CD24⁺ Ovarian Cancer Cells Are Enriched for Cancer-Initiating Cells and Dependent on JAK2 Signaling for Growth and Metastasis

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Abstract

Ovarian cancer is known to be composed of distinct populations of cancer cells, some of which demonstrate increased capacity for cancer initiation and/or metastasis. The study of human cancer cell populations is difficult due to long requirements for tumor growth, interpatient variability, and the need for tumor growth in immune-deficient mice. We therefore characterized the cancer initiation capacity of distinct cancer cell populations in a transgenic murine model of ovarian cancer. In this model, conditional deletion of Apc, Pten, and Trp53 in the ovarian surface epithelium (OSE) results in the generation of high-grade metastatic ovarian carcinomas. Cell lines derived from these murine tumors express numerous putative stem cell markers, including CD24, CD44, CD90, CD117, CD133, and ALDH. We show that CD24⁻ and CD133⁻ cells have increased tumor sphere-forming capacity. CD133⁺ cells demonstrated a trend for increased tumor initiation while CD24⁺ cells versus CD24⁻ cells had significantly greater tumor initiation and tumor growth capacity. No preferential tumor-initiating or growth capacity was observed for CD44⁺, CD90⁻, CD117⁺, or ALDH⁺ versus their negative counterparts. We have found that CD24⁻ cells, compared with CD24⁺ cells, have increased phosphorylation of STAT3 and increased expression of STAT3 target Nanog and c-myc. JAK2 inhibition of STAT3 phosphorylation preferentially induced cytotoxicity in CD24⁺ cells. In vivo JAK2 inhibitor therapy dramatically reduced tumor metastases, and prolonged overall survival. These findings indicate that CD24⁺ cells play a role in tumor migration and metastasis and support JAK2 as a therapeutic target in ovarian cancer.

Introduction

Ovarian cancer is the fifth leading cause of cancer-related death among women (1). Most ovarian cancer patients present with advanced-stage disease such that treatment with surgery and chemotherapy results in a median progression-free survival of only 16 to 22 months and a 5-year survival rate of only 27% (2). Contributing to the poor outcome in ovarian cancer may be the significant cellular heterogeneity in ovarian cancer.

Our group and others have characterized distinct ovarian cancer cell populations in human ovarian cancer cell lines and patient specimens. Human cancer cell populations defined by various combinations of Hoechst dye exclusion, CD133, aldehyde dehydrogenase (ALDH), CD44, CD117, CD24, and Epcam, have all been reported to have increased ability to initiate cancer and/or promote metastasis in vivo (3–11). The study of the distinct cancer cell populations that make up human cancer is very challenging due to interpatient variability, the need for testing tumor initiation in immunosuppressive mice, and long-term growth requirement for in vivo studies. Murine tumor models provide means to study the biology of heterogeneous cancer cell populations in tumors arising as a consequence of well-defined genetic mutations in an immunocompetent microenvironment.

There are currently several genetic murine models of ovarian cancer, which use ovular bursal injection of an adenovirus expressing Cre recombinase (AdCre) to induce Cre-mediated deletion of specific tumor-suppressor genes in the ovarian surface epithelium (OSE; refs. 12–14). Wu and colleagues (15) developed a model of a high-grade endometrioid ovarian carcinoma, which develops after inactivation of Apc, Pten, Trp53. In this model, the addition of Tp53 mutation appears to be associated with a type I to type II ovarian cancer progression, with tumor-bearing mice dying rapidly (within weeks) due to widely metastatic disease in a manner similar to that of patients with advanced-stage ovarian cancer patients (16, 17). Genetic analysis of these tumors demonstrated gene expression patterns similar to human disease.

