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Characterization and Targeting of the Aldehyde Dehydrogenase Subpopulation in Ovarian Cancer

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Ovarian cancer, aldehyde dehydrogenase, ALDH1A1, cancer stem cell

Abstract on next page.
In the first year of our grant, we have demonstrated that ALDH-positive cells from the A2780cp20 and SKOV3TRip2 cell lines have approximately 50-fold increased tumorigenicity compared to ALDH-negative cells. Additionally, tumors that formed after ALDH-positive cells were injected were composed of both ALDH-positive and ALDH-negative cells, demonstrating multipotentiality of these cells. By contrast, tumors that formed after injection of ALDH-negative cells were composed of only ALDH-negative cells, demonstrating that tumorigenicity is not absolute, but ALDH-negative cells lack such differentiating capacity. In separate experiments, mice injected intraperitoneally with A2780cp20 or SKOV3TRip2 cells were treated with ALDH1A1-targeting siRNA incorporated into DOPC liposomes, with or without chemotherapy. While downregulation of ALDH1A1 alone did not have a significant effect on tumor growth, it did sensitizze these normally-resistant cell lines to cisplatin or paclitaxel, respectively. Finally, a cohort of high-grade epithelial ovarian cancer patient specimens were examined, and we noted that patients with higher density of ALDH-positive cells had shorter progressive-free survival than those with smaller percentages of ALDH1A1. These studies demonstrate that ALDH1A1-positive cells are more aggressive, contribute to poor patient outcomes, and contribute to chemoresistance, but these effects can be reversed by downregulating ALDH1A1 expression.
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Characterization and targeting of the ALDH subpopulation in ovarian cancer
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University of Alabama at Birmingham, Birmingham, AL
Ovarian Cancer Academy OC093443 2010-2011 Annual Report

INTRODUCTION:

While most ovarian cancer patients initially respond to chemotherapy, most will ultimately recur and succumb to disease, suggesting that there is a subpopulation of cells within a heterogeneous tumor that has either inherent or acquired resistance to chemotherapy\(^1\). Recently subpopulations of cancer cells in solid tumors have been observed to have properties of stem cells, and therefore designated as “cancer stem cells” (CSC’s) or tumor initiating cells (TIC’s) \(^2\,^3\). The intent of this project is to characterize whether ovarian cells that express aldehyde dehydrogenase (ALDH1A1) have cancer stem cell properties, and if targeting ALDH1A1 would lead to a reversal of the chemoresistant properties. Characteristics of cancer stem cell that will be assessed include tumorigenicity experiments, evidence of multipotentiality, and enhanced resistance to chemotherapeutics. The effects of ALDH1A1 downregulation will be determined both \textit{in vitro} and \textit{in vivo}, using small interfering RNA (siRNA) encapsulated in nanoparticles that allow efficient in vivo delivery. If our hypotheses are confirmed, we will have identified a subpopulation of ovarian cancer cells that might survive initial chemotherapy and contribute to resistance, and further more may find a clinically feasible novel methodology to target these cells to improve outcomes in this devastating disease.

BODY:

Task 1: Determine tumorigenicity of ALDH1A1 subpopulations

The goal of task 1 was to determine the tumorigenicity of ALDH1A1 subpopulations. We first injected, in limiting dilutions, sorted ALDH1A1-positive and ALDH1A1-negative populations (based on the ALDEFLUOR assay) of previously-collected and stored primary ovarian cancer specimens. Viability of these cells based on PI exclusion appeared good. Unfortunately, tumors failed to grow in either population. Therefore we changed our initial focus to examine sorted populations from two cell lines with ALDH1A1 activity, A2780cp20 and SKOV3TRip2 (Figure 2B-D of appended manuscript).

We sorted ALDH1A1-positive and negative populations from the A2780cp20 cell line using the ALDEFLUOR assay and injected cells intraperitoneally into NOD-Scid mice in limiting dilutions to determine tumor initiating potential (for methods, see appended manuscript) \(^4\). As summarized in Table 1, ALDEFLUOR-positive cells exhibited increased tumorigenic potential, with 100% tumor initiation after injection of 100,000, 25,000, or 5,000 cells, and 1 tumor established after 1,000 cells injected. ALDEFLUOR-negative cells were also able to form tumors, although at a lower rate: two of 5 mice formed tumors after injection of 25,000 or 100,000 cells, and no tumors formed after injection of 5,000 or 1,000 cells. Mice were followed for 1 year after injection, and thorough necropsies were performed in remaining mice to confirm

\[1\]
that tumors failed to develop. The TD50, or dose of cells required to permit tumor formation in 50% of animals, was 50-fold lower with ALDEFLUOR-positive cells.

Table 1. Tumorigenicity of ALDH1A1-positive and ALDH1A1-negative cells.

<table>
<thead>
<tr>
<th>A2780cp20 cells injected IP</th>
<th>1 mil</th>
<th>250k</th>
<th>100k</th>
<th>25k</th>
<th>5k</th>
<th>1k</th>
<th>Serial transplantation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDEFLUOR-negative</td>
<td>5/5</td>
<td>4/5</td>
<td>2/5</td>
<td>2/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>ALDEFLUOR-positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5/5</td>
</tr>
</tbody>
</table>

Perhaps more striking was the make-up of these tumors. One requirement of a tumor-initiating population is that they have the capacity to give rise to heterogeneous tumors, composed of both stem cell and non-stem cell populations, therefore demonstrating multipotent differentiating potential. This was noted in tumors that formed after injection of ALDEFLUOR-positive cells. In all 16 of these tumors, a strongly-positive ALDH1A1 population was noted in the minority of the sample, on average 4.7% of the tumor (range 2.4-6.1%, Figure 4A of appended manuscript). However, no ALDEFLUOR-positive cells were found in the tumors that formed after injection of ALDH1A1-negative cells (Figure 4B). This was confirmed with IHC (Figure 4C,D). This argues against the idea that tumors formed because of contamination with ALDEFLUOR-positive cells, or that ALDH1A1 expression is simply induced by the tumor microenvironment regardless of the capacity of the cells.

This difference in the capacity to generate ALDEFLUOR-positive cells was also noted in vitro. SKOV3TRip2 cells sorted into ALDEFLUOR-positive and negative populations were cultured separately, and the ALDEFLUOR assay performed on the different populations at 24, 48, and 72 hours (Figure 4E,F). Of the ALDEFLUOR-positive cells, the population gradually reverted to 75.3%, 54.2%, and 51.4% ALDEFLUOR-positive, respectively for each timepoint. However, the ALDEFLUOR-negative cells could not produce any ALDEFLUOR-positive cells.

Future studies in Task 1 will include confirmation that ALDH1A1-positive cells have enhanced tumorigenicity from patient samples. In order to increase the viability and tumor-generating capacity of cells injected, we will use primary xenografts for this purpose. As describe in further detail under Task 2, these xenografts are in the process of being established.

Task 2: Determine if ALDH1-positive cells survive chemotherapy in the tumor microenvironment.

After obtaining IRB and IACUC approval, we have begun collecting primary ovarian cancer specimens and transplanting them directly in SCID mice. Our protocols have evolved in the last several months. Initially we used NOD-SCID mice, but because of the time often required for tumor formation, many were dying due to spontaneous development of lymphoma. Changing to use of SCID mice has significantly reduced this problem.

We have examined which sites of implantation are optimal for xenograft formation. We have implanted and compared growth in four sites: 1) subcutaneous, 2) subrenal capsule, 3) intraperitoneal, and 4) mammary fat pad. After attempts in 17 patients, these respective sites have yielded take rates of 76%, 12%, 12%, and 58%, respectively. In the last 10 patients, a xenograft has grown from at least one site in 9 patients (90%). We are in the process of further
developing our protocols to where these xenografts can reliably be maintained, expanded to more mice, and frozen for later reimplantation.

