Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness

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The recent identification of “side population” (SP) cells in a number of unrelated human cancers and their normal tissue sources has renewed interest in the hypothesis that cancers may arise from somatic stem/progenitor cells. The high incidence of recurrence attributable to multidrug resistance and the multiple histologic subtypes of Mullerian origin (serous, mucinous, and endometrioid) affects >22,000 women in North America per year, and accounts for >16,000 deaths per year with a projected 5 year mortality rate exceeding 70% (14). Aggressive surgical cytoreduction followed by chemotherapy results in complete clinical response in 50–80% of patients with stage III and IV disease. However, the majority of patients will relapse and become drug-resistant (14–16). Various types of membrane-spanning ATP-binding cassette transporters, such as the multidrug-resistant gene 1 and breast cancer-resistance protein 1 (BCRP1), contribute to the drug resistance of many cancers, including ovarian cancer, by pumping lipophilic drugs out of the cell (17). Within bone marrow, researchers have defined a subset of verapamil-sensitive BCRP1-expressing cells with the ability to efflux the lipophilic dye Hoechst 33342. This subset has been described as the SP (18). The functional and phenotypic characteristics of ovarian cancer predict a stem cell etiology, and the availability of tumor cells in ascites permits their study by flow cytometry.

Here we show that distinct histologic types of genetically engineered mouse ovarian cancer cells (MOVCAR 7 and 4306) have a proportionately large SP, making them a model to study ovarian cancer stem cell biology. A similar, albeit very small, SP was also identified in human ovarian cancer cell lines (IGROV-1, SK-OV3, and OVCAR-3) and in patient primary ascites cells. We used the MOVCAR 7 cell line to demonstrate that SP cells can reconstitute colonies in vitro, form tumors earlier than NSP cells in vivo, and remain responsive to Mullerian Inhibiting Substance (MIS). Our findings suggest that the SP phenotype may be a marker for ovarian cancer stem cells and that one of the advantages of MIS may be its ability to inhibit proliferation of both stem and nonstem cancer cells as compared with the lipophilic chemotherapeutic doxorubicin, which more effectively inhibited the NSP. These findings, if corroborated in further studies of human specimens, may provide an explanation for the ability of transporter substrates such as anthracyclines to cytoreduce but essentially never cure recurrent ovarian cancer. More importantly, identification of the ovarian cancer stem

cancer stem cells | breast cancer-resistance protein 1

Recentiely, two human primary cancers, leukemia and breast, and several human cancer cell lines, such as central nervous system, gastrointestinal tumors, and retinoblastoma, were shown to possess "side population" (SP) cells that have been described as cancer stem cells (1–5). Cancer stem cells, like somatic stem cells, are thought to be capable of unlimited self-renewal and proliferation. Multipotent cancer stem cells may explain the histologic heterogeneity often found in tumors (6–9). In addition, cancer progression and metastasis may involve tumor stem cell escape from innate somatic niche regulators. Quiescent somatic stem cells residing in specific tissue niches until activation by injury or other stimuli have been described in skin and hair follicles, mammary glands, intestines, and other organs (10). The evolving evidence that somatic stem cells contribute to normal tissue repair and regeneration suggests the potential for multipotent somatic stem cells in the ovary responsible for regulated surface epithelial repair after ovulatory rupture and possibly the generation of oocyte nurse cells for folliculogenesis (11). Ovarian somatic stem cells would be expected to divide asymmetrically, yielding both a daughter cell that proceeds to terminal differentiation for epithelial repair and an undifferentiated self-copy. Repeated asymmetric self-renewal sets the stage for somatic stem cells or their immediate progenitors to accrue mutations over time, which might ultimately lead to their transformation into cancer stem cells and malignant progression.