In this study, we characterized cell lines and primary tumors from the Apc⁻/Pten⁻/Trp53⁻ ovarian tumor model for cells with ovarian cancer-initiating cell (CIC) activity. Tumors generated in this model have an endometrioid histology, but in the presence of a p53 mutation, have a high-grade metastatic phenotype reminiscent analogous to that seen in patients with high-grade serous cancer (15). We demonstrate that cells with
expression of the cell surface marker CD24 have greater sphere-forming capacity, ability to passage, and ability to initiate tumors in vivo. Similar to the observation in hepatocellular carcinomas, CD24+ CIC demonstrate preferential phosphorylation of STAT3 and expression of Nanog and CD24+ cells are preferentially sensitive to inhibition of STAT3 phosphorylation with a JAK2 inhibitor. Finally, we show that JAK2 therapy in vivo using this tumor model prevents tumor metastasis. This study supports other work demonstrating CD24+ cells as a CIC population with increased metastatic potential and suggests that targeting JAK2 could reduce ovarian tumor metastasis.

Materials and Methods

Cell culture

Murine ovarian endometrioid adenocarcinoma cell lines were derived as previously described (18). Briefly, the W2476T tumor cell line was established by mechanically dispersing ovarian tumor tissues with sterile scalpels followed by digestion at 37°C with 0.05% Trypsin–EDTA for 20 minutes. Cells were cultured for five passages in DMEM containing 10% FBS and 1% penicillin/streptomycin in an incubator with 3% O2; 5% CO2. During the first five passages of primary culture, nonadherent cells were discarded, and only adherent cells were passaged. W2476T cells display epithelial (cobblestone) morphology in culture. Cells were maintained and grown in RPMI containing 10% of FBS and 1% of penicillin/streptomycin (Gibco) at 37°C and 5% CO2. To create W2476T-Luciferase–expressing cells, W2476T cells were transduced with Luciferase-expressing lentiviral construct (provided by the UMCC Vector core).

Isolation of CICs from W2476T cell line and primary Apc–; Pten–; Tp53– tumors

Primary Apc–; Pten–; Tp53– tumors were mechanically dissected into single-cell suspension as previously described (5). Cells from primary tumor suspensions or the W2476T cell lines were then isolated using fluorescence-activated cell sorting (FACS). Briefly, primary ovarian tumor or W2476T cell line single-cell suspensions were counted and incubated with primary antibodies CD24-PerCP Cy5.5, CD117-APC, and CD133-PE (ebioscience), CD44-Pacific Blue (BioLegend), CD90-PE (BD Pharmingen) for 30 minutes at 4°C. Cells were then stained with propidium iodide (PI) or DAPI as a viability stain. For ALDH1+ samples, ALDH enzymatic activity was defined using the ALDEFLUOR kit (STEM-CELL Technologies) as previously described (5). FACS was performed with approximately 1 x 10⁶ cells using FACSAria (Recon Dickinson) under low pressure in the absence of UV light. Live cells were selected on the basis of both forward versus side-scatter profiles and the absence of PI or DAPI stain and ALDEFLUOR/DEAB–treated cells were used to define negative gates for ALDH1.

Sphere assays

Sphere culture was performed as previously described (5, 19, 20) with FACs-isolated CD24+/−, CD44+/−, CD90+/−, CD117+/−, CD133+/−, and ALDH1+/− cell populations plated in triplicate in either 6-well or 24-well ultralow attachment plates in serum-free DMEM/F12, epidermal growth factor (EGF) 20 ng/mL, gentamycin 20 μg/mL, insulin 5 μg/mL, 1% penicillin/streptomycin (Gibco), hydrocortisone 1 ng/mL, β-Mercaptoethanol 100 μmol/L (Sigma), and basic fibroblast growth factor (bFGF) 10 ng/mL (Millipore). Either 300 or 2,000 cells (depending on the availability of cells to perform the experiment triplicates) were seeded in ultralow attachment plates (Sigma). Primary spheres were counted on day 5 and dissociated enzymatically with warm 0.025% Trypsin–EDTA (Gibco) for 2 minutes at 37°C and then passed five times through a sterile Pasteur pipette to isolate single cells. The cells obtained from dissociation were analyzed microscopically for single cellularity and then replated in ultralow attachment plates. Secondary spheres were counted on day 5 after passaging.