In 10 mice, we have had enough mice develop tumors that that have been randomized to treatment with saline, maximum-tolerated-dose (MTD) combination carboplatin plus paclitaxel, and low-dose carboplatin plus paclitaxel (10% of MTD dosing). After a positive response was obtained in the MTD dosing, tumors were collected in multiple formats. Now that an adequate cohort is available, we will begin testing these tumors to see if they are enriched with putative cancer stem cells, to include ALDH1A1 expression.

**Task 3: Target ALDH1 with siRNA in vivo**

There are no known inhibitors of ALDH1A1 for *in vivo* studies. Therefore, after IACUC approval, we utilized a method for delivery of siRNA *in vivo* using DOPC nanoparticles. We and others 5-9 have previously demonstrated delivery of siRNA incorporated into DOPC nanoliposomes to the tumor parenchyma with subsequent target downregulation. In this study nude mice were injected intraperitoneally with either SKOV3TRip2 or A2780cp20 cells and randomized to four treatment groups to begin 1 week after cell injection: 1) control siRNA in DOPC, delivered IP twice per week; 2) docetaxel 35 mg, delivered IP weekly (for SKOV3TRip2 model) or cisplatin 160 µg, delivered IP weekly (for A2780cp20 model); 3) ALDH1A1-siRNA in DOPC, IP twice per week; or 4) ALDH1A1-siRNA in DOPC plus docetaxel (for SKOV3TRip2) or cisplatin (for A2780cp20). After four weeks of treatment, mice were sacrificed and total tumor weight recorded. Immunohistochemical analysis confirmed reduced ALDH1A1 expression with ALDH1A1-siRNA/DOPC treatment compared to controls but not with chemotherapy alone. In SKOV3TRip2 xenografts (Figure 5F in appended manuscript) there was a non-significant reduction in tumor growth with docetaxel treatment of 37.0% (p=0.17) and with ALDH1A1 siRNA treatment of 25.0% (p=0.38) compared to control-DOPC. The observation that ALDH1A1 downregulation alone significantly decreased SKOV3TRip2 growth *in vitro* but was less pronounced *in vivo* suggests that tumor microenvironment factors such as supporting stromal cells may be able to protect cells from ALDH1A1 depletion. However, the combination of ALDH1A1 siRNA and docetaxel resulted in significantly reduced growth, by 93.6% compared to control siRNA (p<0.001), by 89.8% compared to docetaxel plus control siRNA (p=0.003), and by 91.4% compared to ALDH1A1 siRNA (p=0.002). In A2780cp20 (Figure 5G in appended manuscript), there was a similar non-significant reduction in tumor weight with cisplatin alone of 43.9% (p=0.32) and with ALDH1A1 siRNA treatment of 57.0% (p=0.19). These effects may be even less significant than the mean tumor weights suggest, given the presence of two especially large tumors in the control siRNA group. However, again combined therapy showed a sensitization to chemotherapy with ALDH1A1 siRNA, with combination therapy reducing growth by 85.0% compared to control siRNA (p=0.048), by 73.4% compared to cisplatin plus control siRNA (p=0.013), and by 65.3% compared to ALDH1A1 siRNA alone (p=0.039). Given the minimal effects of either single agent and the consistent finding of significant improvement with combined therapy, these data suggest a synergy between ALDH1A1 downregulation and both taxane and platinum chemotherapeutic agents, though formal dose-finding experiments would be required to definitively prove synergy.

Further analysis of these tumors will include an exploration of mechanisms whereby ALDH1A1 might be sensitizing tumors to both platinum and taxane chemotherapeutic agents.
Task 4: Evaluate mechanisms of ALDH1-mediated chemoresistance

We have sorted the A2780cp20 cell lines based on ALDH1A1 activity, as defined by the ALDEFLUOR assay. mRNA was extracted by the Trizol method, and submitted to our core facility for microarray analysis with the Illumina Human 12 chip. These data are not yet available. Future work on this task will include statistical analysis of these gene sets, and validation of genes of interest.

We have attempted to transfect the ALDH1A1-negative A2780ip2 cell line with a construct producing ALDH1A1. The construct was obtained through Addgene (plasmid #11610), produced in the laboratory of Dr. Steven Johnson. Our initial confirmation that selected surviving cells had ALDH1 production was negative. We are in the process of repeating the transfection, in order to determine the effects of forced overexpression of ALDH1A1 in a null line.

**KEY RESEARCH ACCOMPLISHMENTS:**

- ALDH-positive cells from the A2780cp20 and SKOV3TRip2 cell lines have approximately 50-fold increased tumorigenicity compared to ALDH-negative cells.
- Tumors that form after ALDH-positive cells are injected are composed of both ALDH-positive and ALDH-negative cells, demonstrating multipotentiality of these cells.
- Tumors that form after injection of ALDH-negative cells were composed of only ALDH-negative cells, demonstrating that tumorigenicity is not absolute, but ALDH-negative cells lack such differentiating capacity.
- Treatment of tumor-bearing mice with ALDH1A1-targeting siRNA incorporated into DOPC liposomes had a minimal effect on tumor growth.
- Treatment of tumor-bearing mice with ALDH1A1-targeting siRNA in DOPC sensitized normally-resistant cell lines to cisplatin or paclitaxel.
- High-grade epithelial ovarian cancer patient specimens with higher density of ALDH-positive cells have a shorter progressive-free survival than those with smaller percentages of ALDH1A1.
- With increased experience of direct implantation of fresh patient tumors into SCID mice, we now have an approximate 80% success rate in generation of primary xenografts

**REPORTABLE OUTCOMES:**

- Publication in Molecular Cancer Therapeutics, manuscript chosen for issue highlights and cover art:
Abstracts presented:


Grants awarded for which data generated by this work contributed preliminary data:


Funding applied for and not accepted:


- CDMRP DOD Consortium Award, 2010: Collaborator, “Early Events in Ovarian Cancer Pathogenesis.”


- CDMRP DOD OCRP Translational Leverage Award, 2011: Principle Investigator, “Utilization of primary xenograft and nanoparticle siRNA resources to test personalized therapeutics in ovarian cancer.”

Assets:

- Established reliable method of developing primary ovarian cancer xenografts into SCID mice that may be an important tool for future studies, such as examination of methodologies for testing personalized medicine.

- Have approximately 15 primary xenografts maintained in mice without having ever been cultured *in vitro*. 
CONCLUSIONS:

Our findings have demonstrated that ALDH1A1-positive cells are more tumorigenic than ALDH1A1-negative cells, contribute to poor patient outcomes, and contribute to chemoresistance. Importantly, these effects can be reversed by downregulating ALDH1A1 expression with nanoparticle-delivered siRNA. We have also shown that tumors that form after injection of ALDH1A1-negative cells are exclusively composed of ALDH1A1-negative cells, eliminating the possibility that tumors form simply due to contamination of the sorted cells with ALDH1A1-positive cells. A limitation of these findings to identifying the most significant ovarian cancer subpopulation is that ALDH1A1-negative cells are also tumorigenic, and therefore ALDH1A1 is not exclusively the tumorigenic population. Additional studies will attempt to delineate the contribution of ALDH1A1 as well as other subpopulations to the survival of cancer cells after chemotherapy in vivo, such as CD133 and CD44. Addition work in the coming years will also attempt to define the important signaling components within ALDH1A1-positive cells that contribute to the chemoresistant and tumorigenic phenotype.