Epithelial ovarian cancer, thought to emanate from the surface epithelium of the ovary (12, 13), consists of various histologic subtypes of Mullerian origin (serous, mucinous, and endometrioid), affects >22,000 women in North America per year, and accounts for >16,000 deaths per year with a projected 5 year mortality rate exceeding 70% (14). Aggressive surgical cytoreduction followed by chemotherapy results in complete clinical response in 50–80% of patients with stage III and IV disease. However, the majority of patients will relapse and become drug-resistant (14–16). Various types of membrane-spanning ATP-binding cassette transporters, such as the multidrug-resistant gene 1 and breast cancer-resistance protein 1 (BCRP1), contribute to the drug resistance of many cancers, including ovarian cancer, by pumping lipophilic drugs out of the cell (17). Within bone marrow, researchers have defined a subset of verapamil-sensitive BCRP1-expressing cells with the ability to efflux the lipophilic dye Hoechst 33342. This subset has been described as the SP (18). The functional and phenotypic characteristics of ovarian cancer predict a stem cell etiology, and the availability of tumor cells in ascites permits their study by flow cytometry.

Here we show that distinct histologic types of genetically engineered mouse ovarian cancer cells (MOVCAR 7 and 4306) have a proportionately large SP, making them a model to study ovarian cancer stem cell biology. A similar, albeit very small, SP was also identified in human ovarian cancer cell lines (IGROV-1, SK-OV3, and OVCAR-3) and in patient primary ascites cells. We used the MOVCAR 7 cell line to demonstrate that SP cells can reconstitute colonies in vitro, form tumors earlier than NSP cells in vivo, and remain responsive to Mullerian Inhibiting Substance (MIS). Our findings suggest that the SP phenotype may be a marker for ovarian cancer stem cells and that one of the advantages of MIS may be its ability to inhibit proliferation of both stem and nonstem cancer cells as compared with the lipophilic chemotherapeutic doxorubicin, which more effectively inhibited the NSP. These findings, if corroborated in further studies of human specimens, may provide an explanation for the ability of transporter substrates such as anthracyclines to cytoreduce but essentially never cure recurrent ovarian cancer. More importantly, identification of the ovarian cancer stem

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Abbreviations: BCRP1, human breast cancer-resistance protein 1; Bcrp1, mouse breast cancer-resistance protein 1; MIS, Mullerian Inhibiting Substance; MDR1, MIS type II receptor; MTT, methythiazolletetrazolium; NSP, non-SP; SP, side population.

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Identification of SPs in Mouse Ovarian Cancer Cell Lines. To determine whether mouse ovarian cancer cell lines contain candidate cancer stem cells, Hoechst 33342 was used to sort for the SP phenotype. The serous adenocarcinoma-recapitulating MOVCAR 7 and 8 cell lines were developed by using the MIS type II receptor (MISRII) promoter to drive the SV40 T antigen (19). The endometrioid carcinoma-recapitulating 4306 cell line was developed from conditional LSL-K-rasG12D+/Ptenflpflp mice in which the ovarian surface epithelium was infected with adenovirus expressing Cre recombinase (20). Flow cytometry demonstrated a very high percentage of Hoechst\(^{\text{low}}\) SP cells in the MOVCAR 7 and 4306 cell lines (Fig. 1 \(A\) and \(B\)), whereas SP was not detected in MOVCAR 8. Verapamil, a BCRP1 inhibitor (18), effectively eliminated the SP in both MOVCAR 7 and 4306 cells (Fig. 1 \(C\) and \(D\)). The average first sort percentage of SP cells was 6.28\% \((n=6)\) for MOVCAR 7 and 1.83\% \((n=4)\) for 4306 cells, which is elevated relative to the SP found in other somatic and malignant sources (3, 18, 21). Colocalization of Hoechst\(^{\text{low}}\) and Bcrp1 immunoreactive MOVCAR 7 and 4306 cells confirmed the presence of SP cells (Fig. 1 \(E-J\)). Bcrp1 mRNA was detected by qualitative RT-PCR in SP cells (data not shown). Thus, MOVCAR 7 and 4306 cells possess SPs with Hoechst efflux characteristics reminiscent of those defined in hematopoietic stem cells.