Tumor initiation studies with cell lines and primary tumor cells

Mice were housed and maintained in the University of Michigan Unit for Laboratory Animal Medicine and all studies were performed with the approval of the University Committee on the Use and Care of Animals. For screening cell line experiments, 5 x 10⁵ W2476T-Luc cells were FACS-sorted for each cancer stem cell marker and were injected subcutaneously into the contralateral axilla of CIEA NOG (NOD/Shi-scid/IL-2R α null mice; Taconic) mice. Two weeks after injection, tumor growth was monitored weekly using in vivo bioluminescence imaging and tumor measurement using calipers as previously described (19). Mice were imaged using an IVIS Image System 200 Series (Xenogen Corporation) approximately 10 minutes after injection of α-luciferin. The bioluminescence images were collected using sequential mode until reaching peak values and analyzed by LivingImage 3.0 software (Xenogen Corporation). Mice were followed until tumors met euthanasia guidelines. Tumors were then harvested, weighed, and snap-frozen for histologic analysis, extraction of RNA and protein. Tumor volumes were calculated using the L x W x W/2 formula.

For CD24 cell limiting dilution studies, 1,500, 600, or 200 CD24+ or CD24− W2476T cells were injected into the contralateral flanks of mice euthanized as above. Confirmatory experiments were then performed with 200 CD24+ or CD24− W2476T cells injected into the flanks of separate mice. Animals were monitored, as indicated above, for tumor development for up to 6 months.

For primary murine ovarian cancer cell experiments, Apc−; Pten−; Tp53− tumor-bearing mice were generated via intra-bursal AdCre injection as previously described (16, 17). Apc−/Pten−; Tp53− murine ovarian tumors were harvested and cells were dissociated and sorted using FACS for CD24+, CD24− as stated above. CD24− and CD24+ (5 x 10⁴ or 5 x 10⁵) primary murine ovarian cancer cells were injected into individual NOG mice. Tumor growth was then monitored weekly using calipers for up to 5 months.

In vivo therapeutic response

Apc−/Pten−; Tp53− tumor-bearing mice were generated via intra-bursal AdCre injection as previously described (16, 17). Mice were monitored following AdCre injection until ovarian tumors were approximately 500 mm³ (~4 weeks). Mice were paired by tumor size and then randomized to treatment with cisplatin alone (once a week 2 mg/kg × 2 weeks and vehicle daily) or cisplatin + daily TG101209 (50 mg/kg in 40 μL DMSO; n = 6/group). We chose a reduced dose of TG101209 (a 4-fold reduction vs. previous studies) as i.p. dosing would increase local tumor related toxicity. Mice were monitored in conjunction with staff in the Unit of Laboratory and Animal Management and euthanized when the animal appeared (i) ill with signs of weight loss, (ii) had...
significant bowel distention, (iii) or primary ovarian tumors were felt to be approximately 1,000 mm\(^3\). For treatment of "early-stage" tumors, therapy was initiated 7 days after injection of AdCre. Apc\(^{−/−}\);Pten\(^{−/−}\);Trp53\(^{−/−}\) mice were treated with 40 \(\mu\)L of DMSO alone or TG101209 single-agent therapy daily for 21 days \((n = 11\) controls; \(n = 14\) TG101209). A subset of animals \((n = 4/\text{group})\) was sacrificed 1 hour after the final TG101209 therapy and tumors were harvested for the assessment of p-Stat3.

### RNA isolation and qRT-PCR

A subpopulation of W2476T cells was isolated by FACS and RNA was extracted using PureLink RNA Mini Kit (Ambion) per the manufacturer's recommendations. RNA integrity was confirmed on the Agilent 2100 BioAnalyzer. Quantitative real-time PCR was performed for 40 cycles using SYBR green (Applied Biosystems) as recommended by the manufacturer, with primers at 100 nmol/L concentrations. Expression was normalized to Transferrin. All transcripts were confirmed using 3% agarose gel electrophoresis.

**Western blotting**

To detect differential expression of pSTAT3 in CD24\(^{−}\) versus CD24\(^{+}\) cells, approximately 3 \(\times\) 10\(^5\) W2476T cells and CD24\(^{-}\) sorted cells were incubated overnight at 37°C and 5% CO\(_2\). Protein lysates were obtained after 1 hour incubation with TG10209 or Stat6 (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad) using Spectra max Stattic (Sigma; ref. 22).