REFERENCES:


APPENDICES:


- Appendix 2: Curriculum Vitae, Charles N. Landen, Jr.
Targeting Aldehyde Dehydrogenase Cancer Stem Cells in Ovarian Cancer


*Mol Cancer Ther* 2010;9:3186-3199. Published OnlineFirst October 1, 2010.
Targeting Aldehyde Dehydrogenase Cancer Stem Cells in Ovarian Cancer

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Abstract

Aldehyde dehydrogenase-1A1 (ALDH1A1) expression characterizes a subpopulation of cells with tumor-initiating or cancer stem cell properties in several malignancies. Our goal was to characterize the phenotype of ALDH1A1-positive ovarian cancer cells and examine the biological effects of ALDH1A1 gene silencing. In our analysis of multiple ovarian cancer cell lines, we found that ALDH1A1 expression and activity was significantly higher in taxane- and platinum-resistant cell lines. In patient samples, 72.9% of ovarian cancers had ALDH1A1 expression in which the percentage of ALDH1A1-positive cells correlated negatively with progression-free survival (6.05 vs. 13.81 months; \( P < 0.035 \)). Subpopulations of A2780cp20 cells with ALDH1A1 activity were isolated for orthotopic tumor–initiating studies, where tumorigenicity was approximately 50-fold higher with ALDH1A1-positive cells. Interestingly, tumors derived from ALDH1A1-positive cells gave rise to both ALDH1A1-positive and ALDH1A1-negative populations, but ALDH1A1-negative cells could not generate ALDH1A1-positive cells. In an \( \text{in vivo} \) orthotopic mouse model of ovarian cancer, ALDH1A1 silencing using nanoliposomal siRNA sensitized both taxane- and platinum-resistant cell lines to chemotherapy, significantly reducing tumor growth in mice compared with chemotherapy alone (a 74%–90% reduction; \( P < 0.015 \)). These data show that the ALDH1A1 subpopulation is associated with chemoresistance and outcome in ovarian cancer patients, and targeting ALDH1A1 sensitizes resistant cells to chemotherapy. ALDH1A1-positive cells have enhanced, but not absolute, tumorigenicity but do have differentiation capacity lacking in ALDH1A1-negative cells. This enzyme may be important for identification and targeting of chemoresistant cell populations in ovarian cancer. \( \text{Mol Cancer Ther}; 9(12); 3186–99. \) ©2010 AACR.

Introduction

Ovarian cancer was expected to be diagnosed in 21,550 women in 2009 and take the lives of 14,600 women (1). Although ovarian cancer is among the most chemosensitive malignancies at the time of initial treatment (surgery and taxane/platinum-based chemotherapy), most patients will develop tumor recurrence and succumb to chemoresistant disease (2). An understanding of the mechanisms mediating survival of subpopulations of ovarian cancer cells is necessary to significantly improve outcomes in this disease.

In many malignancies, a subpopulation of malignant cells termed cancer stem cells or tumor-initiating cells has been hypothesized to represent the most tumorigenic and treatment-resistant cells within a heterogeneous tumor mass. Defined by their enhanced ability to generate murine xenografts and give rise to heterogeneous tumors that are composed of both tumor-initiating cell and non-tumor-initiating cell populations, these cells may also be more chemoresistant and depend on unique biological processes compared with the majority of tumor cells (3, 4). In ovarian cancer, many of these properties have been identified in populations of CD44/c-kit–positive cells (5), CD133-positive cells (6–8), and Hoechst-excluding cells (the side population; ref. 9).

Among several markers that have been used to identify cancer stem cells, aldehyde dehydrogenase-1A1
(ALDH1A1) has been a valid marker among several malignant and nonmalignant tissues (10–20). It holds the attractive distinction of not only being a potential marker of stemness but potentially playing a role in the biology of tumor-initiating cells as well (10). ALDH1A1, 1 of 17 ALDH isoforms, is an intracellular enzyme that oxidizes aldehydes, serving a detoxifying role, and converts retinol to retinoic acid, mediating control on differentiation pathways. The ALDH1A1 population defines normal hematopoietic stem cells, being used to isolate cells for stem cell transplants in patients. Using the ALDEFLUOR assay, a functional flow cytometric assay that identifies cells with active ALDH1A1, tumor-initiating cell-enriched populations have been identified in multiple malignancies (20), including breast (11–14), colon (15, 16), pancreas (17), lung (18), and liver (19). Whether or not the ALDH1A1-active population is enriched for tumor-initiating cells has not been demonstrated for ovarian cancer. More importantly, although ALDH1A1 is implicated in chemoresistance pathways, it is not known whether targeting ALDH1A1 can sensitize resistant cells to chemotherapy and therefore represent a potential target for cancer stem cell–directed therapy. We sought to characterize expression of ALDH1A1 in ovarian cancer cell lines and patient samples, determine whether it contains tumor-initiating cell properties, and examine whether targeting ALDH1A1 sensitizes cells to chemotherapy in both in vitro and in vivo ovarian cancer models.

Materials and Methods

Cell lines and culture
The ovarian cancer cell lines SKOV3ip1, SKOV3-TRip2, HeyA8, HeyA8MDR, A2780ip2, A2780cp20, IGROV-AFI, and IGROV-cp20 (21, 22) were maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum (Hyclone). SKOV3TRip2 [taxane-resistant, a kind gift of Dr. Michael Seiden (23)] and HeyA8MDR were maintained with the addition of 150 nmol/L of paclitaxel. The HIO-180 SV40-immortalized, nontumorigenic cell line derived from normal ovarian surface epithelium was a kind gift of Dr. Andrew Godwin. All cell lines were routinely screened for Mycoplasma species (GenProbe detection kit) with experiments done at 70% to 80% confluent cultures. Purity of cell lines was confirmed with STR genomic analysis, and cells used were always less than 20 passages from the stocks tested for purity.

Whole genomic analysis
RNA was extracted from 3 independent collections of SKOV3ip1 and SKOV3TRip2 cells at 80% confluence with the RNAasy Mini kit (Qiagen). It was subjected to microarray analysis using the Illumina HumanRef-8 Expression BeadChip, which targets ~24,500 well-annotated transcripts. Microarray data were normalized by the cubic-spline method (24) using the Illumina BeadStudio software. The significance of differentially expressed genes was determined by Student’s t test followed by correction for false discovery (25). A heat map was generated using Cluster 3.0 and Java TreeView software. The array data have been registered with GEO (accession #GSE23779) for public access.

Western blot analysis
Cultured cell lysates were collected in modified radioimmunoprecipitation assay lysis buffer with protease inhibitor cocktail (Roche) and subjected to immunoblot analysis by standard techniques (26) using anti-ALDH1A1 antibody (BD Biosciences) at 1:1,000 dilution overnight at 4°C, or anti–β-actin antibody (Sigma Chemical) at 1:2,000.

Immunohistochemical staining and clinical correlations
Immunohistochemical (IHC) analysis was done on formalin-fixed, paraffin-embedded samples, using standard techniques (26). For ALDH1A1, antigen retrieval was in citrate buffer for 45 minutes in an atmospheric pressure steamer, using anti-ALDH1A1 antibody (BD Biosciences) at 1:500 dilution in Cyto-Q reagent (Innovex Biosciences) overnight at 4°C. Primary antibody detection was with Mach 4 HRP polymer (BioCare Medical) for 20 minutes at room temperature, followed by diaminobenzidine incubation. After IHC staining, the number of tumor cells positive for ALDH1A1 was counted and expressed as a percentage of all tumor cells by an examiner blinded to clinical outcome. Patient samples were categorized as having low (<1%), intermediate (1%–20%), or high (21%–100%) ALDH1A1 expression. The IHC analysis was done on samples collected at primary debulking surgery on 65 untreated patients with stage III–IV, high-grade papillary serous adenocarcinoma; with institutional review board approval, clinical information was collected. Progression-free and overall survival were plotted with the Kaplan–Meier method for patients in each group of ALDH1A1 expression and compared with the log-rank statistic by using PASW 17.0.

