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Müllerian inhibiting substance preferentially inhibits stem/progenitors in human ovarian cancer cell lines compared with chemotherapeutics

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Cancer stem cells are proposed to be tumor-initiating cells capable of tumorigenesis, recurrence, metastasis, and drug resistance, and, like somatic stem cells, are thought to be capable of unlimited self-renewal and, when stimulated, proliferation and differentiation. Here we select cells by expression of a panel of markers to enrich for a population with stem cell-like characteristics. A panel of eight was initially selected from 95 human cell surface antigens as each was shared among human ovarian primary cancers, ovarian cancer cell lines, and normal fimbria. A total of 150 combinations of markers were reduced to a panel of three—CD44, CD24, and Epcam—which selected, in three ovarian cancer cell lines, those cells which best formed colonies. Cells expressing CD44, CD24, and Epcam exhibited stem cell characteristics of shorter tumor-free intervals in vivo after limiting dilution, and enhanced migration in invasion assays in vitro. Also, doxorubicin, cisplatin, and paclitaxel increased this enriched population which, conversely, was significantly inhibited by Müllerian inhibiting substance (MIS), whereas the lipophilic chemotherapeutic agent doxorubicin more significantly inhibited the NSP cells (4).

In the present study, we performed flow cytometry and FACS to screen for stem cell markers and identified stem/progenitor cell enriched populations as defined by functional assays of colony formation and invasion in vitro, and by limiting dilution implantation in vivo, in a number of human ovarian cancer cell lines. In an attempt to define other markers specific to enriched stem/progenitor cell populations in these epithelial cancers for later use in screening human patients, 130 markers were screened and those amenable to flow cytometry and FACS (n = 95) (Fig. 1), and conserved between patient primary ovarian cancer cells in ascites, human ovarian cancer cell lines, and the epithelium of normal human fimbria, were selected for further study (Fig. 1). Eight surface markers conserved in all three sources were tested in various combinations and, of these, CD44+CD24+Epcam+ most consistently enriched for a population capable of colony growth.

The “triple-positive” (3−) cells also had a shorter tumor-free interval in vivo when xenotransplanted by limiting dilution, and migrated better in invasion assays in vitro than did triple-negative (3−) cells. Further, we assessed the effects of chemotherapeutic agents and MIS on survival of these ovarian cancer cell populations enriched or not enriched for stem-like cells. From these studies, we demonstrated that, although chemotherapeutic agents significantly inhibited viable nonenriched populations, they consistently enhanced stem/progenitor cell enriched populations. By contrast, MIS and its anthrapyrazolone small molecule agonist, SP600125, preferentially inhibited the stem/progenitor cell-enriched population. These differential results suggest that chemotherapeutic drugs and MIS should in the future be studied to determine if they can function in rationally selected combinations

As evidence is accumulating to indicate that cancer could be a stem cell disease (1–4), it is becoming increasingly important to be able to identify cancer stem/progenitor cells and to develop treatment modalities that specifically target the stem cell enriched population, coupled with treatments effective against the larger population not enriched for stem cells. The concept of cancer stem cells has opened new areas of research in carcinogenesis, but has the more immediate translational potential of uncovering new treatment targets.

We previously identified somatic label-retaining cells with stem cell features in ovarian surface epithelium (5), and with others (6, 7), postulate that somatic stem cells or their immediate progenitors can revert to cancer stem cells (8). It is also possible that the stem cells may remain the same, but that signals which control the stem/progenitor cell activity may change. Several recent studies have demonstrated that cancer stem cells may confer chemotherapeutic resistant ovarian tumor growth and metastasis (2, 9).

Ovarian cancer is diagnosed in approximately 25,000 new cases per year in the United States and is associated with a 50% mortality rate (10, 11); more than 90% of cases are epithelial in origin (12, 13). Epithelial ovarian cancers fall into four main subtypes: mucinous, endometrioid, clear cell, and serous (14). Serous ovarian carcinomas (15, 16) account for the overall high ovarian cancer-related mortality. Fewer than 25% are detected at an early stage; thus, there is an urgent need for better predictive molecular markers that characterize early oncologic transformation (17) to permit earlier detection, to uncover additional therapeutic targets, and to change therapeutic protocols.

