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Characterization of ovarian cancer cell lines as *in vivo* models for preclinical studies

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Abstract

Objective—The value of cell lines for pre-clinical work lies in choosing those with similar characteristics. Selection of cell lines is typically based on patient history, histological subtype at diagnosis, mutation patterns, or signaling pathways. Although recent studies established consensus regarding molecular characteristics of ovarian cancer cell lines, data on *in vivo* tumorigenicity remains only sporadically available, impeding translation of *in vitro* work to xenograft models.

Methods—We introduced 18 ovarian cancer cell lines into athymic nude mice through subcutaneous, intraperitoneal, and ovary intrabursal routes, and observed tumor development over 6 weeks. We also profiled cell line gene expression and identified differentially expressed gene sets based on their ability to form tumors in the subcutaneous or intraperitoneal locations. Representative cell lines were further subjected to proteomic analyses.

Results—Ovarian cancer cell lines showed variable ability to grow in mice when implanted subcutaneous, intraperitoneal, or intrabursal. While some cell lines grew well in both SC and IP locations, others showed a strong propensity to grow in one location only. Gene expression profiles suggested that cell lines showing preference for IP growth had gene expression patterns more similar to primary tumors.

Conclusions—We report the tumorigenicity of 17 human ovarian cancer cell lines and one mouse cell line in three distinct anatomical locations, and associated gene networks. Growth patterns and histopathology, linked to molecular characteristics, provide a valuable resource to the

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research community, and better guide the choice of cell lines for *in vitro* studies to translate efficiently into xenograft testing.

1. Introduction

Ovarian cancer is a disease that is often diagnosed in late stages(1), with metastasis throughout the peritoneal cavity(2). The initial pattern of spread of ovarian cancer follows that of the peritoneal fluid. Tumor deposits travel to the omentum and the peritoneal surface of intestines and liver. In later stages, tumors leave the peritoneal cavity, traveling typically to lymph nodes in the retroperitoneum and into the parenchyma of the liver.

Ovarian cancers include 5 pathologically defined histologic categories. Serous cancers are the most common, and are predominantly high grade. Recent studies support a fallopian tube origin for high grade serous cancers, especially in women who carry a hereditary mutation in BRCA genes(3). Low grade serous cancers may arise in stepwise fashion from fallopian tube fimbriae or ovarian surface epithelium(4). Endometrioid cancers may arise from endometrial surface epithelium shed in retrograde fashion onto the ovaries and peritoneal tissue(5). Clear cell cancers are also associated with endometriosis that has similarly deposited in the peritoneal cavity. Mucinous cancers are even rarer, and may arise from a diverse set of tissue that has localized to an ovarian surface niche(6).

Primary treatment of ovarian cancer includes surgical cytoreduction and cytotoxic chemotherapy with platinum (cisplatin or carboplatin) and taxane (paclitaxel or docetaxel). Unfortunately, relapses are common following initial treatment. Relapses that occur within a year of completing therapy suggest the existence of cancer cells that are resistant to platinum therapy, while those that occur after one year are considered platinum sensitive(7).

Cell lines have been developed from cancers at each point in this disease trajectory, and diagnosed histology. These cell lines are predominantly studied *in vitro*, under standard cell culture conditions. Mouse xenografts have variable success, and have been established in subcutaneous or intraperitoneal locations. Identification of factors associated with disease subtypes and clinical outcome in ovarian cancer cell lines has led pathway-targeted therapeutic advances. As with other systems, most available ovarian cancer cell lines were generated decades ago and variable information exists as to these parameters. Recent studies have reported comprehensive data gathered for the most commonly available ovarian cancer cell lines (8, 9). These comprehensive studies used molecular characteristics such as gene expression patterns, mutations or copy number variation to classify cell lines as representative of particular histologic subtypes. Here, we selected cell lines that represented different subtypes of ovarian cancer, in order to characterize the *in vivo* growth of different models. Clear cell ovarian cancer cell lines have been well characterized in prior studies, but were not included in our panel (10). We included cell lines to represent high grade serous, low grade serous, endometrioid, and undifferentiated to allow comparisons and contrasts among molecular and histologic characteristics. We sought to characterize the growth patterns of 17 commonly used human ovarian cancer cell lines and one mouse line in each of these anatomical locations. We also profiled gene expression and phosphorylated proteins in order to interrogate distinct signaling pathways in these cell lines.