For dual staining of ALDH1A1 and CD68 (for macrophages), staining for ALDH1A1 was done first as previously, followed by exposure to anti-CD68 antibody (1:4,000; Dako) and goat anti-mouse-AP (Jackson Immunoresearch). AP was developed with Ferangi blue chromagen kit (Biocare Medical). For dual staining of ALDH1A1 and hypoxic tumor regions, mice bearing SKOV3TRip2 xenografts were injected with 60 mg/kg of Hypoxyprobe-1 reagent (HPI, Inc.). Tumor sections in FFPE were subjected to antigen retrieval as above, followed by exposure to fluorescein isothiocyanate (FITC)-conjugated anti-hypoxyprobe-1 mouse antibody (1:50) overnight at 4°C. This was detected with HRP-conjugated anti-FITC antibody (1:500, Jackson Immunoresearch) and DAB resolution. Endogenous murine
IgG was then blocked with anti-mouse IgG F(ab')2 fragments (Jackson ImmunoResearch), and ALDH1A1 stained as above using AP-conjugated anti-mouse IgG and Ferangi Blue chromagen.

**ALDEFLUOR assay and tumorigenicity in limiting dilutions**

Active ALDH1A1 was identified with the ALDEFLUOR assay according to manufacturer’s instructions (StemCell Technologies). The ALDH1A1-positive population was defined by cells with increased FITC signal, with gates determined by diethylenediaminobenzaldehyde (DEAB)-treated cells (DEAB being an inhibitor of ALDH1A1 activity). For tumorigenicity experiments, the ALDEFLUOR-positive population from A2780cp20 cells were sorted with a FACS Aria II flow cytometer (BD Biosciences) and reanalyzed to confirm at least 95% positivity. Collected cells were washed and resuspended in Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution (HBSS; Gibco) and injected intraperitoneally into NOD-SCID mice in limiting dilutions. Mice were followed for 1 year or until tumors formed, then sacrificed and tumor confirmed histologically. For flow cytometric analysis of these tumors, xenografts were dissociated mechanically with a scalpel, passed through a 70-μm filter to collect single-cell suspensions, with the remaining clumped cells cultured in 0.5 mg/mL of collagenase and 0.0369 mg/mL of hyaluronidase (Calbiochem) for 30 minutes at 37°C. These chemically digested cells were again filtered through a 70 μm filter, added to the initial collection and subjected to the ALDEFLUOR assay. ALDEFLUOR-positive cells or negative cells were then injected into additional mice (n = 5) to examine maintenance of tumorigenicity.

**Primary xenograft development**

With institutional IRB and IACUC approval, excess of freshly collected omental metastases from advanced stage ovarian cancer patients were acquired after tissue required for diagnosis and management had been sequestered. 3 to 4-mm³ sections were cut and implanted subcutaneously on the dorsal aspect of NOD-SCID mice. Adjacent sections were submitted for histologic analysis to confirm tumor. Tumors were measured in 2 dimensions twice per week. After progressive growth was noted, mice with formed tumors were treated with vehicle or cisplatin (7.5 mg/kg weekly by intraperitoneal administration for Accreditation of Laboratory Animal Care. For all in vivo experiments, trypsinized cells were suspended in HBSS and 10⁶ cells injected intraperitoneally into 40 mice per experiment. After 1 week, mice were randomized to: a) control siRNA/DOPC, b) control siRNA/DOPC plus chemotherapy, c) ALDH1A1-targeting siRNA/DOPC, or d) chemotherapy plus ALDH1A1-targeting siRNA/DOPC. SiRNA/DOPC dose was 5 μg twice per week in a volume of 100 μL intraperitoneally. Chemotherapy doses were docetaxel 35 μg intraperitoneally weekly for SKOV3Trp2, or cisplatin 160 μg intraperitoneally weekly for A2780cp20. Mice were treated for 4 weeks before sacrifice and tumor collection. SiRNA was incorporated into 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) neutral nanoliposomes as previously described (28), lyophilized, and reconstituted in 0.9% saline for administration.

**Assessment of cell viability with chemotherapy IC₅₀ and cell-cycle analysis**

To a 96-well plate, 2,000 cells per well were exposed to increasing concentrations of docetaxel or cisplatin in triplicates. Viability was assessed by 2-hour incubation with 0.15% MTT (Sigma) and spectrophotometric analysis at OD₄₅₀ (optical density at 450 nm). For effects of siRNA on IC₅₀, cells were incubated with siRNA for 24 hours in 6-well plates and then replated in 96-well plates, and chemotherapy was administered after 12 hours to allow attachment. IC₅₀ was determined by finding the dose at which the drug had 50% of its effect and calculated by the following equation: IC₅₀ = [(OD₄₅₀max − OD₄₅₀min)/2] + OD₄₅₀min. Test of synergy was according to the Loewe additivity model (27) and calculated by the following equation: combination index (CI) = [D₁/D₁c] + [D₂/D₂c] (where CI ≥ 1 suggests an additive effect, <1 suggests synergy, and ≥1 suggests antagonism). For cell-cycle analysis, cells were transfected with siRNA as described previously for 72 hours, trypsinized, washed in PBS, and fixed in 75% ethanol overnight. Cells were then centrifuged, washed twice in PBS, and reconstituted in PBS with 50 μg/mL of propidium iodide. Propidium iodide fluorescence was assessed by flow cytometry, and percentage of cells in each cycle was calculated by the cell-cycle analysis module for FlowJo.

**Orthotopic ovarian cancer model and in vivo delivery of siRNA**

For orthotopic therapy experiments using ovarian cancer cell lines, female athymic nude mice (Ncr-nu) were purchased from the National Cancer Institute and cared for in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care. For all in vivo experiments, trypsinized cells were suspended in HBSS and 10⁶ cells injected intraperitoneally into 40 mice per experiment. After 1 week, mice were randomized to: a) control siRNA/DOPC, b) control siRNA/DOPC plus chemotherapy, c) ALDH1A1-targeting siRNA/DOPC, or d) chemotherapy plus ALDH1A1-targeting siRNA/DOPC. SiRNA/DOPC dose was 5 μg twice per week in a volume of 100 μL intraperitoneally.
Statistical analysis

Comparisons between treatment groups of tumor weight was carried out with the 2-tailed Student’s *t* test, if tests of data normality were met. Those represented by alternate distribution were examined by Mann–Whitney *U* statistic. Differences between groups were considered statistically significant at *P* < 0.05. The number of mice per group (*n* = 10) was chosen as directed by a power analysis to detect a 50% decrease in tumor growth with β error of 0.2. Progression-free and overall survival in patients with 3 categories of ALDH1A1 staining were compared by plotting with the Kaplan–Meier method and assessing for statistical differences with the log-rank statistic, using PASW 17.0 software.

**Results**

**Expression profiling of chemoresistant ovarian cancer cell lines**

To discover genes mediating taxane resistance, expression profiling of parental SKOV3ip1 and taxane-resistant SKOV3TRip2 cells was done with microarray analysis using the Illumina HumanRef-8 Expression BeadChip. The SKOV3TRip2 cell line was previously generated through progressive exposure to paclitaxel (designated SKOV3TR; 23) and then passaged intraperitoneally in mice for 2 generations to select populations with enhanced tumorigenicity. Similarly, SKOV3ip1 were derived from SKOV3 parental cells to select for cells with enhanced tumorigenicity. We found 34 genes to be upregulated more than 10-fold in SKOV3TRip2 (Fig. 1), among which was ALDH1A1, with a 92.7-fold increase (*P* = 0.0025). Twenty genes were more than 10-fold increased in SKOV3ip1. SKOV3TRip2 cells were confirmed to have approximately 3,000-fold increased resistance to docetaxel, as measured by MTT IC₅₀ (62.5 nmol/L vs. 0.02 nmol/L; Fig. 2A).