“Side population” (SP) cells identified by Hoechst 33342 dye exclusion in a wide range of cancers were found to be enriched for cancer stem cells (1, 18, 19). We found that SP cells form larger tumors and have higher tumorigenic propensity than do non-SP (NSP) cells, but did so in mouse ovarian cancer cell lines (MOV-CAR7). These stem/progenitor cells were inhibited by Müllerian inhibiting substance (MIS), whereas the lipophilic chemotherapeutic agent doxorubicin more significantly inhibited the NSP cells (4). These findings predict that chemotherapeutic agents and MIS may differentially affect populations in human ovarian cancer that are relatively chemoresistant and demonstrate stem cell characteristics.

In the present study, we performed flow cytometry and FACS to screen for stem cell markers and identified stem/progenitor cell enriched populations as defined by functional assays of colony formation and invasion in vitro, and by limiting dilution implantation in vivo, in a number of human ovarian cancer cell lines. In an attempt to define other markers specific to enriched stem/progenitor cell populations in these epithelial cancers for later use in screening human patients, 130 markers were screened and those amenable to flow cytometry and FACS (n = 95) (Fig. 1), and conserved between patient primary ovarian cancer cells in ascites, human ovarian cancer cell lines, and the epithelium of normal human fimbria, were selected for further study (Fig. 1). Eight surface markers conserved in all three sources were tested in various combinations and, of these, CD44+CD24+Epcam+ most consistently enriched for a population capable of colony growth. The “triple-positive” (3−) cells also had a shorter tumor-free interval in vivo when xenotransplanted by limiting dilution, and migrated better in invasion assays in vitro than did triple-negative (3−) cells. Further, we assessed the effects of chemotherapeutic agents and MIS on survival of these ovarian cancer cell populations enriched or not enriched for stem-like cells. From these studies, we demonstrated that, although chemotherapeutic agents significantly inhibited viable nonenriched populations, they consistently enhanced stem/progenitor cell enriched populations. By contrast, MIS and its anthrapyrazolone small molecule agonist, SP600125, preferentially inhibited the stem/progenitor cell-enriched population. These differential results suggest that chemotherapeutic drugs and MIS should in the future be studied to determine if they can function in rationally selected combinations


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to assure that both populations are effectively targeted (20, 21). Identification of a readily definable stem cell like population that is amenable to isolation by flow cytometry will aid in the development of future therapeutic strategies.

**Results**

In Fig. 1B, the left arm indicates that unseparated cells were cultured and treated with chemotherapeutic agents, MIS, or vehicle alone for 3 d. Flow cytometry analyzed 50,000 cells for viability and for percent of cells in side population relative to NSP or expressing Epcam+, CD44+, and CD24+ relative to those not expressing these three markers.

The right arm reflects cells separated by FACS and analyzed after using 150 combinations of eight markers. Combinations with the most robust colony growth in all cell lines were chosen for functional testing in vitro by invasion and proliferation assays, and by in vivo assays to determine tumor-free interval after injection of cells subjected to limiting dilutions.

**SP of Ovarian Cancer Cell Line Is Enhanced After Treatment with Chemotherapeutic Agents.** The verapamil-sensitive SP of human ovarian cancer cell lines was selected by exclusion of the DNA-binding Hoechst 33342 dye (Fig. S1), SKOV-3, OVCAR-5, and IGROV-1, treated for 3 d with doxorubicin, showed an increased percentage of SP cells (Fig. S2 A and C) and a decreased percentage of NSP cells. OVCAR-5 and IGROV-1 cells treated with cisplatin also significantly increased the percentage of SP cells in OVCAR-5 (Fig. S2 B and D) and in IGROV-1 while decreasing that of NSP cells. Fuchsia also caused an increase in the percentage of SP cells in OVCAR-5 and SKOV-3 cells (Fig. S2 B and D). Chemotherapeutic agents dose-dependently reduced the total number of cells after 3 d of treatment (Table S1).