2. Materials and methods

2.1 Cell lines

Cell lines were obtained as gifts, from ATCC or NCI-60 as described in Table 1. Cells were cultured in RPMI medium containing 10% FBS and Pen-Strep and grown under standard conditions, with the following exceptions: MDAH-2994 and ACI-8 were cultured in DMEDM/F12 with 10% FBS and Pen-Strep, TOV-112D was cultured in ATCC Complete Medium with 15% FBS and Pen-Strep. Cell lines were authenticated via Short Tandem Repeat analysis by the Molecular Detection Group, Leidos, Frederick National Laboratory, and compared to ATCC (www.atcc.org), Cell Miner (<http://discover.nci.nih.gov/cellminer>) profile. Cell Lines ACI-8 and ACI-23 were authenticated by J. Risinger prior to gifting. Cells used for tumor induction were tested by MTBM as required by the NCI ACUC Committee and confirmed to contain no mouse viruses.

2.2 Xenografts

Mice were housed and observed according to approved NCI-ACUC guidelines; body weights were taken twice weekly for 5–6 weeks or as required by humane endpoints for all mice.

Subcutaneous: Cells ($1-2 \times 10^6$) were counted and prepared as suspensions in 0.5ml PBS for subcutaneous (flank) injections into 6–8 week old athymic nu/nu female in groups of 5 mice. Subcutaneous tumor caliper measurements were taken twice weekly in mice exhibiting palpable subcutaneous tumors until 5–6 weeks or humane endpoints. Tumor volumes were calculated according to the formula $V = \frac{1}{2} (\text{length} \times \text{width}^2)$.

Intraperitoneal: Cells ($1-2 \times 10^6$) were counted and prepared as suspensions in 0.5ml PBS intraperitoneal injections into 6–8 week old athymic nu/nu female in groups of 5 mice. Tumor burden for mice injected IP was assessed by histopathology pathology analysis after necropsy and collection of peritoneal wall, spleen, liver and diaphragm.

Intrabursal: For ovarian intrabursal injections, 2.5×10^5 cells/5 μ l were introduced to the right side ovarian bursa as described (11), and 5ul PBS introduced into the contralateral ovary of each mouse. At necropsy, the uterus and ovaries were removed for observation. Ovaries having received cell injections and the contralateral ovaries (PBS-injected) were dissected from the uterus and weighed. Ovary wet weight was used as a measure of tumor burden.

2.3 Histopathology and immunohistochemistry

Tissues from IP injected mice were collected at the end of the experiment, FFPE processed and H&E stained. Pathology evaluation was performed to assess xenograft presence in spleen, liver, diaphragm and peritoneal wall or other grossly involved organs. Invasiveness of tumor tissue into organs was scored using a semi-quantitative scale. Fixed Tissues were also stained for tumor markers p53, WT1, PAX8, ARID1A, CA125, PTEN, Cyclin E1, Cytokeratin 7, ER, PR and Ki67 using standard immunochemistry conditions. Marker presence in tumor tissues was assessed using a semi-quantitative intensity scale and localization. Immunohistochemistry (IHC) analysis if PTEN, PAX8, WT1, CA125 and

CyclinE1 was determined by the presence or absence of nuclear or cytoplasmic staining specific to the marker. An immunohistochemical reactivity score (IRS) was determined for Ki67, and CK7. The IRS score was the sum of the overall IHC staining score and the intensity score. IHC staining score consisted of: 0 (<10%), 1 (11–50%), 2 (51–80%), 3 (>80%) and the staining intensity was designated by 0, no stain; 1, weak intensity; 2, moderate intensity; 3, strong intensity. TP53 staining was quantified using the Aperio Imaging Analysis Toolbox, under Aperio nuclear algorithm. The IRS score is reported as for Ki67 and CK7.

2.4 Microarray Analysis

Ovarian cancer cell lines were cultured in standard 2D conditions, and RNA was harvested using (RNeasy, Qiagen) per manufacturer's instructions. Gene expression was profiled by Affymetrix U133plus2.0 arrays. CEL files were imported into GeneSpring software (Agilent), probe levels were normalized by the GC-RMA algorithm, and then the entities with intensity values below 100 were filtered out before significant analysis. Based on the relative degree of tumor formation in mice when injected in specific locations, each cell line was classified as High, Med, or Low. Cell lines were grouped as follows. SC grouping: High (4/4 mice with tumors), Med (1–3/4), or Low (0/4). IP grouping: High (3/3 or 4/4), Med (2–3/4) or Low (0–1/4) based on microscopic tumor formation. Differentially expressed genes were identified by applying a cut-off of 2 fold change with a p-value of less than 0.01. Differentially expressed gene sets then imported into Ingenuity Pathway Analysis software to identify significant pathways.

