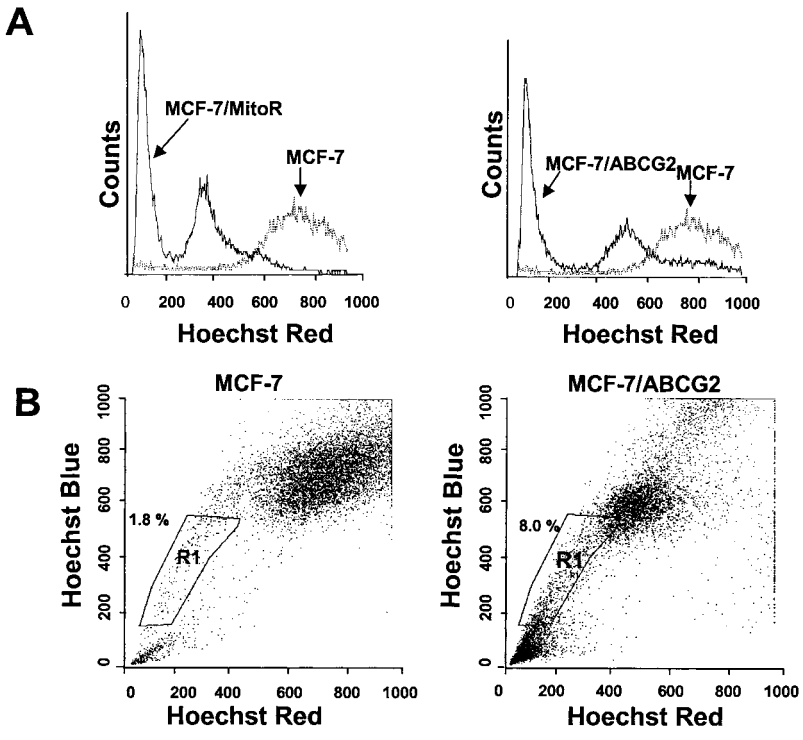

Fig. 2 FACS analysis was performed after incubation of MCF-7/MitoR cells, wild-type MCF-7 cells, and ABCG2-transduced MCF-7 cells (*A*) after incubation with 4 μg/ml Hoechst 33342 for 45 min as described in "Materials and Methods." *B*, Hoechst 33342 staining on MCF-7 and MCF-7/ABCG2 cells. *R1*, Hoechst staining-resistant area.

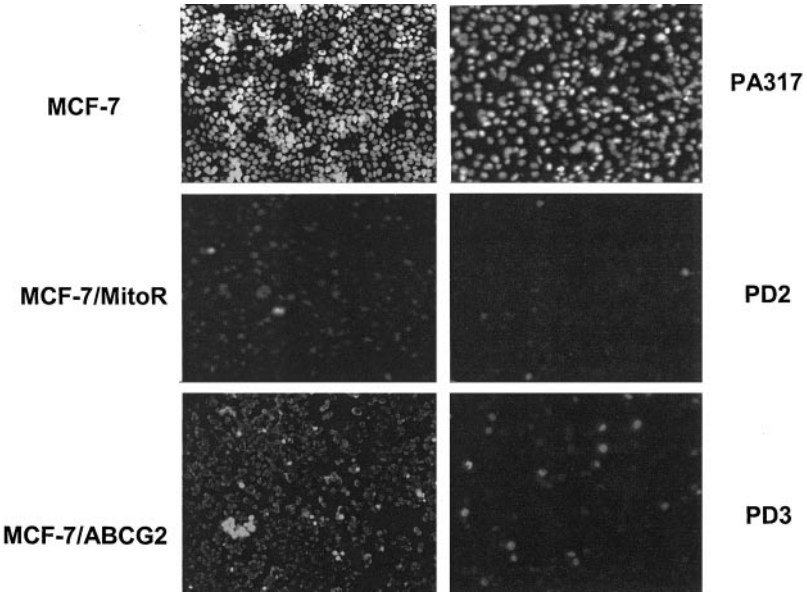

Fig. 3 Fluorescence microscopic analysis of Hoechst 33342 staining of cell lines was performed as described in "Materials and Methods." Cells were examined by fluorescence microscopy (excitation, 350 nm; emission, 450 nm).

33342 and sorted into a distinct population of SP cells (0.1%) and non-SP cells (Fig. 4*A*). ABCG2 RNA was examined in SP cells and non-SP cells using a sensitive RT-PCR assay. As shown in Fig. 4*B*, SP cell populations isolated from two different murine bone marrows (SP1 and SP2) showed higher levels of ABCG2 RNA compared with those detected in NSP cells (NSP1 and NSP2). As a control, actin RNA levels were equal in all samples.