Ex Vivo Growth of SP and NSP Cells. Growth characteristics of the SP and NSP cells were consistent with previous findings for cancer stem cells (21). MOVCAR 7 and 4306 cells were sorted by flow cytometry and equal numbers of SP and NSP cells cultured. SP cells from both cell lines formed characteristic compact circular colonies with a cobblestone appearance and survived numerous passages (Fig. 2 \(A\) and \(C\); \(n=9\)). NSP cells from both cell lines were sparse and failed to proliferate beyond 1–2 weeks (Fig. 2 \(B\) and \(D\); \(n=9\)). These differences were not a consequence of prolonged Hoechst retention in the NSP cells because propidium iodide was used to gate out all nonviable cells. Serial sorting and reanalysis (total passages \(3\)) of SP cells demonstrated enrichment of the SP and the presence of NSP cells (Fig. 2 \(E-J\)). Bcrp1 mRNA was detected by qualitative RT-PCR in SP cells when recovered and serially sorted in culture.

SP Cells Are in G1 Cell Cycle Arrest and Resistant to Doxorubicin in Vitro. By definition, SP cells should express high levels of BCRP1 and thus be able to efflux the lipophilic dye Hoechst 33342 and some lipophilic anticancer drugs, including those used in the treatment of ovarian cancer (18, 22). The lipophilic anticancer drug doxorubicin is a substrate of the BCRP1 transporter, whereas the
lipophilic microtubule inhibitor paclitaxel is not (23). To investigate the functional significance of the Bcrp1 transporter found in MOVCAR 7 SP cells, we tested their response to doxorubicin and paclitaxel, as compared with that observed in the NSP, by methylthiazol tetrazolium (MTT) proliferation assays (Fig. 3). MOVCAR 7 SP cells demonstrated decreased inhibition by doxorubicin and G1 cell cycle arrest. MOVCAR 7 cells were sorted for in vitro growth-inhibition analysis against doxorubicin and paclitaxel. SP cells showed 30% inhibition (A) by doxorubicin (*, P < 4.2 \times 10^{-4}) and 85% inhibition (B) by paclitaxel (*, P < 6.7 \times 10^{-10}) compared with vehicle-treated controls. NSP cells were inhibited by doxorubicin and paclitaxel by 81% and 88% versus vehicle-treated controls (**, P < 3.2 \times 10^{-11} (A); **, P < 5.1 \times 10^{-10} (B)). (A) NSP cells were significantly more inhibited by doxorubicin than by SP cells (81% versus 30% growth inhibition; **, P < 1.6 \times 10^{-5}). Cell cycle analysis of three populations was performed as shown in C. HoechstHigh NSP and HoechstMid NSP cells (D and E) demonstrate a predominance of S phase, 69.3% (average = 45.3%) and 68.9% (average = 51.5%), respectively, and decreased G1-arrested cells, 23% (average = 53%) and 15.9% (average = 39%), compared with HoechstLow NSP cells (P < 0.0047 (F)). HoechstLow SP cells demonstrate a predominance of G1-arrested cells, 63% (average = 65.8%), and decreased S phase replicating cells, 33.4% (30.57%). All experiments were performed in triplicate.

Quiescence is one of the defining characteristics of somatic stem cells (24). Cell cycle analysis of three sorted populations, HoechstHigh SP, HoechstMid NSP, and HoechstHigh NSP (Fig. 3C), revealed that the HoechstMid and HoechstHigh NSP cells had a higher percentage of cells in S phase (Fig. 3D and E), compared with HoechstLow SP (Fig. 3F). In contrast, HoechstLow SP cells demonstrate a predominance of cells in the G1 phase (Fig. 3F) compared with the HoechstMid and HoechstHigh NSP cells (Fig. 3D).