2.5 Proteomics

For phospho-protein arrays, cells were harvested using cell scraper after 24 hours in standard culture conditions. PathScan RTK signaling antibody array kit was used to detect activated signaling nodes, per manufacturer's instructions (Cell signaling, #7982, Danvers, MA).

For flow cytometry, cells were counted and processed for ALDH enzymatic activity using the ALDEFLUOR kit (STEMCELL Technologies, Vancouver, BC, Canada). Briefly, 1×10^6 cells were incubated in 200ul aldefluor assay buffer with 1ul substrate. Immediately upon adding substrate, half of each sample was transferred to a separate tube for incubation with 1ul DEAB (negative control). Cells were incubated at 37°C for 45 minutes protected from light. Cells not exposed to DEAB were subsequently stained 1:11 with CD133-APC (Miltenyi Biotec, San Diego, CA) and incubated for 30 minutes at 4°C protected from light. Flow cytometry was performed on a BD FACS Calibur using BD CellQuest Pro software (BD Biosciences, San Diego, CA) and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR).

3. Results

3.1 Growth patterns of ovarian cancer xenografts

We attempted to establish subcutaneous xenografts with 17 distinct human ovarian cancer cell lines and one well characterized mouse cell line, ID8 (Table 1). The mouse line and three of the human lines had also been labeled with GFP-luciferase construct for imaging,

and these were also included. Mice tolerated subcutaneous inoculation well and continued to thrive throughout the course of the study, showing less than 3 percent average weight loss at 9–10 days and a subsequent 7% weight gain at the end of the experiment, comparable to control mice (Figure 1A). The subcutaneous xenografts grew with varying efficiency in the mice (Figure 1B and 1C, Table 2). All mice were sacrificed after 6 weeks, according to our approved animal use protocol. The average tumor volume increase was most prominent in cell lines HeyA8, MDAH-2774, and Igrov1.

Twelve of the human ovarian cancer cell lines were engrafted into the intraperitoneal location in a second cohort of mice. Again, the mice tolerated the inoculation, losing less than 3% weight by day 10; average weight gain was 9%, including mice that developed ascites (Figure 1D). Interestingly, some cell lines showed differential ability to form tumors in either the subcutaneous and intraperitoneal locations (Table 2). For example, Hey A8 formed tumors in either location, Igrov1 grew well in subcutaneous location but poorly in the peritoneal location, and Ovar3 did not grow subcutaneously but did form measurable tumors when inoculated intraperitoneally. After euthanasia and necropsy, abdominal tissues were harvested and fixed for analysis of cell line growth patterns *in vivo*, specifically by observing gross lesions and conducting pathologic analysis of microscopic xenograft presence and tumor invasiveness. Peritoneal wall, spleen, liver, and diaphragm were examined as typical locations for xenograft metastasis. Hematoxylin and eosin stains were evaluated for signs of tumor deposit, either adherent to the surface of the organs, or invasive into the organ (Figure 1E). Lesions were quantified and scored by a veterinary pathologist (LTL) (Table 2). Mice receiving intraperitoneal injections were also assessed for the appearance of ascites (Figure 1F).

Given the technical challenges of intrabursal injection, we focused on the cell lines that grew poorly in the subcutaneous location including Caov3, Ovar3, Ovar4, OV90 and also included cell lines Caov4, Skov3-GL, Ovar8, and ACI-23 for comparison. We reasoned that the latter four cell lines that had grown well in subcutaneous grafts would have a high chance of growing in the intrabursal location, and aimed to identify this orthotopic location as an option for quantifying localized tumor mass for the cell lines that grew poorly in other locations. Each mouse had tumor cells injected into the intrabursal sac of one ovary, and saline into the contralateral bursa (see Methods). On average, mice did not lose significant weight following survival surgery the intrabursal inoculation procedure. The immediate weight gain we observed following the procedure is likely due to tissue edema caused by manipulation and administration of topical analgesics (Figure 1G). Tumor burden for mice injected in the intrabursal sac was assessed after necropsy by measuring the wet weight of the ovaries (Figure 1H). Strikingly, OV90 and Caov4 were highly tumorigenic in intrabursal location. In contrast, highly tumorigenic Skov3-GL cell line in subcutaneous did not form tumors in intrabursal within the 6 week period, while Ovar8 and ACI-23 cell lines were able to form measurable tumors in both subcutaneous and intrabursal locations. Interestingly, cell lines Ovar3, Ovar4, and Caov3 were tumorigenic by intraperitoneal location only within the time period of these studies.