**Drug treatment assays**

W2476T cells \((5 \times 10^5)\) were seeded and treated with increasing concentrations of TG101209 \((0, 0.6, 1, \text{ and } 1.3 \text{ \mu mol/L})\) were administered on day 1 only. Cell number as a percentage of untreated control was assessed with a Cell Countess after 24 hours or FACS evaluated for CD24 expression as above.

**Cells in suspension**

For drug treatment in spheres, W2476T cells were first sorted for CD24-positive and -negative populations and then subjected for sphere assay. Twenty-four hours later, cells were treated with Stat6 or TG101209 for 3 consecutive days and then spheres were counted and subjected to passaging studies as above on day 5 after sorting.

**p-Stat3 quantification**

p-Stat3 density was assessed as previously described (23). Briefly, five independent sections from TG101209 and DMSO-treated tumors \((n = 4/\text{each})\) were stained with anti-p-Stat3 (NEED AB 1:50) and signal detected with DAB as above. Ten high-powered fields from each section were then photographed and DAB stain quantified as previously described using Olympus biologic suites software. Results were compared using a two-sided Student \(t\) test.

**In vitro invasion assays**

W2476T cells \((5 \times 10^5)\) were plated in T25 flasks and pretreated with TG101209 0.6 \text{ \mu mol/L} or DMSO for 24 hours. Cells were trypsinized and plated \(25 \times 10^5\) per chamber in RPMI containing 2\% of FBS and TG101209 \((0.6 \text{ \mu mol/L})\) or DMSO in rehydrated BD BioCoat Matrigel Chamber (BD Biosciences). RPMI containing 10\% of FBS was used as a chemoattractant. Cells were incubated for 24 hours at 37°C and 5% CO\(_2\) and then cells were fixed with cold methanol and stained with Vectashield Mounting Medium for fluorescence with DAPI H-1200 (Vector Laboratories). Five sections from each Matrigel chamber were photographed and then scored for DAPI pixel density using Olympus Microsuite Biological Suite Software. DAPI labeling for TG101209-treated cells and DMSO controls were compared using a two-sided Student \(t\) test.

**Migration assay**

W2476T cells were treated for 24 hours with 0.6 \text{ \mu mol/L} of TG101209. Next day, cells were sorted for CD24\(^{−}\) and CD24\(^{+}\) cells as above and then resuspended in RPMI with 2\% of FBS and 0.6 \text{ \mu mol/L} of TG101209 or vehicle control. Then, cells were loaded in the left end of the migration chip; chips contain 20, 1-mm long channels with a concentration of approximately 1 cell per channel. The right side of the channel was loaded with RPMI 5\% FBS (to create a chemoattractant gradient) containing 0.6 \text{ \mu mol/L} of TG101209. Twenty-four hours later, the movement of cells in the migration channels was measured. Three devices were used per condition in two independent experiments.

**Biostatistics**

All in vitro assays, including FACS analysis of CIC markers, tumor sphere assays, qRT-PCR, cytotoxicity, and invasion assays, are a result triplicate assays in three independent experiments. Data were compared using a two-sided Student \(t\) test with Microsoft Excel software. For murine tumor studies, final tumor volumes/weights were also compared using a two-sided Student \(t\) test as above. For survival studies, the Kaplan–Meier curves were generated and compared using the Mantel–Cox test and \(P\) values were obtained using Prism GraphPad (GraphPad Software).

**Results**

To characterize the various cell populations in the murine Apc\(^{−}\);Pten\(^{−}\);Trp53\(^{−}\) ovarian cancer tumor model, we first used flow cytometry to analyze the expression of commonly reported CIC markers in a tumor cell line (W2476T) derived from the Apc\(^{−}\);Pten\(^{−}\);Trp53\(^{−}\) ovarian cancer tumor model (Fig. 1A and B, i). We detected substantial expression of CD24 (20%–30%), CD44 (22%–35%), and more limited expression of CD90, ALDH, CD117, and CD133 (0.5%–2% each). We also evaluated mechanically dissected Apc\(^{−}\);Pten\(^{−}\);Trp53\(^{−}\) primary tumors and performed FACS analysis. Primary tumors had low viability (20%). Surviving primary cells had higher expression of CD24 (55%–85%) and CD44 (48%–68%) and low expression of CD90 (1%–3%), CD117 (0%–1%), and CD133 (0.5%–1%; Fig. 1B, ii). However, immunohistochemical (IHC) analysis of primary tumors demonstrated significantly lower numbers of CD24\(^{−}\) and CD44\(^{−}\) cells with each labeling 5% to 10% of tumor cells (Fig. 1C). The discrepancy between FACS results and IHC analysis suggests that the process of mechanically dissecting tumors into single cells may enrich for cells expressing CD24 and CD44.