As shown in Fig. 5, immunohistochemistry using a polyclonal rabbit antibody directly against ABCG2 shows that ABCG2-transduced cells (MCF-7/ABCG2) stain positive for ABCG2 protein compared with the nontransduced parental MCF-7 cell line. Similarly, we have found that murine bone marrow SP cells stain positive for ABCG2 expression compared with NSP cells (Fig. 5).

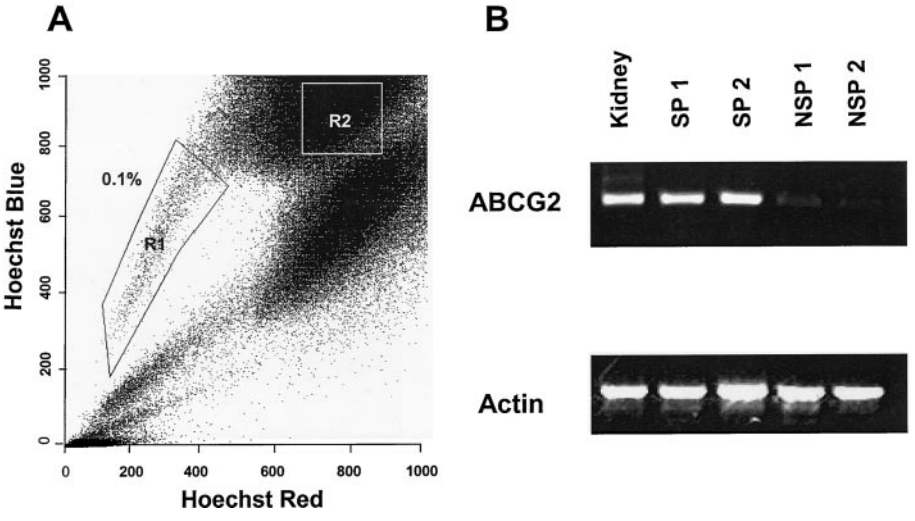

Fig. 4 HSCs were collected from a BALB/c mouse and separated into SP and NSP populations after incubation with Hoechst 33342 for 90 min as described in "Materials and Methods." RNA was obtained from SP cells, NSP cells, and mouse kidney tissue, and ABCG2 RNA levels were determined by nested RT-PCR as described in "Materials and Methods." As a control, the amounts of actin RNA were equal in each of the samples.

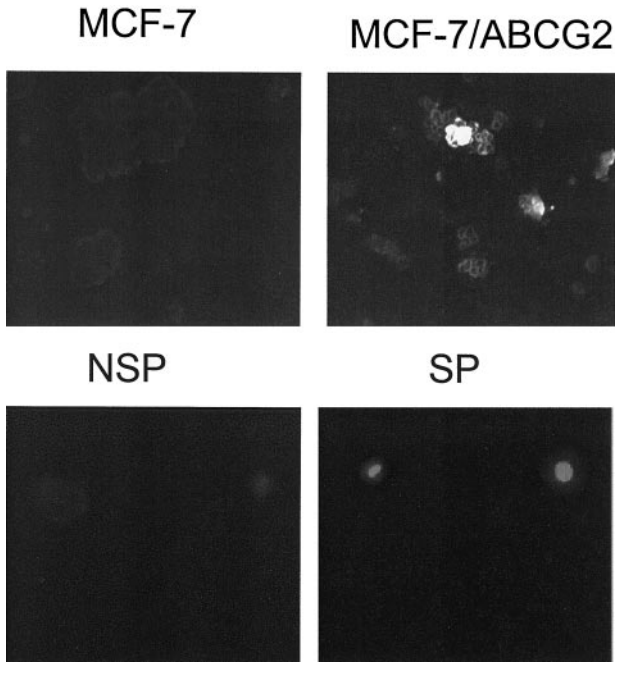

Fig. 5 Immunofluorescent staining of MCF-7, MCF-7/ABCG2, murine NSP, and murine SP cells was performed as described in "Materials and Methods" using anti-ABCG2 and antirabbit FITC as secondary antibody (excitation, 494 nm; emission, 519 nm).

Discussion

HSCs are pluripotent cells capable of regenerating all hematopoietic cell lineages. The isolation of HSCs from a number of species, including mouse and human, has been facilitated by the observation that these cells actively efflux fluorescent dyes such as rhodamine 123. Other studies have shown that rhodamine 123 is transported by the multidrug resistance *MDR1* gene product (20) and that decreased rhodamine 123 accumulation observed in both human CD34+ HSCs (20) and murine c-kit$^+$ stem cells (12) was associated with increased MDR1 expression in these cells.