3.2 Histology and markers

We analyzed the morphology and protein markers in the cell lines growing in vitro and from intraperitoneal tumors that were able to grow. Cell lines growing in attached conditions in vitro took on a variety of morphologies (Figure 2A). Cells from attached cultures were collected, fixed in formalin and paraffin embedded for immunohistochemistry, as was the tissue collected from intraperitoneal inoculation of cell lines. Histologically, the intraperitoneal tumors appeared as undifferentiated carcinoma, without clear morphology of any human histologic subtype. We stained for p53, WT1, PAX8, and CA125 as markers of high grade serous ovarian cancer (Figure 2B, Table 3). We attempted to measure Cyclin E1, and found it to be present at relatively constant levels in all cell lines, without clear evidence of amplification. Loss of ARID1A or PTEN were sought as markers of endometrioid ovarian cancer. These markers were present in all cell lines except two of the xenografts (PEO1 and ACI-8) appeared to lose staining, discrepant with their appearance from in vitro cultured samples. Ki67 was included as a marker of proliferation.

3.3 Gene expression analysis

Cell lines were categorized as High, Medium or Low based on their ability to form tumors in the subcutaneous or intraperitoneal locations (Table 2). For the grouping of the subcutaneous location, the cut-off of 4 out of 4 mice with tumors, 1–3 out of 4, or 0 out of 4 was applied to group as High, Medium, or Low, respectively. For the intraperitoneal location, High group was assigned when the presence of microscopic tumor formation in 3 out of 3 or 4 out of 4 mice, Medium in 2–3 out of 4, or Low in 0–1 out of 4. By this relative group assignment, each group contains at least 3 cell lines which allows significant analysis in gene expression profiling. Differential gene expression was determined by comparing profiles from cell lines in the three categories. Pairwise comparisons were performed –High vs. Low, High vs. Medium, and Medium vs. Low – for both anatomical locations (Figure 3A). Genes were selected as differentially expressed based on fold-change >2.0 and p-value >0.01 (Supplementary data 1). As indicated, several genes stood out as differentially expressed between the high and low tumorigenic cell lines, but these genes differed in the SC and the IP comparisons. For example, CGN was low in the SC group, but ADAMTS9 and SERPINB5 were down in the IP comparison. Overlap between the comparisons occurred, since these might suggest important biological processes (Figure 3B). For subsequent analyses, the Medium category was excluded, since these were cell lines that could not clearly be classified as either High or Low. Interestingly, there was essentially no overlap in the differentially expressed gene sets between subcutaneous injected comparison of High vs Low and intraperitoneal injected comparison of High vs Low (Figure 3C). Only one gene common in this comparison was *C19ORF40* with reduced expression in highly tumorigenic groups of both SC and IP locations. Interestingly, this gene is amplified in 30 (10%) of 311 cases in The Cancer Genome Atlas dataset and only one case shows deletion (12). The gene panels were examined across 11 cancers in TCGA, including ovarian cancer (Figure 3C). Interestingly, the SC gene pattern did not show significant correlations between the ovarian cancer dataset and most cancer types, and were strongly anti-correlated to basal breast cancers and uterine carcinosarcoma ($p<0.01$). On the other hand, the IP gene pattern in ovarian cancer was positively correlated with uterine carcinosarcoma, glioblastoma, and AML, and showed reverse correlation to most of the other cancers. Taken together, the cell

lines showing preference for IP growth were segregated by gene expression patterns that may be more represented in primary cancers.

3.4 Exploratory protein analyses

As an initial attempt to profile proteomics of ovarian cancer cell lines to correlate with in vivo tumorigenic activity, we analyzed protein lysates from selected four cell lines on phosphoprotein arrays (Supplementary figure 1A, 1B). We reasoned that this baseline profile could be related to in vivo tumorigenicity and may be applicable to drug sensitivity screens. Based on visual examination of significant difference among samples, Src and S6 ribosomal protein activities were mostly varied. Quantification of phosphorylation demonstrated high p-Src in highly tumorigenic cell line MDAH-2774, intermediate levels in moderately tumorigenic TOV-112D and Caov3, and absent in poorly tumorigenic ACI-8. Conversely, the ACI-8 and Caov3 cell lines showed relatively higher levels of p-S6 ribosomal protein compared to low to absent p-S6 in TOV-112D and MDAH-2774. Since S6 ribosomal protein is a downstream target of the mTOR pathway, ACI-8 and Caov3 would be reasonable cell lines to establish preclinical studies. However, both cell lines showed very limited tumorigenic activities in different anatomical locations. Not surprisingly, most highly activated protein on the protein array was Src in MDAH-2774 cells. Therefore, our preliminary proteomics data suggest feasibility of this assay to link with in vivo tumorigenic activity and possible with identification of drug sensitivity, although this will require more intensive validation prior to implementation.