**MIS and Its Anthrapyrazolone Agonist, SP600125, Diminish SP Cells.** The receptor-mediated ligand MIS, compared with vehicle alone, decreased the percentage of SP cells (Fig. S3 A and C) in the human ovarian cancer cell lines known to be responsive to MIS in vitro or in vivo, namely OVCAR-5 (20) and IGROV-1 (21, 22). SP600125, an anthrapyrazolone, discovered in a small molecule screen to act as an MIS agonist in MIS type II receptor (23, 24), but not in BMP type II receptor transfected cells, also significantly reduced the percentage of SP cells in OVCAR-5 and IGROV-1 (Fig. S3 B and D and Table S1). SP600125 also decreased the NSP of IGROV-1 cells, but at higher doses (Fig. S3D). Thus, different from chemotherapeutic agents, MIS and its agonist SP600125, after 3 d of treatment in culture, significantly reduce the ratio of viable SP cells of a number of human ovarian cancer cell lines.

**Enrichment of Cancer Stem Cell Populations in Human Ovarian Cancer Cell Lines by Selection with a Marker Panel Compatible with Flow Cytometry.** Primary ascites from three patients with ovarian cancer were screened in flow cytometry for relative binding of 95 fluorescently labeled surface markers. After negative selection for CD45 and CD31 cells, the panel was narrowed to 24 cell surface markers reconfirmed in five ovarian cancer cell lines (Fig. L4). A panel of eight surface markers consistently found in both primary cancers and cancer cell lines, as well as normal Fallopian tube infundibular cells (Fig. L4), was further tested in more than 50 different combinations (Table S2) for their ability to form colonies (25) in each of three cell lines, OVCAR-5, IGROV-1, or SKOV-3 cells (Fig. 1B, right arm). The CD44+CD24+EpCam+ population formed more large colonies after 14 d than did other combinations (Fig. 2A and B and Table S2; each combination represents $n$ = 3–5 experiments). Moreover, we analyzed the association of SP and the CD44+CD24+EpCam+ population, and found that the 3+ cell percentage is increased in the SP cells (Fig. S4) and that SP cells are dramatically increased in the 3+ cells of OVCAR-5, indicating that there is an overlap between SP and CD44+CD24+EpCam+ cells.

**Triple-Positive Cells Invade Matrigel More Effectively than Do 3− Cells.** CD44+CD24+EpCam+ cells in serum-free media invade through an extracellular matrix-coated membrane insert after incubation for an additional 72 h in serum containing media. When stained and then quantitated by ImageJ software (National Institutes of Health), 3+ cells showed significantly more invasion through the matrix than did 3− cells ($P < 0.05$; $n = 3$; Fig. 2B, Right).

**Triple Positive Cells Injected into the Right Flank of NOD/SCID Mice Grow Tumors Earlier than Did Triple Negative Left Flank Cells.** CD44+CD24+EpCam+ cells grew tumors earlier at comparable cell dilutions ($10^3$ and $10^5$) than did 3− cells. Tumor-free intervals were then subjected to Kaplan-Meier analysis and log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests for significance. The tumor free interval was shorter ($P = 0.015$ for $10^5$ cells, $P = 0.049$ for $10^3$ cells; $n = 5$ animals for each) for the 3+ cells compared with 3− cells (Fig. 2C).

**Marker Panel-Selected Cells Are Diminished by MIS and SP600125.** After treatment with chemotherapeutic agents or MIS, remaining viable populations of OVCAR-5 cells were selected by flow...
cytometry for 3+ markers (Fig. 1B, left arm), and the remaining cell numbers determined and compared with those cells that were 3−. There was a dramatic expansion of the ratio of 3+ OVCAR-5 cells (Table S4, right column) when treated with doxorubicin (4.52–39.9%) or cisplatin (2.03–10.41%) (Fig. 3A and B and Table S4) whereas the total number of viable cells slightly decreased. By comparison, both the total numbers of viable unseparated cells and 3+ separated cells decreased significantly when treated with MIS or SP600125 (Fig. 3C and D and Table S4).