In Vivo Growth Characteristics of MOVCAR 7 SP and NSP Cells. To assess in vivo tumorigenicity of MOVCAR 7 SPs and NSPs, viable propidium iodide-negative SP and NSP cells were sorted and injected into the dorsal fat pad of nude mice (Fig. 4A and Table 1, which is published as supporting information on the PNAS website). Tumors appeared in three of three animals at 10 weeks after injection of 5.0 \times 10^5 SP cells, whereas animals injected with an equal number of NSP cells had no detectable tumors (zero of three) at that time (Fig. 4B). Tumors appeared in two of three of the NSP animals only after 14 weeks. Tumors appeared at 7 weeks in animals injected with 7.5 \times 10^5 SP cells, whereas NSP-injected animals had no detectable tumors (zero of three) at that time and only appeared after 10 weeks in two of three animals (Table 1). To investigate whether the appearance of tumors in the NSP could possibly be explained by incomplete sorting, we reanalyzed the sorted populations by using identical gating and found 82.6% SP cell purity (Fig. 4C; NSP contamination = 2.63% or \sim 13,150 SP cells in a total of 5 \times 10^5 cells per animal; 19,750 SP cells in a total of 7.5 \times 10^5 cells per animal) and 92.3% NSP cell purity (Fig. 4D; SP contamination = 1.72% or \approx 8,600 SP cells in 5 \times 10^5 cells per animal; 12,900 SP cells in 7.5 \times 10^5 cells per animal). In addition, the NSP tumors dissected from animals at euthanization showed verapamil-sensitive SP cells (Fig. 4E and F), suggesting that SP cells have the potential to initiate earlier tumor growth at lower numbers. In a parallel experiment, preinjection analysis demonstrated an SP fraction equal to 0.21% (\sim 12,600 SP cells per animal) of the 6 \times 10^5 sorted cells per animal injected into 50 nude mice (data not shown). The average time to appearance of these tumors was \sim 9 weeks; in close agreement with our 7.5 \times 10^5 NSPs (\sim 12,900 SP cells) injected animals and corroborating our speculation that a very small population of SP cells has the potential to initiate tumor growth in vivo.

MOVCAR 7 and 4306 SP Cells Respond to MIS in Vitro. MIS has been shown to inhibit MOVCAR 7 both in vitro and in vivo (25). Thus, we investigated whether MIS inhibits MOVCAR 7 and 4306 SP and/or NSP cells in vitro. We first confirmed that SP and NSP cells possess an intact MIS signal transduction pathway, previously shown to be required for MIS responsiveness in the embryonic urogenital ridge (26). By using anti-MISRII antibody we observed that MOVCAR 7 and 4306 cells express the MISRII receptor by epifluorescent and confocal microscopy (Fig. 5B and C; 4306 cells...
We then confirmed the presence of MISRII, MISRI (Alk 2 and 3), and Smad 1/5/8 mRNA by RT-PCR in sorted SP and NSP cells (SP in Fig. 5D; NSP and 4306 cells are the same but not shown), suggesting these cells would likely respond to MIS.

MOVCAR 7 and 4306 SP and NSP cells were sorted, incubated for 24 h, and treated with 10 μg/ml MIS for MTT proliferation assays. MOVCAR 7 SP and NSP cells responded to MIS after initial sorting of the neat population. MOVCAR 7 SP cells were inhibited by 86%, whereas NSP cells were inhibited by 93% compared with vehicle controls (Fig. 5E). In contrast, only 4306 SP cells showed a significant inhibition of 37% by MIS (Fig. 7, which is published as supporting information on the PNAS web site). However, because NSP cells could not reliably be maintained in culture for serial sorting, we evaluated the ability of MIS to inhibit the SP alone after enrichment in both cell lines. MOVCAR 7 serial sorting followed by MTT showed 93% inhibition after sort 2 and 94% inhibition after sort 3 (Fig. 5F and G). Serial sorting of 4306 cells followed by MTT showed 60% inhibition after sort 2 (Fig. 7), and no inhibition after sort 3 was observed (17% inhibition; P = 0.054). Thus, MIS inhibits MOVCAR 7 and 4306 SP cells in vitro.

Human Ovarian Cancer Cell Lines and Primary Patient Ascites Cells Have SPs. To determine the prevalence of SP cells in human ovarian cancer, we evaluated the cell lines OVCAR 3, OVCAR 8, SK-OV-3, and IGROV-1, as well as ascites from six ovarian cancer patients (see cell line and patient demographics in Table 2, which is published as supporting information on the PNAS web site). Patient ascites cells were obtained directly from the operating theatre and analyzed within 96 h. We detected verapamil-sensitive SP cells in IGROV-1 (Fig. 6A), OVCAR 3 (data not shown), and SK-OV-3 (21), but not in OVCAR 8 (Fig. 6B). Viable human ascites cells, selected as CD45−CD31−, were found to exhibit verapamil-sensitive SP cells in four of six patients (Fig. 6C and D). Thus, an appreciable number of human ovarian cancer cell lines and primary ovarian cancer ascites cells possess SP cells.