In several models, the tumorigenicity of cell lines has been linked to the fraction of cells with stem-like properties. CD133 expression and ALDH1 activity have both been associated with ovarian cancer cells that have propensity to grow as tumors in mice. We proceeded to measure the expression of these putative markers on selected ovarian cancer cell lines. Interestingly, our preliminary data show that two cell lines with measurable tumorigenicity in the intrabursal location (OV90 and ACI-23) both expressed high levels of CD133 (Supplementary figure 1C). On the other hand, two cell lines that did not produce measurable tumors within 6 weeks of intrabursal inoculation (OVCAR8 and CAOVS3) expressed low CD133 (Supplementary figure 1C). ALDH1 activity did not segregate with the highly tumorigenic cell lines, suggesting that CD133 may be a more reliable marker for the intrabursal location. Future studies are needed to investigate this phenomenon in more depth.

4. Discussion

Ovarian cancer cell lines show variable ability to grow in nude mice, when implanted either subcutaneously, intraperitoneal, or intrabursal. The information gathered in this study can help select cell lines to study *in vitro*, in order to select lines for subsequent *in vivo* experiments. This current work complements the literature in establishing cell lines as in vivo models (13, 14). The use of established cell lines provides a convenient tool that is easily accessible to study in most laboratories, without the need for maintaining colonies of transgenic animals. One must have confidence, however, that the lines provide an adequate model of the human disease under study.

Our results are largely concordant with previously published studies (13, 14). Specifically, we concur that IGROV1 grows well in the subcutaneous location, and expresses PAX8 but not CK7. Notable exceptions are our results with OVCAR3 and OVCAR8 in the subcutaneous location, where we had opposite results (13, 14). Cell line behavior is extremely sensitive to conditions of cell culture prior to engraftment and to the manner of xenograft inoculation. For example, tumorigenicity can be increased by using matrigel. In addition, passage number and source can give rise to different strains of cell lines. In this study, our goal was not to maximize the tumorigenicity of every cell line, but rather to compare relative abilities to form tumors in distinct locations. All of our cell lines were authenticated prior to beginning these experiments. We used the same number of cells (1×10^6) and the same timeframe (6 weeks) for each cell line in order to define relative tumorigenicity. Cell culture conditions did vary according to cell line, as noted in the Methods, but were modified in most cases to decrease this variable across cell lines prior to xenografting. For this reason, our cell culture conditions were slightly different from previously published conditions in some cases. These details may contribute to the differences between our results and those from previously published reports.(8, 9, 15)

While some cell lines grew well in both SC and IP locations, others showed a strong propensity to grow in one location only. This implies an underlying biology that sets up a signaling program for proliferation within a particular microenvironment. Human ovarian cancers grow predominantly in the peritoneal cavity, metastasizing to sites outside the abdomen less commonly and usually after several relapses. Additionally, mouse studies have demonstrated that even with hematogenous dissemination, ovarian cancer cells prefer to home to the intraperitoneal space (16). Therefore, the intraperitoneal environment likely provides an situation that better mimics the original microenvironment from which the cell lines were derived. We examined gene expression profiles of cell lines grown in standard 2D culture conditions, as those used prior to inoculation into mice. Indeed, these gene expression profiles suggest that the cell lines showing preference for IP growth had differential expression of genes that may be more representative of patterns in primary tumors (12, 17).

Notably, CGN, a gene with a role in the formation of tight junctions, was downregulated in tumors that grew well subcutaneously (18). Transcriptional regulator NR2F2 and DEAF1 were noted to be overexpressed. While NR2F2 has been shown to increase recurrences, DEAF1 has not been previously associated with cancer. ITGB4, a mediator for cell-cell or cell-matrix was highly upregulated in these cell lines and targeting ITGB4 has been shown to decrease invasiveness of ovarian cancer. In the IP comparison, genes MSRB3 and MUC1 were upregulated in highly tumorigenic cell lines. Overexpression of MSRB3 has been hypothesized to decrease levels of p53/p27 and MUC1 has been shown to increase metastasis and progression. It should be noted that the metastatic effects of MUC1 has yet to be entirely elucidated and further research is required. In the IP comparison, CCDC92 (19), MSRB3 and MUC1 were upregulated in highly tumorigenic cell lines, while tumor suppressor genes ADAMTS9, an inhibitor of angiogenesis (20), and SERPINB5, a blocker of tumorigenic potential (21) were downregulated. As with MUC1, SERPINB5 requires further research to establish its effects on ovarian cancer. These patterns further support the IP location as a superior model for ovarian cancer because gene expression resembles that of