The ability to grow in suspension is an important attribute of ovarian cancer cells. Ovarian cancer cells commonly present in cancer-associated ascites are believed to be a source of both chemotherapy resistance and metastases (24). We next tested the ability...
of FACS-isolated cells expressing the specific cell markers characterized above to generate tumor spheres. CD24$$^{+}$$ and CD133$$^{+}$$ cells generated more primary tumor spheres, and demonstrated a greater ability to passage to form secondary spheres (Fig. 1D).

CD24 identifies a population of cells enriched for tumor initiation capacity

*In vivo* tumor initiation remains the gold standard for the identification of CIC. We therefore sorted luciferase W2476T-Luc cells for each marker and their negative counterparts and assessed tumor initiation capacity. Specifically, CD24$$^{+}$$ versus CD24$$^{-}$$, CD44$$^{+}$$ versus CD44$$^{-}$$, CD90$$^{+}$$ versus CD90$$^{-}$$, CD117$$^{+}$$ versus CD117$$^{-}$$, and ALDH$$^{+}$$ versus ALDH$$^{-}$$ cells were FACS isolated and then 5,000 cells of each population were injected subcutaneously in the axilla of NOG mice ($n = 5$) and tumor formation monitored by bioluminescence. In each mouse, marker positive cells were injected in the right axilla and marker negative cells in the left axilla. CD24$$^{+}$$ cells demonstrated tumor formation earlier and formed statistically larger tumors than CD24$$^{-}$$ cells (Fig. 2A and B). CD133$$^{+}$$ versus CD133$$^{-}$$ cells demonstrated a trend for increased tumor growth (Fig. 2A and B, ii). Neither CD44, CD90, CD117, nor ALDH marker positive versus negative populations were noted to have significantly different tumor growth capacity between marker positive versus marker negative populations ($P > 0.20$; Fig. 2A; Supplementary Fig. S1).

Given the increased tumor growth rate of CD24$$^{+}$$ cells, we next performed limiting dilution tumor initiation studies with 1,500, 600, and 200 CD24$$^{+}$$ versus CD24$$^{-}$$ cells injected into contralateral flanks of mice and monitored for up to 3 months. Two hundred CD24$$^{+}$$ cells had a greater rate of tumor initiation (5
of 5) compared with CD24− cells (2 of 5; Fig. 2C) and Table 1. To confirm these studies, we repeated assays with 200 CD24+ versus CD24− cells injected into separate mice and followed for 5 months. Once again, CD24+ cells demonstrated greater tumor initiation (9 of 10) capacity versus CD24− cells (5 of 10). From the cell line tumor initiation studies using the "extreme limiting dilution analysis" (25) software, we estimate tumor-initiating cell frequency of 1 in 133 (range, 72–238) for CD24+ cells and 1 in 668 (range, 357–1,252) for CD24− cells (P = 0.0000517).

Interestingly, FACS analysis of tumors derived from both CD24+ and CD24− cell initiated tumors had both CD24− and CD24+ cells (Supplementary Fig. S2). Not surprisingly, given the presence of both CD24− and CD24+ cells in all tumors, tumors from both CD24+ and CD24− cells were capable of serial passage through at least three generations.

Next, we similarly assessed the tumor initiation rates of CD24+ cells freshly isolated from Apc−/Pten−/Trp53− tumors. Likely due to the poor viability of primary cells after mechanical cell isolation, significantly higher cell numbers (50,000) were necessary to initiate tumors. However, once again CD24+ cells showed greater
tumor initiation rates compared with CD24− cells counterparts (Table 1).