**ALDH1A1 expression in ovarian cancer cell lines**

To confirm an increase in ALDH1A1 expression/activity in SKOV3TRip2 and examine expression in other ovarian cancer cell lines, 4 pairs of parental and chemoresistant cell lines were examined: SKOV3ip1/SKOV3TRip2; HeyA8/HeyA8MDR (multidrug resistant); A2780ip2/A2780cp20 (10-fold increased cisplatin resistance); and IGROV-AF1/IGROV-cp20 (5-fold increased cisplatin resistance). In addition, an immortalized, nontransformed cell line derived from normal ovarian surface epithelium, HIO-180, was examined. We found that expression of total ALDH1A1, as measured by Western blot analysis, was in each case higher in the chemoresistant cell line, with the exception of HeyA8/HeyA8MDR, in which ALDH1A1 was low to absent in both (Fig. 2B). To examine whether ALDH1A1 was not only present but also active, we subjected cells to flow cytometric analysis using the ALDEFLUOR assay. This functional assay predominantly identifies active ALDH1A1 by conversion of a chemical to a fluorochrome. The presence of a subpopulation of ALDH1A1-active cells could be readily identified in SKOV3TRip2 (58% of the total population) and A2780cp20 (2.2%) but not in their parental cell line (Fig. 2C). Furthermore, the strong shift in fluorescent signal in some cells suggests that there was not simply a general increase in expression in all cells but rather separate populations of ALDH1A1-positive and -negative cells. This was confirmed by immunohistochemistry, which showed distinct populations of ALDH1A1-positive or -negative cells in A2780cp20 and SKOV3TRip2 cells but not in the parental A2780ip2 and SKOV3ip1 cells in culture (Fig. 2D). Finally, we observed that this heterogeneous profile was maintained in tumors. After intraperitoneal injection of SKOV3TRip2 cells into nude mice and collection of the resulting orthotopic tumor implants, IHC staining of for ALDH1A1 showed both positive and negative ALDH1A1 subpopulations (Fig. 2E). To examine whether this heterogeneity in expression was due to differential expression in hypoxic regions, a tumor-bearing mouse was injected with hypoxyprobe reagent and sacrificed after 30 minutes. The tumor was stained with ALDH1A1 and antihypoxyprobe antibody. We found that the ALDH1A1-positive cells were not preferentially localized to hypoxic regions in the tumor, with only 1.5% of ALDH1A1-positive cells concurrently positive for hypoxyprobe and only 3.3% of hypoxyprobe-positive cells also positive for ALDH1A1 (*P* < 0.01; Fig. 2F).

**ALDH1A1 expression in human ovarian cancer specimens**

To determine the pattern of ALDH1A1 expression and possible correlations with chemoresistance in patients, we next examined ALDH1A1 expression in 65 untreated, high-grade papillary serous stage III–IV ovarian cancer patient specimens (patient characteristics in Table 1). We found a wide range of expression patterns (Fig. 3A). There was no ALDH1A1 in tumor cells in 27.1% of samples. ALDH1A1 expression was noted in 1% to 20% of cells in 44% of tumors, representing the largest cohort of expression patterns. As in xenografts from cell lines, expression was typically strong in some cells and negative in others, signifying distinct heterogeneity in the tumor. There was no distinct histologic pattern to the location of the positive cells (such as around vasculature or on the leading edge of the tumor), but positive cells did tend to cluster together. The remaining tumors (28.9%) all had between 21% and 100% staining, with 10% of all patients having strong ALDH1A1 expression in nearly 100% of their tumor cells. To confirm that ALDH1A1 expression was not being mistakenly identified in tumor-infiltrating macrophages, several snap-frozen samples were dual stained for ALDH1A1 and CD68. Although images are not as detailed as those from paraffin-embedded samples, dual staining clearly shows that the majority of macrophages (blue) are ALDH1A1 negative and therefore the heterogeneous ALDH1A1 positivity in tumors is not simply due to detection of macrophage infiltration (Fig. 3B).
Figure 1. Comparison of whole genome expression profiling between SKOV3TRip2 and SKOV3ip1 cell lines. Total RNA from the SKOV3TRip2 and SKOV3ip1 cell lines were subjected to whole genome expression profiling using the Illumina platform. The genes with a greater than 10-fold increase in SKOV3TRip2 are shown in red, whereas those with a greater than 10-fold increase in SKOV3ip1 are shown in green. FC, fold change.
Correlation of ALDH1A1 expression with clinical outcomes

To determine whether ALDH1A1 expression correlated with clinical outcomes, we compared progression-free survival and overall survival from patient samples described earlier (and in Table 1) in cohorts with no ALDH1A1 expression, 1% to 20% expression, and greater than 20% expression, as this grouping allowed similar
Once tumors were established and were used so that tumor growth and response could be measured. Once tumors were established and were used so that tumor growth and response could be measured. Once tumors were established and were used so that tumor growth and response could be measured.

Preferential survival of ALDH1A1-positive cells with cisplatin treatment

To determine whether the ALDH1A1-positive cells have preferential survival in the tumor microenvironment with platinum treatment, we established mouse xenografts from primary patient samples by subcutaneously implanting a freshly collected tumor specimen into NOD-SCID mice. A subcutaneous rather than orthotopic model was used so that tumor growth and response could be accurately measured. Once tumors were established and growing, and achieved a size of approximately 1 cm³, intraperitoneal administration of 7.5 μg/kg of cisplatin weekly was initiated whereas only vehicle was administered to controls (Fig. 3D). When tumors grew to a size of 2 cm³ in controls, having remained stable with cisplatin treatment, they were harvested and sections stained for ALDH1A1 expression. Baseline expression of ALDH1A1 in the implanted tumor was seen in approximately 1% of cancer cells and similar levels were found in growing xenografts in untreated mice (Fig. 3E). A significant increase in the percentage of ALDH1A1-positive cells was, however, noted in cisplatin-treated xenografts to 38% (P < 0.001; Fig. 3E). Consistent with this, the ALDEFLUOR assay on the dissociated tumor showed that 0.6% of cells from untreated tumors were ALDEFLUOR positive whereas 17.6% of cells from cisplatin-treated tumors were ALDEFLUOR positive. Because the treated xenograft in this case did not regress, but rather remained stable in size, cisplatin exposure may have induced ALDH1A1 expression in surviving cells in addition to preferential killing of ALDH1A1-negative cells.