other characteristics noted in highly metastatic disease. The difference in gene expression between in vitro cultures and in vivo xenografts is the subject of ongoing studies in our laboratory.

Limited proteomic analysis of ovarian cancer cell lines identified that Src, a regulator of proliferation/cell adhesion/cell migration, was highly activated in some cell lines that grew well in the intraperitoneal location. This is consistent with previous findings of focal adhesion and cell surface receptor signaling as top networks in primary ovarian cancers, associated with clinical features (22). In contrast, S6 ribosomal protein phosphorylation was very low in the highly tumorigenic cell line, but relatively higher in the cell lines that were less able to grow in mice. While these data are exploratory and hypothesis-generating, our observations provide thought-provoking ideas that could guide further research. The significance of these findings require further experimental investigation.

Human xenografts into mice are widely used due to their relative accessibility for the study of metastasis patterns, and the in vivo response to drug combinations. The subcutaneous route of inoculation allows ease of monitoring tumor size, and thus response to experimental intervention in the mouse. Human ovarian cancers rarely metastasize to the skin or soft tissue, however, making the subcutaneous location a less representative model. The intraperitoneal location may be a more representative location but presents a challenge to accurate quantitation of tumor burden. The intrabursal route of inoculation may provide a more orthotopic location from which researchers can measure localized tumor growth and evaluate patterns of metastasis throughout the peritoneal cavity. In ongoing and future studies we plan a comprehensive assessment of each of these cell lines when injected into the mouse ovarian bursa. Such labor intensive studies are required to generate reproducible and reliable models of human ovarian cancer, in order to test novel approaches to therapies targeting tumor growth and/or metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Human cell line xenografts provide a valuable tool for studying cancer biology and response to treatments.
- Characterizing growth conditions in mice can speed translation of in vitro work to in vivo testing.
- The tumorigenicity of 17 human ovarian cancer cell lines is variable, and may reflect differing biology of each individual model.

Figure 1. Inoculation of ovarian cancer cell lines

Each ovarian cancer cell line was injected SC into 4 mice, and monitored for 6 weeks. **A.** Mice were weighed twice weekly. Shown is the average weight for each group as compared to initial body weights; average body weights increased by 7% across all mice. **B.** Palpable tumors were detectable at variable onsets ranging from day 8 to day 30 after inoculation. Tumor size was measured by caliper; calculated tumor volumes grew to 11 - 220 mm³ after 6 weeks. Shown is the average measurement for each group. **C.** Representative appearance of subcutaneous tumors in the study. **D.** Each ovarian cancer cell line was injected IP into 4 mice, and monitored for 6 weeks. Mice were weighed twice weekly. Shown is the average weight for each group as compared to initial body weights; average body weights increased by 9% across all mice. **E.** Representative invasive and non-invasive lesions, shown by H&E stains of examined tissues. Arrows indicate areas of tumor invasion into tissue (10X magnification). **F.** Representative appearance of mice receiving intraperitoneal cell injections and developing abdominal ascites in the study. **G.** Each ovarian cancer cell line was injected into the ovarian intrabursal sac of 5 mice, and monitored for 6 weeks; contralateral ovarian intrabursal sac was injected with vehicle only. Mice were weighed twice weekly. Shown is the average weight for each group as compared to initial body weights; average body weights increased by 19% across all mice. **H.** At the end of the study, mice were euthanized and ovaries and uterus removed. Ovaries were dissected and weighed. Average ovary weights and S.E.M. of 5 mice are shown. Error bars represent mean $\pm$ S.E. **I.** Ovaries and uterus photographed. Shown are the cell-line injected ovary (left) and vehicle-injected ovary (right) from representative mice.

Figure 2. Morphology and immunohistochemical analysis of cell lines in vitro and in vivo

A. Cell lines were photographed growing in 2-dimensional attached culture conditions to demonstrate morphologic features. **B.** Immunohistochemical staining of p53, PTEN and CK7 were scored according to intensity and percent cells positive. Shown are representative images for negative, weak or focal, and strongly positive staining. **C.** Shown are representative immunohistochemistry images for positive staining of Ki67, CA125, WT1, PAX8, ARID1A and CyclinE1.