Over the past several years, several other members of the *ABC* gene superfamily have been implicated in the development of MDR including the *MRP* gene family and, more recently, the *MXR/BCRP/ABCP1* gene now referred to as the *ABCG2* gene (5, 8). *ABCG2* is expressed in prokaryotic and eukaryotic organisms, and increased *ABCG2* gene expression was identified previously in human placenta (8) and in several cell lines selected for resistance to chemotherapeutic agents, including mitoxantrone, anthracyclines, and camptothecins (4–6, 21). The *ABCG2* gene product is presumed to function as an ATP-dependent membrane transporter, translocating molecules across a cell membrane against a concentration gradient (22). The role of this gene in drug resistance was confirmed by gene transfection studies that demonstrated that *ABCG2* gene overexpression is associated with enhanced drug efflux and development of drug resistance (5, 23). These studies also noted that *ABCG2* gene-transfected cells displayed decreased rhodamine 123 uptake (23), suggesting the possibility that this gene, similar to *MDR1*, may also be expressed in HSCs.

Another fluorescent dye, Hoechst 33342, has been used to identify a relatively small fraction of bone marrow and peripheral blood cell populations referred to as SP cells (11). The SP phenotype, characterized by reduced Hoechst 33342 staining, identifies a subset of stem cells in multiple mammalian species (19). Of interest are studies demonstrating that transfection of the *MDR1* gene into murine bone marrow cells resulted in an expansion of Hoechst 33342 dull SP cells (18), suggesting an important role for *MDR1* gene expression in the maintenance of the SP phenotype. These studies also suggested the possibility that the *MDR1* gene product was involved in Hoechst 33342 transport. However, Hoechst 33342 transport studies using verapamil, a potent inhibitor of the *MDR1* transporter, have indicated that Hoechst transport involves gene products (19) other than the *MDR1* gene product (P-glycoprotein).

In this study, we have shown that MCF-7/MitoR cells, which overexpress the *ABCG2* gene, also have reduced Hoechst 33342 uptake, suggesting a role for the *ABCG2* gene in Hoechst

33342 transport. To address this possibility, a retroviral vector expressing ABCG2 (G1-ABCG2) was constructed. G1-ABCG2 infection of MCF-7 cells resulted in marked overexpression of ABCG2 protein and development of resistance to mitoxantrone. This confirms previous studies that overexpression of ABCG2 in cells is sufficient to cause drug resistance. Furthermore, PA317 packaging cells producing the G1-ABCG2 retroviral vector as well as MCF-7 cells infected with G1-ABCG2 vector developed reduced Hoechst 33342 uptake compared with control cells. Thus, expression of the *ABCG2* gene confers resistance to mitoxantrone as well as resistance to Hoechst 33432 uptake. Finally, we isolated SP cells from murine bone marrow and showed by RT-PCR that these cells have increased expression of ABCG2 RNA relative to non-SP cells.

Recent studies by Zhou *et al.* (24) have also indicated that *ABCG2* is expressed in SP stem cells isolated from bone marrow cells. In this report, Zhou *et al.* also found that *ABCG2* overexpression is a conserved feature of SP cells isolated from several tissues including skeletal muscle and liver. Furthermore, these workers found that transduction of bone marrow cells with a vector expressing *ABCG2* gene, followed by passage in culture for an additional 12 days, resulted in an increase in the SP population from 0.05% to 62.5%. Thus, both studies indicate that *ABCG2* expression results in diminished intracellular accumulation of Hoechst 33342 and increased SP phenotype.

SP cells isolated from a variety of tissues using differential Hoechst staining have been found to have features of pluripotent stem cells. Thus, the finding that the *ABCG2* gene can result in decreased Hoechst 33342 accumulation suggests that increased *ABCG2* expression may be a common feature of pluripotent stem cells. The function of the *ABCG2* gene in stem cells is unclear. Because ABC transporters are known to transport a variety of toxic lipophilic compounds, the expression of *ABCG2* and other ABC transport genes may protect stem cells from cytotoxic agents. It is also possible that high expression of ABC transporters, as suggested by Bunting *et al.* (18), may be critical to maintaining a quiescent state in stem cell populations. Perhaps expression of transporters similar to the *ABCG2* gene may be necessary for stem cells to regulate the uptake of small hydrophobic signaling molecules involved in cellular differentiation. In summary, these studies indicate that the *ABCG2* gene product is involved in regulating Hoechst 33342 accumulation in cells including pluripotent hematopoietic SP stem cells. Additional studies are needed to determine the function of *ABCG2* gene expression in hematopoietic and other pluripotent stem cell populations.

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