**Tumor-initiating capacity of ALDH1A1-positive ovarian cancer cells**

In breast and other cancers, the ALDH1A1-active cancer cells have been shown to represent a tumor-initiating population (10–19). To determine whether this were the case in ovarian cancer, we sorted ALDH1A1-positive and -negative populations from the A2780cp20 cell line using the ALDEFLUOR assay and injected cells intraperitoneally into NOD-SCID mice in limiting dilutions to determine tumor-initiating potential. As summarized in Table 2, ALDEFLUOR-positive cells exhibited increased tumorigenic potential, with 100% tumor initiation after the injection of 100,000, 25,000, or 5,000 cells, and 1 tumor was established after the injection of 1,000 cells. ALDEFLUOR-negative cells could form tumors, although at a lower rate: 2 of 5 mice formed tumors after the injection of 25,000 or 100,000 cells and no tumors formed after the injection of 5,000 or 1,000 cells. Mice were followed for 1 year after injection and thorough necropsies were performed in remaining mice to confirm that tumors failed to develop. The TD50, or dose of cells required to permit tumor formation in 50% of animals, was 50-fold lower with ALDEFLUOR-positive cells. Perhaps, more striking was the makeup of these tumors. One requirement of a tumor-initiating population is that it has the capacity to give rise to heterogeneous tumors, composed of both stem cell and non–stem cell populations, therefore

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**Table 1. Characteristics of patients tested for ALDH1A1 expression (n = 65)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Percentage or average (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>62.2 (34–89)</td>
</tr>
<tr>
<td>Caucasian race</td>
<td>71%</td>
</tr>
<tr>
<td>Pretreated with chemotherapy</td>
<td>0%</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>74%</td>
</tr>
<tr>
<td>IV</td>
<td>26%</td>
</tr>
<tr>
<td>Ca125</td>
<td>3,071 (161–9,600)</td>
</tr>
<tr>
<td>Ascites</td>
<td>87%</td>
</tr>
<tr>
<td>Optimal debulking</td>
<td>74%</td>
</tr>
<tr>
<td>Papillary serous histology</td>
<td>100%</td>
</tr>
<tr>
<td>Platinum/taxane primary therapy</td>
<td>96%</td>
</tr>
<tr>
<td>Progression-free survival, mo</td>
<td>14.2 (1.7–108)</td>
</tr>
<tr>
<td>Overall survival, y</td>
<td>2.5 (0.2–11.8)</td>
</tr>
<tr>
<td>ALDH1A1 staining</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>27.1%</td>
</tr>
<tr>
<td>1%–20% of cells</td>
<td>44.0%</td>
</tr>
<tr>
<td>21%–100% of cells</td>
<td>28.9%</td>
</tr>
</tbody>
</table>

Abbreviation: Ca125, cancer antigen 125

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**Table 2. Tumorigenicity of ALDEFLUOR-positive and negative cells**

<table>
<thead>
<tr>
<th>A2780cp20 cells injected intraperitoneally</th>
<th>1,000,000</th>
<th>250,000</th>
<th>100,000</th>
<th>25,000</th>
<th>5,000</th>
<th>1,000</th>
<th>Serial transplantation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDEFLUOR negative</td>
<td>5/5</td>
<td>4/5</td>
<td>2/5</td>
<td>2/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>ALDEFLUOR positive</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>1/5</td>
<td>5/5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
demonstrating multipotent differentiation potential. This was noted in tumors that formed after the injection of ALDEFLUOR-positive cells. In all 16 of these tumors, a strongly positive ALDH1A1 population was noted in the minority of the sample, on average 4.7% of the tumor (range 2.4%–6.1%; Fig. 4A). However, no ALDEFLUOR-positive cells were found in the tumors that formed after the injection of ALDH1A1-negative cells (Fig. 4B). This was confirmed by the IHC analysis (Fig. 4C and D). This argues against the idea that tumors are formed because of contamination with ALDEFLUOR-positive cells or that ALDH1A1 expression is simply induced by the tumor microenvironment regardless of the capacity of the cells. This difference in the capacity to generate ALDEFLUOR-positive cells was also noted in vivo. SKOV3TRip2 cells sorted into ALDEFLUOR-positive and -negative populations were cultured separately, and the ALDEFLUOR assay was done on the different populations at 24, 48, and 72 hours (Fig. 4E and F). Of the ALDEFLUOR-positive cells, the population gradually reverted to 75.3%, 54.2%, and 51.4% ALDEFLUOR-positive cells, respectively, for each time point. However, the ALDEFLUOR-negative cells could not produce any ALDEFLUOR-positive cells.

To confirm that the ALDEFLUOR-positive cells from tumors maintained tumorigenicity, these populations...
were sorted and reinjected intraperitoneally into mice and continued to form tumors at 100% rate with 25,000 cells injected. However, ALDEFLUOR-negative cells from the tumors forming after ALDEFLUOR-negative cells were injected did not form tumors. Taken together, these studies show that ALDEFLUOR-positive cells have increased but not absolute tumorigenicity, but they do have a differentiation capacity and maintenance of the tumorigenic phenotype that is absent in ALDEFLUOR-negative cells.

In an effort to determine whether ALDEFLUOR-positive cells, freshly collected from ovarian cancer patients, have similar tumorigenicity, we have sorted ALDEFLUOR-positive and -negative cells from 5 separate ovarian cancer patients, dissociating tumors metastatic to the omentum at the time of primary debulking surgery. In this cohort, 1.5% to 17.8% of cells were ALDEFLUOR positive. A total of 25,000 ALDEFLUOR-positive cells, 100,000 ALDEFLUOR-negative cells, or 100,000 unsorted cells were injected intraperitoneally into 5 mice per group per patient. Unfortunately, no tumors formed in any mice, highlighting the difficulty of tumorigenicity studies in primary ovarian cancer samples dissociated to single cell suspensions.

To preliminarily determine whether there is an overlap between the ALDEFLUOR-positive population and other
markers of putative stem cells in ovarian cancer, these 5 samples were also profiled for CD44, c-kit, and CD133.

We were not able to identify a convincing positive c-kit population from any sample. CD133-positive cells made up an average of 3.1% of total tumor cells (range, 0.6%–5.7%) and were greater than 80% of ALDEFLUOR positive in all 5 samples (mean, 86.7%; range, 81.5%–100%). CD44 was more commonly expressed, representing an average of 45.7% of tumors (but with a very broad range of 2.4%–98.2%). Of the CD44-positive cells, 75.4% were also ALDEFLUOR positive (range, 46.6%–88.8%). Similarly, the SKOV3TRip2 line has 82% CD44-positive cells, and of these, 74% were ALDEFLUOR positive. Although a great number of samples will need to be examined to fully delineate whether multiple marker–positive cells can more accurately define the most pure tumorigenic cell, there is certainly overlap in marker expression. There are both double-positive CD44/ALDEFLUOR and CD133/ALDEFLUOR-positive populations that may prove more discerning as cancer stem cell populations, and ongoing studies could assess this distinction. Interestingly, the A2780cp20 cell line is completely negative for CD44 and the HeyA8 cell line is negative for ALDH1A1/ALDEFLUOR, despite the fact that both are highly tumorigenic. This highlights the fact that these cannot be the sole mediators of tumorigenicity in mice.

**Downregulation of ALDH1A1 sensitizes ovarian cancer cells to chemotherapy**

Given the association of ALDH1A1 expression with chemoresistant cell lines and a decreased progression-free survival in ovarian cancer patients, we asked whether downregulation of ALDH1A1 could sensitize resistant cells to chemotherapy. Two different siRNA constructs were identified that reduced ALDH1A1 expression by greater than 80% (Fig. 5A). Reduction in the ALDEFLUOR population was confirmed (Fig. 5B). SKOV3TRip2 or A2780cp20 cells were exposed to ALDH1A1-targeting siRNA (ALDH1A1 siRNA) or control siRNA for 24 hours before replating and adding increasing concentrations of docetaxel or cisplatin, respectively. Cell viability 4 days after the addition of chemotherapy was assessed with the MTT assay. In SKOV3TRip2 cells, siRNA-ALDH1A1 alone reduced viability by 49% (Fig. 5C; \( P < 0.001 \)). Downregulation of ALDH1A1 also reduced the docetaxel IC\(_{50}\) from 178 to 82 nmol/L. In A2780cp20, the effects of ALDH1A1 downregulation alone were modest (Fig. 5D); reduced viability by 15.9%, \( P = 0.040 \) but sensitization to cisplatin was considerable, with a decrease in the IC\(_{50}\) from 5.1 to 2.0 \( \mu \)mol/L. Tests for synergy suggest moderate synergy in each cell line (CI = 0.82 for SKOV3TRip2 and 0.75 for A2780cp20). The contrasting effects of ALDH1A1-siRNA alone are consistent with the number of ALDH1A1-active cells in these cell lines, with SKOV3TRip2 cell lines having 50% to 60% of ALDEFLUOR-positive cells and A2780cp20 having just 2% of 3%. To determine how ALDH1A1 downregulation alone may affect cell growth, cell-cycle analysis was done in a separate experiment. We found that ALDH1A1 downregulation induced an accumulation of SKOV3TRip2 cells in S and G\(_2\) phases (\( P < 0.001 \); compared with control siRNA) but had only minimal effects on the cell cycle of A2780cp20 cells (Fig. 5E).