Effects of Chemotherapeutic Agents and MIS and Its Agonist on Cell Survival of 3+ and 3− Cells. We tested the effect of chemotherapeutic agents and MIS on the survival of 3+ and 3− populations in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Separated OVCAR-5 cells plated at 2,000 cells per well were treated with different doses of doxorubicin for 3 d. Doxorubicin slightly inhibited the survival of 3+ cells (P < 0.05), but more strongly inhibited 3− cells (P < 0.01) (Fig. 4A, Upper), as did cisplatin (Fig. 4A, Lower). Similar results were seen on cells separated by FACS into NSP (Fig. S5A), which were more inhibited than SP by doxorubicin and cisplatin. These results indicate that chemotherapeutic agents preferentially inhibit 3− and NSP (Fig. S5A), and that 3+ and SP cells show relative resistance to chemotherapeutic agents.

The 3+ and 3− cells from OVCAR-5 cells were treated with different doses of MIS for 3 d. In contrast, MIS significantly inhibits 3+ cells at lower doses than those required to inhibit 3− cells, although both were significant (Fig. 4B, Upper, for 3 d; Fig. S5C, after 7 d). SP600125 also significantly inhibited growth of 3+ cells (P < 0.01), but required higher doses to inhibit 3− cells, although both were significant (Fig. 4B, Lower, and Fig. S5D). Survival of SP cells was more inhibited by MIS and SP600125 compared with NSP cells (Fig. S5B).

Discussion
It is essential to identify ovarian cancer populations enriched for tumor-initiating cells and, at the same time, to elucidate the molecular mechanisms that regulate normal self-renewal or differentiation, and to determine how such mechanisms can be co-opted or changed to contribute to transformation and drug resistance and whether modulation of these mechanisms will affect patient outcome. SP cells have been described as being enriched for cancer stem cells and characterized in several mammalian tissues or cancers by efflux of lipophilic substrates, including the cell-permeable DNA-specific bisbenzimidazole dye Hoechst 33342 and some chemotherapeutic agents (1, 4, 5, 19, 26–28). Having identified SP cells in mouse ovarian cancer cell lines (4) and shown that they were more tumorigenic than NSP cells, we now show...
that SP populations existing in human ovarian cancer cell lines (OVCAR-5, IGROV-1, and SKOV-3) form more colonies than NSP cells and are resistant to chemotherapeutic agents (doxorubicin, cisplatin, and paclitaxel), but more sensitive to MIS and its agonist, SP600126.

To consider other therapeutic agents that can target SP cells and NSP cells, we turned to extracellularly active receptor mediated agents and tested the effects of MIS, as mouse ovarian surface epithelium expresses MIS RII receptors (21, 29, 30), as do human ovarian cancer cell lines and primary ovarian cancer ascites cells (20). We found that MIS and its small molecule MISRII receptor-dependent agonist, SP600125 (23), significantly decreased SP cell percentages in OVCAR-5, IGROV-1, and SKOV-3.

As little is known about markers more specific for the stem cell population of human ovarian cancer, we performed a screen of 95 flow cytometry-compatible cell surface markers that were conserved in human primary ovarian cancer ascites and in human ovarian cancer cell lines (OVCAR-3, OVCAR-5, OVCAR-8, SKOV-3, and IGROV-1; Fig. 1A). Eight of these (Epcam, CD24, CD44, CD90, CD105, CD133, E-cadherin, and SP) were preserved in normal human infundibulum from 16 patients undergoing excision for benign disease, as fimbria removed prophylactically with ovaries in patients with familial breast cancer were found to be the site of occult tumors bearing characteristics of serous cystadenocarcinoma of the ovary (31–33). Normal infundibulum as a surrogate for ovarian surface epithelium contained a significant SP after enzymatic digestion of the human normal fimbria, and therefore was used to screen cancer stem cell surface markers that could be subsequently used in human patient ascites.