Figure 3. Differential gene expression analysis based on SC and IP tumorigenicities of ovarian cancer cell lines

Cell lines were grouped based on tumor growth in SC xenografts and IP injection. **A.** Highlighted genes are those with fold change >2.0 and p-value <0.01 in either direction (red=up; green=down) based on class comparison. **B.** Up- and Down-regulated genes in each comparison were compared separately and the numbers of overlapped probe sets were shown in Venn diagram. **C.** Differentially expressed genes (probe sets) from SC High vs Low and IP high vs Low were compared, showing essentially no overlap between the genes in the SC and the IP datasets. The gene sets were examined in 11 cancers from TCGA. Calculated correlations between ovarian cancer and each of 10 other cancers are shown.

Table 1**Ovarian cancer cell lines used in this study**

* Histology refers to original citation of each cell line(23–37). Note that more recent studies have disputed these classifications, on a molecular basis. Abbreviations: GL, GFP and Luciferase; G1, grade 1 (well differentiated); G2, grade 2 (moderately differentiated); G3, grade 3 (poorly differentiated); N/A, not available; WT, wild type

Cell line	Source	Histology*	Reference	Tissue site	Prior therapy	p53 protein
HeyA8	E. Kohn, NCI	serous G2	23	papillary deposit	Radiotherapy	P72R *
HeyA8-GL	E. Kohn, NCI	serous G2	23	papillary deposit	Radiotherapy	P72R *
Igrov1	NCI-60	serous G3	24	primary ovarian	None	Y126C *
A2780	E. Kohn, NCI	adenoca	25	"primary tumor"	None	WT
A2780-GL	E. Kohn, NCI	adenoca	25	"primary tumor"	None	WT
Ovar5	NCI-60	adenoca G1	26	N/A	None	Absent *
Ovar8	NCI-60	adenoca G3	26	N/A	Multi-chemo	d (Y126-K132) *
Skov3	ATCC/ HTB-77	serous	27	ascites	ThioTEPA	Absent
Skov3-GL	E. Kohn, NCI	serous	27	ascites	ThioTEPA	Absent
Ovar3	E. Kohn, NCI	serous G2	28	ascites	Multi-chemo	Mutant
Caov3	ATCC/ HTB-75	adenoca	29	N/A	N/A	Mutant
Ovar4	E. Kohn, NCI	adenoca G2	26	N/A	Multi-chemo	WT
Caov4	ATCC/HTB-76	adenoca	30	fallopian tube	N/A	Mutant
PEO1	J. Brenton, UK	serous	31	ascites	Multi-chemo	Mutant **
PEO4	J. Brenton, UK	serous	31	ascites	Multi-chemo	Mutant **
OV90	ATCC/CRL11732	adenoca	32	ascites	None	Mutant
ACI-8	J.I Risinger, MSU	N/A	33	N/A	N/A	N/A
ACI-23	J.I Risinger, MSU	N/A	33	ovary	None	WT
MDAH-2774	ATCC/CRL-10303	endometrioid	34	N/A	N/A	WT
TOV-112D	ATCC/CRL-11731	endometrioid	34	N/A	None	Mutant
ID8	K. Roby, Kansas	epithelial ca	35	mouse ovary	None	N/A
ID8luc2	K. Roby, Kansas	epithelial ca	35	mouse ovary	None	N/A

*
Reference: Kim et al. *Oncotarget*. 2014 Dec 30;5(24):12788-802.
**
Reference : *Oncogene*. 2010 September 2; 29(35): 4905-4913.

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Table 2

Ovarian cancer cell line growth in mice

Ovarian cancer cell lines were classified as High, Medium or Low, based on their ability to form tumors in mice when injected in specified locations. Cell lines were grouped as follows. SC grouping: High (4/4 mice with tumors), Med (1–3/4), or Low (0/4). IP grouping: High (3/3 or 4/4), Med (2–3/4) or Low (0–1/4) based on microscopic tumor formation.