There are no known inhibitors of ALDH1A1 for *in vivo* studies. Therefore, we used a method for delivery of siRNA *in vivo*, using DOPC nanoparticles. We and others (28–32) have previously shown delivery of siRNA incorporated into DOPC nanoliposomes to the tumor parenchyma with subsequent target downregulation. In this study, nude mice were injected intraperitoneally with either SKOV3TRip2 or A2780cp20 cells and randomized to 4 treatment groups to begin 1 week after cell injection: a) control siRNA in DOPC, delivered intraperitoneally twice per week; b) docetaxel 35 mg, delivered intraperitoneally weekly (for SKOV3TRip2 model) or cisplatin 160 \( \mu \)g, delivered intraperitoneally weekly (for A2780cp20 model); c) ALDH1A1-siRNA in DOPC, intraperitoneally twice per week; or d) ALDH1A1-siRNA in DOPC plus docetaxel (for SKOV3TRip2) or cisplatin (for A2780cp20). After 4 weeks of treatment, mice were sacrificed and total tumor weight recorded. The IHC analysis confirmed reduced ALDH1A1 expression with ALDH1A1-siRNA/DOPC treatment compared with controls but not with chemotherapy alone (Supplementary Fig. 2; too little tissue was available to examine with the ALDEFLUOR assay). In SKOV3TRip2 xenografts (Fig. 5F), there was a nonsignificant reduction of 37.0% in tumor growth with docetaxel treatment (\( P = 0.17 \)) and of 25.0% with ALDH1A1 siRNA treatment (\( P = 0.38 \)) compared with control siRNA/DOPC. The observation that ALDH1A1 downregulation alone significantly decreased SKOV3TRip2 growth in *vitro* but was less pronounced *in vivo* suggests that tumor microenvironment factors such as supporting stromal cells may be able to protect cells from ALDH1A1 depletion. However, the combination of ALDH1A1 siRNA and docetaxel resulted in significantly reduced growth by 93.6% compared with control siRNA (\( P < 0.001 \)), by 89.8% compared with docetaxel plus control siRNA (\( P = 0.003 \)), and by 91.4% compared with ALDH1A1 siRNA (\( P = 0.002 \)). In A2780cp20 (Fig. 5G), there was a similar nonsignificant reduction of 43.9% in tumor weight with cisplatin alone (\( P = 0.32 \)) and of 57.0% with ALDH1A1 siRNA treatment (\( P = 0.19 \)). These effects may be even less significant than the mean tumor weights suggest, given the presence of 2 especially large tumors in the control siRNA group. However, again combined therapy showed a sensitization to chemotherapy with ALDH1A1 siRNA, with combination therapy reducing growth by 85.0% compared with control siRNA (\( P = 0.048 \)), by 73.4% compared with cisplatin plus control siRNA (\( P = 0.013 \)), and by 65.3% compared with ALDH1A1 siRNA alone (\( P = 0.059 \)). Given the minimal effects of each single agent and the consistent finding of significant improvement with combined therapy, these data suggest a synergy between
ALDH1A1 downregulation and both taxane and platinum chemotherapeutic agents, though formal dose-finding experiments would be required to definitively prove synergy.

Discussion

We have found that ALDH1A1 expression and activity are increased in chemoresistant ovarian cancer cell lines.
and in *in situ* primary ovarian cancer xenografts treated with cisplatin. Expression of ALDH1A1 is frequent in ovarian tumors, and patients with low ALDH1A1 expression levels have a more favorable outcome than those with more ALDH1A1-positive cells. ALDEFLUOR-positive cells have increased (but not absolute) tumorigenicity compared with ALDEFLUOR-negative cells and have a differentiating capacity that is not present in the ALDEFLUOR-negative population. Most important, downregulation of ALDH1A1 expression sensitized normally chemoresistant tumors to both docetaxel and cisplatin both *in vitro* and in an orthotopic mouse model of ovarian cancer.

The search for tumor-initiating cells in ovarian cancer has resulted in observations that the CD44+/c-kit+ population has an approximately 5,000-fold increase in tumorigenicity, with tumors forming after the injection of as few as 100 cells from primary tumor, xenograft, or spheroid heterogeneous populations (5), and that the CD133+ population has approximately 20-fold increased tumorigenicity, with tumor formation with as few as 100 to 500 cells from murine xenografts, and tumor formation 4 times faster with CD133+ cells (7). Furthermore, the increased tumorigenicity of CD133+ cells can be inhibited by interfering with binding between CD44 and its ligand hyaluronic acid (6). Other investigators have found equal rates of tumor formation among CD133− and CD133+ cells from the A2780 cell line, but a faster growth rate in CD133+ cells (8). The side population (SP) cells from the MOVCAR cell line also formed tumors more frequently and appeared 3 to 4 weeks sooner than tumors derived from non-SP cells (9). In all of these studies, as in ours, the tumors resulting from the putative tumor-initiating cell population contained both tumor-initiating cell and non-tumor-initiating cell populations, demonstrating multipotentiality. Interestingly, we have seen that cells comprising tumors formed from ALDH1A1-negative cells lack the capacity to generate ALDH1A1-positive cells and do not continue to propagate tumors over multiple generations, suggesting that their multipotentiality is limited. This lack of differentiating capacity has also been noted in ALDEFLUOR-negative cells from breast cancer cell lines (33).

The most appropriate source of tumor cells for tumorigenicity experiments is of some debate. Although it is desirable to use samples freshly collected from primary tumors, sorting these samples and establishing primary xenografts have proven problematic. Ovarian cancer xenografts and cells lines have traditionally been challenging to establish from primary samples. All previously reported studies of ovarian tumor-initiating cells have used selected cells of some sort, either from xenografts of varying generations or from cells grown in differentiation-inhibiting media (to form tumor spheres), to serve as a compromise between freshly collected specimens and cell lines. However, those cells that form tumors in mice even in the first generation almost certainly represent some select portion of the original tumor. That these xenografts still contain only a small percentage of tumor-initiating cells speaks either to the appropriateness of this approach or to the testament that the tumor-forming cells are multipotent, give rise to tumor-initiating cell-negative populations, and remain relatively rare. Use of cell lines is often discouraged because of their homogenous nature. But clearly, even within cell lines, there is heterogeneity in ALDH1A1 expression, as shown by the detection of distinct populations by flow cytometric and IHC analyses (Fig. 2). Distinct ALDEFLUOR-positive and -negative populations have also been found in several breast cancer cell lines, with ALDEFLUOR-positive cells having increased tumorigenicity and differing molecular signatures (33). Therefore, our finding that the ALDEFLUOR-positive population in cell lines has increased tumorigenicity may reflect the more aggressive phenotype of ALDH1A1-active cells but does not represent proof that this population is important to *in situ* ovarian cancers. Evidence that patients with increasing ALDH1A1 expression have poor outcomes suggests this association, but additional tumorigenicity experiments from freshly collected tumors would more appropriately define the ALDEFLUOR population as clinically significant tumor-initiating cells.