Fig. 3. MIS and its agonist SP600125 decrease the percentage of marker-enriched stem cell populations in human ovarian cancer cell lines. OVCAR-5 cells were treated with increasing doses of doxorubicin (A), cisplatin (B), MIS (C), or SP600125 (D) for 3 d and stained with a combination of CD44, CD24, and Epcam antibodies. A total of 50,000 cells were tested by flow analysis for viability. Percentages of marker panel selected populations were determined and fold changes calculated (n = 3 separate experiments performed in triplicate at each dose for each drug).

Fig. 4. Chemotherapeutic agents and MIS differentially affect survival of marker-enriched stem cell populations. CD44/CD24/Epcam 3+ and 3− cells were sorted from OVCAR-5 cells by FACS and tested in MTT assays. (A) Doxorubicin treatment (10, 30, 60 nM) and cisplatin treatment (0.5, 1, 2 μM) inhibited the proliferation of 3− cells compared with 3+ cells (*P < 0.05; **P < 0.01). (B) MIS treatment (37.5, 112.5, 225 nM) and SP600125 treatment (5, 10, 16 μM), by contrast, inhibited proliferation of the 3+ population significantly more than the 3− population (*P < 0.05; **P < 0.01). (Bars indicate SD.)
Several of these markers have been identified in human cancer (34-41). For example, Epcam (CD326) is a glycosylated type I membrane adhesion molecule expressed in a variety of human epithelial tissues, cancers, and stem cells (39). CD44 is the receptor for the extracellular matrix component hyaluronic acid (34, 37). Epcam/CD44 expression profiles are significantly higher in colorectal primary tumors than in normal colonic tissues, and tumors originating from Epcam+/CD44+ cells generated the full morphologic and phenotypic heterogeneity of their parental lesions (36). CD133 (prominin-1) originally used to isolate hematopoietic and endothelial progenitor cells, was subsequently found to be a marker of tumor-initiating cells in a number of other human cancers (9, 35, 42-44). CD24, a mucin-like adhesion molecule, is highly expressed in a large variety of human cancers and contributes to tumor growth and metastases by binding to platelet-derived growth factor beta (45, 46). Like CD44+ cells (47), CD24+ cells (48) from human ovarian tumors were recently reported to be enriched in cancer stem cells and to form tumor xenografts in nude mice, whereas CD24- cells were nontumorigenic. Stem cells from different cancers may have different phenotypes, i.e., breast cancer stem cells are CD44+CD24low and colorectal cancer-derived cell lines are CD44+CD24+ (49). Our findings demonstrate that a phenotype of CD44+CD24+ Epcam+ is enriched for stem/progenitor cells in human ovarian cancer cell lines.

E-cadherin (CD243), a ligand for integrin α-Eβ7, encodes a glycoprotein with five extracellular cadherin repeats. Loss of E-cadherin is associated with epithelial-to-mesenchymal transformation and cancer progression by increasing proliferation, invasion, and/or metastasis (50, 51).

After detecting verapamil-sensitive SP cells in OVCAR-3, SKOV-3, and IGROV-1, in multiple replicates, we used SP for comparison while developing a panel of surface markers that would allow us to recover live cells in which to examine molecular mechanisms and to observe the response of separated cells to drugs, for later use in human patients.

Colonies were used to screen the eight marker panel on three human ovarian cancer cell lines showed that the combination of Epcam+, CD24+, and CD44+ formed more colonies than other marker combinations. It was necessary to use this 3+ panel in combination, as each marker alone was not sufficiently selective (Table S3).