Cell line	Subcutaneous		Intraperitoneal				Intrabursal	
	Tumors	GE Group	Gross	Micro	GE Group	Invasive	Ascites	Tumors
HeyA8	4/4	ND	3/3 *	3/3 *	ND	3/3 *	3/3 *	ND
HeyA8-GL	3/4	ND	ND	ND	ND	ND	ND	ND
Igrov1 **	4/4	High	0/3 *	1/3 *	Low	0/3 *	0/3 *	ND
A2780 **	2/4	Med	3/3 *	3/3 *	High	2/3 *	3/3 *	ND
A2780-GL	0/4	ND	ND	ND	ND	ND	ND	ND
Ovcar5 **	4/4	High	2/4	3/4	Med	2/4	0/4	ND
Ovcar8 **	4/4	High	3/3 *	3/3 *	High	2/3 *	0/3 *	Yes
Skov3 **	4/4	High	4/4	4/4	High	3/4	4/4	ND
Skov3-GL	4/4	ND	4/4	4/4	ND	1/4	0/4	No
Ovcar3 **	0/4	Low	3/4	3/4	Med	1/4	0/4	No
Caov3 **	0/4	Low	0/6 *	5/6 *	Med	0/6 *	0/6 *	No
Ovcar4 **	0/4	Low	3/4	3/4	Med	1/4	0/4	No
Caov4 **	2/4	Med	3/4	4/4	High	2/4	0/4	Yes
PEO1 **	1/4	Med	1/4	1/4	Low	0/4	0/4	ND
PEO4 **	0/4	Low	0/4	0/4	Low	0/4	0/4	ND
OV90 **	0/4	Low	2/4	4/4	High	3/4	0/4	Yes
ACI-8 **	0/4	Low	1/4	1/4	Low	0/4	0/4	ND
ACI-23	3/3 *	ND	0/6 *	3/6 *	ND	0/6 *	0/6 *	Yes
MDAH-2774 **	4/4	High	4/4	4/4	High	4/4	4/4	ND
TOV-112D **	0/4	Low	3/4	3/4	Med	3/4	0/4	ND
ID8	4/4	ND	5/5 *	5/5 *	ND	ND	5/5 *	Yes *

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Cell line	Subcutaneous		Intraperitoneal		Intrabursal	
	Tumors	GE Group	Gross	Micro	GE Group	Ascites
ID8luc2	0/4	ND	ND	ND	ND	ND

GL, GFP and Luciferase; GE, gene expression; ND, not done;

* data from previous studies(38–40);

** Cell lines used in subsequent gene expression analyses.

Table 3
Immunohistochemical markers on cell lines grown in vitro and in vivo

Ovarian cancer cell lines were grown in 2D culture and harvested pellet was formalin fixed and paraffin embedded (pellet). Cell lines were also injected into the peritoneal cavity (xenograft). Cells from each location were stained for the indicated markers, and scored as per Methods. P, present; A, absent; -, not done; number indicates IRS score.

Cell line	specimen source	PTEN	PAX8	ARID1A	CA125	WT1	CyclinE1	CK7	Ki67	p53
HeyA8	pellet	P	P	P	P	P	P	5	6	5
Igrov1	pellet	P	P	P	P	P	P	0	6	4
A2780	pellet	P	P	P	P	P	P	0	6	4
Ovcar5	pellet	P	P	P	A	P	P	0	6	-
	xenograft	P	A	P	P	P	P	5	5	-
Ovcar8	pellet	-	-	-	-	-	-	-	-	6
Skov3	pellet	P	A	P	A	P	P	0	6	1
	xenograft	P	P	P	P	P	P	5	5	-
Ovcar3	pellet	P	P	P	A	P	P	4	6	5
	xenograft	P	P	P	P	P	P	4	4	-
Caov3	pellet	P	P	P	P	P	P	5	6	2
Ovcar4	pellet	-	-	-	-	-	-	-	-	6
	xenograft	P	A	P	A	P	P	2	2	-
Caov4	pellet	P	P	P	A	A	P	2	6	-
PEO1	pellet	P	P	P	P	P	P	5	6	-
	xenograft	P	A	A	A	P	P	0	2	-
PEO4	pellet	P	P	P	P	P	P	6	6	-
OV90	pellet	P	-	P	P	P	P	0	6	-
	xenograft	P	-	P	A	P	P	0	6	-
ACI-8	pellet	P	P	P	P	P	P	6	6	5
	xenograft	P	A	A	A	P	P	0	6	-
ACI-23	pellet	-	-	-	-	-	-	-	-	4
MDAH-2774	pellet	-	-	-	-	-	-	-	-	6
	xenograft	P	P	P	A	P	P	4	4	-

-	5	0	P	P	A	P	P	P		TOV-112D
6	-	-	-	-	-	-	-	-	pellet xenograft	

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