CD24 cells preferentially express stem cell genes and phosphorylate STAT3

CD24 has been reported as a CIC marker in hepatic cancer and CD24+ cells in hepatic cancer had increased expression of Nanog, and Nanog promoted cellular self-renewal via phosphorylation of STAT3 (26). We therefore evaluated pSTAT3 in CD24+ and CD24− cells. CD24+ cells showed increased basal levels of STAT3 phosphorylation compared with the CD24− cells (Fig. 3B). Treatment with either the direct STAT3 inhibitor Stattic, or the JAK2 inhibitor TG101209, could reduce p-STAT3 levels, though TG101209 inhibited pSTAT3 more efficiently in isolated CD24+ cells than Stattic (Fig. 3B). Treatment of unsorted W2476T cells with increasing doses of TG101209 was associated with significant decreases in cell number with TD50 approximate-ly 880 mmol/L (Fig. 3C). In order to determine the functional importance of STAT3 phosphorylation, we treated CD24+ and CD24− cells in a tumor sphere formation assay either Stattic or TG101209. Both Stattic and TG101209 preferentially reduced primary tumor sphere formation of sorted cells and preferen-tially decreased CD24+ primary sphere growth (Fig. 3D, i). TG101209 treatment was associated with a decrease in the ability of secondary tumor sphere formation from both unsorted and CD24+ cells, and essentially eliminated passing potential (Fig. 3D, ii). Consistent with TG101209 preferentially targeting CD24+ cells, FACS evaluation of W2476T cell line with increasing doses of TG101209 demonstrated declining percentages of CD24+ cells (Fig. 3D, iii).

We also characterized the expression of several known stem cell gene/targets of STAT3 phosphorylation in CD24+ and CD24− populations (Fig. 3A). Unsorted W2476T cells and sorted CD44+ and CD44− cells were used as controls (Supplementary Fig. S3). Although no differential expression of any tested marker was noted between CD44+ and CD44− cells, qRT-PCR demonstrated that CD24+ cells, compared with CD24− cells, had increased expression of Nanog, c-myc, and Cyclin D1.

Therapy with TG101209 prevents metastases and improves survival

In order to confirm antitumor activity of inhibiting STAT3 phosphorylation in vivo, we assessed the impact of TG101209 in growth of established Apc+/−;Pten−/−;Trp53−/− ovarian tumors. Tumors were initiated and monitored until they reached approximately 500 mm3. Tumors were paired on the basis of size and then randomized to cisplatin versus cisplatin plus TG101209 for 21 days. Combinatory treatment of cisplatin plus TG101209 improved survival of mice compared with mice treated with cisplatin only (Fig. 4A).

We next treated mice with “early-stage” ovarian cancers with TG101209 alone. AdCre was injected into the ovarian bursa to initiate tumors and then treatment with TG101209 or vehicle was initiated 1 week after injection and maintained for 21 days (Fig. 4B, i). A subset of animals was sacrificed during treatment and IHC confirmed significant reductions in STAT3 phosphorylation in TG101209-treated animals (Fig. 4D). The remaining mice n = 12 were monitored until they became ill or primary tumor volumes reached humane endpoints for euthanasia per institutional guidelines (−1,000 mm3). TG101209-treated mice demonstrated significantly increased survival P = 0.02 (Fig. 4B, ii). In addition, at the time of sacrifice, control mice were ill appearing with evidence of ascites and bowel obstructions. In contrast, TG101209-treated mice were well at the time of euthanasia but were sacrificed because primary tumor volumes met euthanasia guidelines. At necropsy, vehicle-treated mice had widespread metastatic disease with metastatic nodules in the intestine, liver, peritoneum, adipose, and bladder (Fig. 4C, i−iii). In contrast, only 1 of 14 mice treated with TG101209 had demonstrable metastases. IHC evaluation of tumors suggested that TG101209-treated tumors had a slight increase in microvascular density; however, no difference of CD45, CD31, or CD14 infiltrates, or SMA staining was noted.