Mouse and Human Ovarian Cancer Cell Surface Phenotype. To investigate whether ovarian cancer cells express somatic and cancer stem cell surface markers, as well as to identify differential expression between SP and NSP cells, we analyzed mouse and human ovarian cancer cells by flow cytometry. All mouse and human SP cells were gated as negative for CD45 (common leukocyte antigen) and CD31 (platelet endothelial cell adhesion molecule 1/endothelial cells). Compared with NSP cells, the MOVCAR 7 SP cells were enriched in number of cells and intensity of expression of c-kit/CD117 (stem cell factor receptor), whereas 4306 and human SP and NSP cells did not express c-kit. MOVCAR 7 SP and NSP cells strongly express the tumor metastasis marker CD 44 (hyaluronic acid receptor),
whereas 4306 cells and most human ovarian cancer cells do not. MOVCAR 7 and 4306 SP and NSP cells did not express CD24, CD34, CD105, CD133, or Sca-1 (Table 3, which is published as supporting information on the PNAS web site). Human cell lines and ascites cells showed variable expression of the epithelial cell marker epithelial-specific antigen/Ep-CAM (epithelial specific antigen) and CD24 (Table 2 and Fig. 8, which is published as supporting information on the PNAS web site). These markers, aside from c-kit in MOVCAR 7, did not add to the consistent SP phenotype and Bcrp1 immunostaining we have observed in identifying putative ovarian cancer stem cells in both mouse and human.

Discussion

The hypothesis that rare "embryonic rests" are responsible for malignancy was suggested >100 years ago (24), but recent advances in somatic stem cell identification has rejuvenated the investigation of this premise (4, 27–29). The unique asymmetric self-renewal capacities of somatic stem cells make it plausible and probable that mutations in these cells are perpetuated and over time lead to malignancy. Like somatic stem cells, cancer stem cells have the properties of self-renewal, heterologous descendant cells, slow cell-cycle times, and, unlike somatic stem cells, enriched tumor formation (8, 24). Here we demonstrate these properties within a subpopulation of mouse ovarian cancer cells that were isolated by SP sorting. MOVCAR7 and 4306 SP cells are able to self-renew and produce heterologous descendant NSP cells in culture. MOVCAR 7 SP cells are predominantly G0 cell cycle arrested, and the in vivo time to appearance of tumors in animals injected with equal numbers of MOVCAR 7 cells may be shorter in those receiving SP cells. We speculate that the number of SP cells required to initiate tumor formation in vivo is likely quite low, as evidenced by the appearance of tumors in NSP-injected animals at the same time as animals injected with unsorted cells possessing approximately the same number of SP cells. Thus, these isolated mouse SP cells possess the properties ascribed to cancer stem cells and provide a model for comparison with human ovarian cancer.

Ovarian cancer patients initially respond well to surgical cytoreduction and chemotherapy. Chemotherapy alone can yield several logs of tumor cytoreduction but seldom a cure. The majority of patients who respond to primary chemotherapy ultimately develop recurrent, usually drug-resistant, disease that is conceivably due to the ability of ovarian cancer stem cells to escape these drugs. BCRP1, otherwise known as the ABCG2 transporter, confers the ability to not only define a stem cell-like Hoechst 33342-excluding SP but, perhaps more importantly, the drug resistance-associated efflux of many lipophilic chemotherapeutic agents such as mitoxantrone, daunorubicin, doxorubicin, indocarbazoled, and others (22). Here we demonstrate that candidate mouse ovarian cancer stem cells, defined as Hoechst-effluxing, verapamil-sensitive, and BCRP1+ SP cells, are more resistant to doxorubicin, confirming these stem cell-like characteristics as a potential mechanism for drug resistance. In addition, we identified a similar subpopulation of cells in both human ovarian cancer cell lines and primary human ascites cells that could be defined as Hoechst-effluxing, verapamil-sensitive, BCRP1+ SP cells. We propose that these “markers” might be used to detect and isolate patient primary ovarian cancer stem cells for further characterization.