As chemotherapeutic agents have been shown in other cancers to contribute to resistance (37, 52) and to enrich for stem cell characteristics (37), we examined the effects of chemotherapeutic agents and MIS and demonstrated that chemotherapeutic agents preferentially inhibited the population not enriched for tumor initiating population. In contrast, receptor-mediated MIS and its agonist SP600125 inhibited significantly the populations enriched for tumor-initiating cells. The fact that these enriched populations showed resistance to chemotherapeutic agents, but maintained sensitivity to MIS and to SP600125, indicates that there is experimental rationale to use this combination of markers to study primary ascites or tumor from patients and to screen for additional selective inhibitors of human ovarian cancer populations enriched for stem-like cells. Furthermore, there is a need to design individualized treatment which specifically addresses the patient-specific population enriched for tumor initiating cells. This approach will hopefully lead to improved outcomes for patients with ovarian cancer. In addition, the panel-selected epithelial cells can now be used as an experimental tool to examine for variations (53, 54) that highlight mechanistic differences that could serve as future therapeutic targets. Furthermore, this marker panel is immediately translatable to the clinic to select patient-specific treatment strategies.

### Materials and Methods

**Cell Lines, Chemotherapeutic Agents, and MIS.** Human ovarian cancer cell lines—OVCAR-5 (55, 56), SKOV-3 (57, 58), and IGROV-1 (59) cells—were maintained in the pediatric surgical research laboratories as previously described (4, 21). Cells were treated with doxorubicin, cisplatin, paclitaxel (all from NovoPlus), MIS, or SP600125 (Sigma) (23). MI was purified in our laboratory, and its bioactivity assessed in embryonic Müllerian duct regression assays (60, 61). The treatment doses were selected (21) around the IC50 for each drug for each cell line (10-60 nM for doxorubicin, 0.5-2 μM for cisplatin, 0.5-4 nM for paclitaxel, 37.5-225 nM for MIS, and 3-16 μM for SP600125).

**Harvesting of Primary Human Ovarian Cancer Ascites.** Primary ascites were removed therapeutically from patients with ovarian cancer at the Massachusetts General Hospital (institutional review board (IRB) approval 2007-P-001918), or at Dana Farber Cancer Institute (IRB Legacy 02-0251) were enzymatically digested at 37 °C in 0.2% (wt/vol) collagenase type II (type II in DMEM; Gibco-BRL) for 30 to 45 min, followed by dilution with DMEM/F-12 medium. Normal epithelial cells, released by scraping the fimbria, were collected, then verapamil-treated (225 ng/mL for 5 min, resuspended with ammonium chloride solution to lyse red blood cells, diluted with DMEM/F-12 medium, and recentrifuged. Cells were then washed twice in the same media, stained with antibodies for 20 min at 4 °C, washed again, and resuspended in PBS solution for immediate flow analysis.

**Digestion of Normal Fimbria Epithelium.** Normal human Fallopian tube fimbria removed from sixteen patients undergoing surgery for benign uterine disease at the Massachusetts General Hospital (IRB approval 2007-P-001918/4) or at Dana Farber Cancer Institute (IRB Legacy 02-0251) were enzymatically digested at 37 °C in 0.2% (wt/vol) collagenase type II (type II in DMEM; Gibco-BRL) for 30 to 45 min, followed by dilution with DMEM/F-12 medium. Normal epithelial cells, released by scraping the fimbria, were collected, then verapamil-treated (225 ng/mL for 5 min, resuspended with 70-μm nylon mesh; Fisher Scientific), recentrifuged at 225 x g for 5 min, resuspended with ammonium chloride solution to lyse red blood cells, diluted with DMEM/F-12 medium, and recentrifuged. Cells were then washed twice in the same media, stained with antibodies for 20 min at 4 °C, washed again, and resuspended in PBS solution for immediate flow analysis.