Given the decrease in metastasis observed with TG101209 treatment, and the preferential toxicity of TG101209 on CD24+ CIC, we evaluated epithelial–mesenchymal transition (EMT) gene status of CD24+ and CD24− cells. CD24+ cells demonstrated statistically significant increases in expression of the EMT-associated genes Twist1, Snail, and Vimentin (Fig. 5A). No significant difference was observed for epithelial genes (Fig. 5), epithelial genes, such as E-cadherin and cytokeratin-19, were expressed at relatively low levels. Consistent with CD24+ cell-specific depleting effects of TG101209, treatment of W2476T cells with TG101209 resulted in significant reduction in expression of Twist1, Snail, and Vimentin (Fig. 5B).

We next evaluated the impact of TG101209 on cancer cell line invasion capacity using Boyden chamber Matrigel invasion assays. Treatment of W2476T cells with TG101209 was associated with a 2.2-fold decrease in cellular invasion (Fig. 5C). To confirm that reduction in invasion was secondary to JAK2 inhibition and not likely due to off target effects, we performed JAK2 siRNA knockdown (Fig. 5C). qRT-PCR with JAK2 siRNA-treated cells demonstrated a 3.5-fold decrease in JAK2 mRNA (Supplementary Fig. S4). JAK2 siRNA knockdown resulted in a 3.5-fold decrease

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Table 1. Number of tumors initiated from CD24+ and CD24− cell populations from cell line and primary tumors for each cell number. Cells were in contrastral flanks of NOG mice and monitored up to 3 months.

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*Cells injected into separate mice and monitored up to 5 months.
in cellular invasion (Fig. 5C). JAK2 siRNA had no impact on cellular viability relative to controls, suggesting JAK2 knockdown was affecting invasion and not just killing migratory cells (Supplementary Fig. S4). Finally, we assessed the impact of TG101209 on cellular migration in vitro using a microfluidic cell migration device (Fig. 5D). In this device using a serum gradient, cells migrated along a narrow channel and the migrated distance was evaluated via microscopy. Interestingly, TG101209 had no impact on migration of either whole cell line or isolated CD24\(^+\) cells. Taken together, these data support a critical role for JAK2 in ovarian cancer invasion and ultimately metastasis.

**Discussion**

Genetic models of cancer provide a homogeneous and reproducible system to study cancer cell biology in an immunocompetent host. The cancer-initiating capacity of distinct populations of cancer cells has been studied in some murine tumor cell lines (27, 28), and in a K-rasG12D mutation/Pten deletion driven tumor model (29). These studies found that cells enriched for cancer-initiating capacity could be identified by Hoechst dye exclusion (side population cells) or expression of CD24, CD44, and Epcam. We characterized cancer cells in a Apc\(^/-\);Pten\(^/-\);Trp53\(^/-\) murine ovarian cancer model. We found
that CD24+ cells represent a subset of cells with increased tumor sphere-forming and tumor-initiating capacity. CD24+ cells preferentially have increased pSTAT3 and express the stem cell genes Nanog, cyclin-D1, and c-Myc all of which have been previously reported to be regulated by STAT3 (30–33). Cyclin D1 and c-Myc are also upregulated in primary human ovarian tumor spheroids (34).

Interestingly, while CD24+ cells have increased tumor initiation capacity compared with CD24− cells, CD24− cells were capable of initiating tumors. In vitro and in vivo lineage tracking experiments demonstrated that CD24− cells could generate CD24+ cells. There are several possibilities to explain these findings. It is possible that there is a subset of CD24− cells that acts as a cancer stem-like cell that has a slower proliferation rate than the CD24+ cells and thus initiates tumors more slowly than CD24+ cells. Another possible explanation could stochastic events leading to CD24+ cell production of CD24− cells. Finally, the ability of CD24− cells to generate CD24+ cells could be the result of FACS contamination. We have found, and others have similarly reported (35), that FACS sorting has 0.2% to 2% impurity (Supplementary Fig. S5). Double sort purification improves purity, but unfortunately decreases cellular viability. Ultimately, single-cell studies with confirmed purity will be necessary to determine whether this tumor model follows a hierarchical/stem cell model.