We cannot with certainty assert that SP cells are cancer stem cells; however, a subpopulation of cells found in the mouse SPs demonstrate some of the properties of cancer stem cells. Regardless of whether SP cancer cells are truly cancer stem cells or early progenitor cells, expression of the drug-resistance transporter BCRP1 or other multidrug-resistance proteins (30–33) may have a profound impact on selection of individual treatment strategies, clinical outcome, and the design or selection of the next generation of chemotherapeutic agents. For example, the fact that MIS could inhibit human anchorage-independent Mullerian tumors in soft agarose (34) indicated that MIS might act on cancer stem cell-like populations and led us to investigate its efficacy against these candidate cancer stem cells. The evidence that MIS inhibits MOVCAR 7 SP cells in vitro suggests that MIS has the potential to function as an effective adjuvant to current ovarian cancer chemotherapeutic regimens because of its ability to attack this elusive subpopulation of cancer cells. However, MIS inhibits MOVCAR 8 and OVCAR 8 (25, 35), indicating that response to MIS is not dependent on the presence of an SP. Currently, the evaluation of a wide range of chemotherapeutic and molecular-targeted agents are tested in nonselected in vitro culture systems or animal xenografts with efficacy being scored on cell death of what is likely the dominant, drug-sensitive, and perhaps biologically irrelevant NSP cells. A clinical testing model of SP cells or even purer subsets within the SP fraction are predicted to yield a more reliable insight into the development of effective therapeutic agents. Further work is needed and underway to more clearly define primary human ovarian cancer stem cells and their response to MIS in vivo.

Methods

Flow Cytometry. Flow cytometry was performed in the Department of Pathology and Center for Regenerative Medicine Flow Cytometry Laboratory according to their published protocols (36). Mouse and human ovarian cancer SP sorting and immunophenotyping were performed as described in Supporting Methods, which is published as supporting information on the PNAS web site. When testing SPs for multidrug resistance-like BCRP1 sensitivity, verapamil (25–50 μg/ml; Sigma) was also added.

For cell cycle analysis, MOVCAR 7 cells were harvested, sorted for HoechstHigh NSP, HoechstMid, and HoechstLow SP cells, and fixed with 70% ethanol for 24 h. Cells were washed in PBS, stained with 20 μg/ml propidium iodide and 1 mg/ml RNase (Type II; Sigma), and collected on a Life Sciences Research flow cytometer configured with CELLQUEST PRO software (BD Biosciences, Franklin Lakes, NJ).

Cell Lines and Culture. Mouse ovarian cancer cell lines, MOVCAR 7 and 8, were developed by D.C. by using the MISRII promoter to drive the SV40 T antigen (19). The OVCAR 3 and OVCAR 8 human ovarian cancer cell lines were developed by Thomas Hamilton (Fox Chase Cancer Center) (37). The 4306 cell line was developed by D.M.D. from conditional LSL-K-rasHG12D/+; PtenloxP/loxP mice after infection of ovarian surface epithelium with adenovirus expressing Cre recombine. These mice developed invasive endometrioid ovarian cancers 7 weeks after infection, and the 4306 cell line was established from ascites cells (20). IGROV-1 and SK-OV-3 were obtained from American Type Culture Collection (ATCC). Cell lines were maintained in 4% female FBS (MIS-free) and DEMEM with added L-glutamine, 1% penicillin/streptomycin, and 1% insulin-transferrin-selenium (ITS; Gibco) at 37°C, 5% CO2, in T175 flasks within a humidified chamber. All cells recovered from sorting were grown in the same media.