**Flow Cytometry and Cell Number After Treatment of Cell Lines.** Cells were plated in T75 flasks at 10% to 15% confluency (Fig. 18, left arm), then treated with increasing doses of doxorubicin, cisplatin, paclitaxel, MIS, SP600125, or vehicle alone for 3 d. Flow cytometry was performed in the Massachusetts General Hospital Department of Pathology Flow Cytometry Laboratory using a custom designed high-resolution 7-laser LSR (BD Science) (4, 62). Cells were stained with Hoechst dye 33342 (Invitrogen) for 90 min at 37 °C, or antibodies to the markers for 30 min at 4 °C. A total of 50,000 cells were tested by flow analysis for viability using 7-AAD (Sigma) and for verapamil-sensitive (50–100 μg/mL; Invitrogen) exclusion of Hoechst dye or for binding of optimal marker combinations (as detailed later).

**Colonies Formation to Select Optimal Combination of Surface Markers.** Single cell suspensions were selected using a 5-laser FACSDiVa cell sorter (BD Science) and stained with a variety of combinations of antibodies to 95 markers to choose eight markers conserved in human ovarian cancer cell lines, primary ascites, and primary human fimbria epithelial cells and also detected on other cancer stem cells: anti-human CD24-PE (eBioscience), anti-mouse/human CD44-APC/Cy7 or CD44-Alexa Fluor–700 (BioLegend), anti-human CD90-APC (BioLegend), anti-human CD105-PE (Invitrogen), and anti-human CD133-APC (BioLegend), anti-human Epcam-Alexa Fluor–647 or Epcam-APC (BioLegend), and anti-human E-cadherin-FITC (CD324, BioLegend; Fig. 18, right arm). For additional antibodies tested, see link to Stem Progenitor Cell Marker Panel at www.massgeneral.org/children/research/researchers/weil_xiaolong.aspx. Cells sorted from the three cell lines were separated by various markers and selected for viability, plated on six-well plates (500, 1,000, or 2,000 cells/well) and incubated for 14 d at 37 °C, with a media change at day 7. At day 14, cells were washed, fixed with methanol, stained with Giemsa, and analyzed with Imagex (National Institutes of Health).

**Effects of Treatment on Cell Survival.** The SP and NSP populations and the 3+ and 3– populations, after sorting, were tested in a cell survival tetrazolium MTT assay as previously described (21) at 2,000 cells per well in 200 μl of media per well after treatment for 3 d with different doses of chemotherapeutic agents (doxorubicin, cisplatin, paclitaxel), MIS, and the MIS agonist SP600125, then compared with an appropriate vehicle as control.

**Cell Invasion Assay.** In vitro cell invasion assay was performed according to the manufacturer’s protocols of an ECM invasion assay kit (Millipore), comparing 3+ versus 3– populations.

**Tumor-Free Interval or Time to Appearance of Xenotransplanted Tumors After Limiting Dilution.** The 3+ and 3– cell populations were isolated from OVCAR-5 cell lines by FACS, serially diluted (10–16, 102 cells), resuspended in 1:1 PBS/Matrigel (BD Biosciences) at 4 °C in 200 μL and injected s.c. into the right flank and 3– into the left flank of 4- to 6-wk-old female NOD.CB17-Prkdc/
Statistical Analysis. Univariate two-tailed t tests were used (4, 21) to compare Mls and drugs with a vehicle as a control. Kaplan-Meier and log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon analyses were used to compare differences of time to tumor appearance. Analyses were performed using Prism 5 software for Mac OSX (version 5.0a, GraphPad Software).

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Scid/j mice (Jackson Laboratory) under a protocol approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee (IRB approval 2009 N000033/1). Mice were monitored weekly for tumor formation, and time of appearance was recorded after euthanasia by CO2 inhalation. Tumors were measured and then fixed or frozen for further study.

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Statistical Analysis. Univariate two-tailed t tests were used (4, 21) to compare Mls and drugs with a vehicle as a control. Kaplan-Meier and log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon analyses were used to compare differences of time to tumor appearance. Analyses were performed using Prism 5 software for Mac OSX (version 5.0a, GraphPad Software).

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