In this study, we found that in vitro blockade of pSTAT3 was associated with reduced cell numbers and reductions in invasive capacity. This is consistent with prior studies in ovarian cancer, indicating a critical STAT3 phosphorylation in ovarian cancer cell survival (36).
with a JAK2 inhibitor preferentially induced cytotoxicity in CD24+ CICs. This is analogous to studies in liver, breast, and colon cancer where CIC demonstrate preferential phosphorylation of STAT3 (30, 37, 38). Interestingly, it has been reported that the JAK–STAT pathway is upregulated in metastatic ovarian cancer cells versus primary tissue (39) and we observed that in vivo inhibition of STAT3 phosphorylation with a JAK2 inhibitor was associated with near complete loss of metastasis. This strongly implicates this pathway in ovarian cancer metastasis.

Given the preferential phosphorylation of STAT3 in CD24+ cells, it indirectly implicates CD24+ cells in metastasis. Consistent with this observation, we found that CD24+ cells demonstrated a more "mesenchymal" phenotype, with higher expression of Twist, Snail, and Vimentin. Our work is consistent with numerous reports linking CD24 as a marker of ovarian CIC with an EMT phenotype; Gao and colleagues (9) reported that CD24+ primary human ovarian cancer cells in addition to having the stem cell characteristics of quiescence, chemoresistance, and tumor initiation also exhibited an EMT phenotype, with high invasive capacity (40). Suggesting a direct role for CD24 in EMT, shRNA depletion of CD24 suppressed cell invasion and reversed the EMT phenotype (41). In addition, CD24 expression is reported to be a negative prognostic marker in ovarian cancer (42, 43). Although there is increasing evidence of the role of CD24 in metastasis, the exact mechanism remains uncertain.

CD24 also appears to play an important role in signal transduction to promote metastasis. CD24 is reported to physically interact with c-src in both breast and ovarian cancer to regulate STAT3 (44–46). In hepatocellular carcinoma, CD24 has been shown to promote self-renewal through Nanog mediated by STAT3 phosphorylation (30). Our studies suggest that the activation of STAT3 plays a critical role in CD24+ cell metastasis. STAT3 phosphorylation can be mediated by either Src or JAK2 (30, 47). We did not see significant phosphorylation of Src in

Figure 5.
CD24+ W2476T cells have an EMT phenotype. A, qRT-PCR demonstrating preferential expression of the EMT-associated genes Twist, Snail, and Vimentin in CD24+ versus CD24- W2476T cells. B, qRT-PCR demonstrating treatment of W2476T cells with TG101209 reduces expression of Twist, Snail, and Vimentin. C, DAPI immunofluorescence and quantification of Matrigel-invasive W2476T cells in control and TG101209 W2476T-treated cells, as well as scrambled siRNA control, and Jak2 siRNA-transfected W2476T cells. *P < 0.05. D, i, representative photomicrographs and (ii) summation of migration distance for CD24+ versus CD24- W2476T cells with and without TG101209 treatment in a microfluidic cell migration device.
these tumor cells. Thus, our studies implicate a critical role for JAK2.

It is important to note that TG101209, in addition to inhibiting JAK2, also inhibits FLT3 and RET kinases (48). Although further studies will be necessary to determine whether inhibition of either FLT3 or RET is also contributing to the CD24+/CD44+ cell targeting/metastasis-inhibiting role of TG101209, several lines of evidence suggest a direct role for JAK2/STAT3; similar results were obtained in vitro with both Statitc, a direct pSTAT3 inhibitor and TG101209. Furthermore, siRNA studies knocking down the expression of JAK2, mimicked TG101209 therapy.

Finally, while we did not see a significant change in the numbers of tumor-infiltrating white blood cells in our in vitro studies, we cannot rule out an important contribution of TG101209 activity on the tumor microenvironment contributing to inhibition of metastasis.

In conclusion, we show that in a well-established murine model of ovarian cancer, the CD24+/CD44+ cell population is enriched for cancer initiation capacity. These findings should be very useful for future therapeutic studies targeting ovarian CIC. Using this model, we find that CD24+/CD44+ cells have increased susceptibility to JAK2 inhibition and JAK2 inhibition in vivo significantly restricts metastasis. These data indicate JAK2 as an important therapeutic target in epithelial ovarian carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Molecular Cancer Therapeutics

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