Human Primary Ascites Cell Isolation. Primary ascites cells were analyzed from five stage III ovarian cancer patients and one (AC-01) patient with recurrent ascites, who ranged in age from 54 to 71 years (mean, 62.2 years). The study was approved by the Human Studies Committee of Massachusetts General Hospital (Protocol No. FWA0003136), and consent was obtained from each patient on the Gynecology Oncology Service at the time of outpatient paracentesis or before surgery. Ascites harvested at laparotomy or ultrasound-guided paracentesis were placed on ice, centrifuged to isolate the cellular component, and resuspended in media. Erythrocytes were lysed, and cells were cultured in RPMI with 10% female FBS, 1% penicillin/streptomycin, and 1% fungizone. Cells were analyzed by flow cytometry within 96 h for the presence of an SP and surface markers.
Immunostaining of Cultured Cells. Anit-MISRII rabbit polyclonal antibodies (153p/MISRII) were developed for Western blot analysis in the Pediatric Surgical Research Laboratories (35). Immunofluorescence was performed on MOVCAr 7 and 4306 Cells by using 153p as described (22). Images were obtained by using a microscope (Nikon Eclipse E400 microscope, SPOT camera, and SPOT ADVANCE software) or confocal microscopy (Leica TCS NT confocal microscope, CONFOCAL software Version 2.5 Build 1227, and krypton 568-nm laser; Leica, Deerfield, IL).

For BCRP1 immunostaining, cells were double-labeled in suspension with Hoechst 33342 and BCRP1 antibody as described in ref. 5 and as detailed in Supporting Methods.

Reverse Transcriptase PCR. Total RNA from MOVCAr 7 and 4306 SP and NSP cells was extracted by using the Qiagen (Valencia, CA) RNeasy Mini Kit (catalog no. 74104) according to the manufacturer’s instructions, and 0.5 μg of RNA was reverse transcribed into cDNA by using Superscript II reverse transcriptase as directed by the manufacturer (Invitrogen). All RT-PCRs were run for 30 cycles with an annealing temperature of 57°C and separated on 2% agarose gels. Mouse PCR primers are as in Table 4, which is published as supporting information on the PNAS web site.

Growth Inhibition by MIS in Vitro. MTT assay was used to assess proliferation inhibition. MOVCAr 7 and 4306 cells were harvested, sorted for SPs and NSPs, and plated in the inner wells of 96-well plates at 1,000 cells per well in 200 μl of medium per well. Twenty-four hours after plating, each set of 10 wells of SP or NSP cells was treated with 10 μg/ml recombinant human MIS (25), 4 nM paclitaxel (6 mg/ml; NovaPlus, Irving, TX), a 4-nM doxorubicin hydrochloride injection (2 mg/ml; NovaPlus), or media alone. At day 5 or 7 of incubation, cell viability was quantified by measuring mitochondrial activity (38) on an ELISA plate reader at an absorbance of 550 nm to generate an OD proportional to the relative abundance of live cells in a given well.

Growth of MOVCAr 7 SP Cells in Vivo. MOVCAr 7 SP and NSP cells were sorted and injected into T and B cell-deficient 6-week-old female Swiss nude mice in equal numbers (first experiment, 5, 10^4; second experiment, 7, 5 × 10^3) into the dorsal fat pad between the scapulae. Mice were housed in the Edwin L. Steele Laboratory for Tumor Biology under American Association for Laboratory Animal Science guidelines with the approval of the MGH Animal Care and Use Committee (protocol no. 2005N000384).

Purification of Recombinant Human MIS. The human MIS gene was transfected into CHO cells, amplified, purified, and maintained in a dedicated facility in the Pediatric Surgical Research Laboratories for use in this study as described in ref. 39. MIS levels were measured by using human MIS-specific ELISA (40). MIS was purified by a combination of lectin affinity chromatography and FPLC anion-exchange chromatography (39). The MIS purified by this method causes regression in the organ culture bioassay for MIS (41, 42).

Statistical Analysis. In MTT assays, MIS-, doxorubicin-, and paclitaxel-treated and untreated samples were analyzed by using the univarient two-tailed Student t test, with P < 0.05 conferring statistical significance. All experiments were performed in triplicate.

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