The importance of tumorigenicity in defining cancer stem cells has also been debated. Although tumor formation with 100 to 500 ALDEFLUOR-positive cells and a lack of tumor formation with the injection of 10⁵ ALDEFLUOR-negative cells definitely reflect an aggressive phenotype, the biologic processes required for xenograft formation—survival under stressful experimental conditions, adhesion, time to proliferation, and variations in host immunocompetence—may not reflect the true population that cancer stem cell research seeks to identify. Our ultimate goal should be to identify the subpopulations in parent tumors that survive chemotherapy and therefore are more likely to cause recurrence. Stem cells that survive chemotherapy should exhibit chemoresistance to be clinically relevant. In breast cancer, for example, the CD44+/CD24− population is highly tumorigenic. However, Tanei et al., who studied tissue obtained before and after neoadjuvant chemotherapy, found that despite a positive response to treatment, the proportion of CD44+/CD24−-negative cells was unchanged. In these samples, however, the ALDH1A1-positive population was significantly increased (34).

ALDH1A1 has previously been proposed to play a role in chemoresistance, having been noted to be higher in proteomic profiling of IGROV platinum-resistant ovarian cancer cells (35), in genomic profiling of multidrug-resistant gastric carcinoma (36), and in cells resistant to cyclophosphamide (37, 38), oxazaphosphorines (39), and now docetaxel and cisplatin. ALDH1A1 oxidizes many intracellular aldehydes into carboxylic acids (40), detoxifying many of the free oxygen radicals generated by chemotherapeutic agents. It stands to reason that a stem cell population should be resistant to multiple chemotherapeutic
agents rather than being specific to one class. This also follows clinically, in that most ovarian cancer patients who develop resistance to platinum agents have resistance to multiple agents (2). ALDH1A1 has been shown to be associated with BRCA1 in breast cancer, in that knockdown of BRCA1 increases the ALDEFLUOR population and ALDEFLUOR-positive cells preferentially contain BRCA1 loss of heterozygosity (41). These findings could also be important to BRCA-mediated ovarian cancer. Despite this body of evidence for the importance of ALDH1A1, it is not fully understood whether any of the additional ALDH isoforms are important to stem cell biology. In our study, ALDH1A1 can be specifically identified with isotype-specific antibodies (as used for the IHC analysis and Western blotting). However, the more important and consistently used identifier of a stem cell population is the ALDEFLUOR assay, which, although primarily dependent on ALDH1A1, may also identify ALDH1A2 and ALDH1A3 isoforms (42) and unpublished data by Stem Cell Technologies. As a therapeutic agent, we have seen positive effects by targeting ALDH1A1 with siRNA, but to maximize the efficacy of therapeutics, the contribution of these additional isoforms will need to be defined with additional studies.

Although our finding of a poor outcome in patients with high ALDH1A1 expression agrees with similar investigations in breast cancer (12, 13) and ovarian cancer (20), one interesting report found that a high ALDH1A1 expression level actually confers a positive prognosis in ovarian cancer (43). This cohort also contained patients with absent, scattered, and diffuse staining. However, this cohort included patients with stage I and II disease and low-grade tumors, and ALDH1A1 expression was higher in these patients [confirming findings from a previous report (44)]. Furthermore, with multivariate analysis, only stage correlated with survival; ALDH1A1 expression no longer predicted outcomes. In ovarian cancer, there is a well-recognized dichotomy in carcinoma, whereby low-grade tumors (which are more often diagnosed at stage I or II) are paradoxically more chemoresistant but have prolonged survival due to slow growth. Given these collective data, and the several mechanisms by which ALDH1A1 has been shown to contribute to chemoresistance, it may be that ALDH1A1 is more frequently expressed in low-grade tumors but participates in chemoresistance to both high-grade and low-grade subtypes.

We have shown that the ALDH1A1-positive population has properties of cancer stem cells, is associated with taxane and platinum resistance, and can be resensitized to chemotherapy with downregulation of ALDH1A1 in vitro and in vivo. Therefore, ALDH1A1 is not just a marker of an aggressive population but also a mediator of the phenotype and a viable target for therapy. As better models are developed to more purely define the true chemoresistant population in de novo patient tumors, the ALDH1A1 population, either alone or in combination with other markers and mediators of resistance, may represent a population that must be targeted to achieve increased response rates and survival in ovarian cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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- Fellow, Gynecologic Oncology, MD Anderson Cancer Center, Houston, TX
  David Gershenson (Chairman) and Diane Bodurka (Program Director), 7/2003 – 6/2007

PRIMARY FACULTY APPOINTMENTS

Clinical Instructor, Department of Obstetrics and Gynecology
University of South Carolina School of Medicine 7/2002 – 6/2003

Assistant Professor, Research, Department of Gynecologic Oncology
University of Texas MD Anderson Cancer Center 7/2007 – 6/2009

Assistant Professor, Department of Obstetrics and Gynecology
University of Alabama at Birmingham 6/2009 – present

BOARD CERTIFICATION

Board certified, American Board of Obstetrics and Gynecology, 12/2003
Board certified, Gynecologic Oncology, 4/2011
LICENCES
Current: 
Alabama 29546 5/27/2009-present
DEA Avail on request 3/25/2005-present
Inactive: 
South Carolina 21944 2002-2004
Texas M0466 2/04/2005-8/31/2010

HONORS AND AWARDS
Medical – University of North Carolina School of Medicine, Chapel Hill, NC
Dean’s List, 1994-1996
Class Co-President, University of North Carolina School of Medicine, 1994-1996
University of North Carolina School of Medicine Merit Scholarship, 1995
NIH Summer Research Fellowship, University of North Carolina SOM, 1995

Residency – Medical University of South Carolina, Charleston, SC
First Place, Resident Oral Presentations, District IV Annual Meeting, ACOG, 2000
Donald F. Richardson Memorial Prize Paper Award, ACOG, 2001
Golden Apple Nominee, Medical University of South Carolina, 2002
Outstanding Resident Teaching Award, MUSC, Dept of OB/GYN, 1999 and 2001
First Place, Resident Oral Presentations, SC/GA Section, ACOG, 2001
Ortho-McNeil Award for Best Resident in Laparoscopy, MUSC, 2002
Resident Research Award for Outstanding Research in Women’s Health, MUSC, 2002

Fellowship – University of Texas MD Anderson Cancer Center, Houston, TX
J.G. Moore Award, Best Presentation by Fellow or Resident, WAGO, 2004
Del and Dennis McCarthy Award for Advances in Surgery, MDACC, 2004
ASCO Fellows Travel Grant, 2005
AACR Scholar-in-Training Award, 2005
Trainee Excellence Award, MD Anderson Alumni and Faculty Association, 2005
Gynecologic Cancer Foundation / Carol’s Cause Outstanding Paper Award, 2006
The Diane Denson Tobola Fellowship in Ovarian Cancer Research, MD Anderson Alumni and Faculty Assn, 2007
Bristol-Myers Squibb Poster Award in Clinical/Translational Research, MD Anderson Trainee Research Day, 2007

Faculty – University of Texas MD Anderson Cancer Center, Houston, TX
Listing in “Guide to America’s Top Obstetricians and Gynecologists”, 2007-present
Reproductive Scientist Development Program Phase I Scholar, 2007-2009
Julie and Ben Rogers Award for Excellence Nominee, 2008

Faculty – University of Alabama at Birmingham, Birmingham, AL
Listing in “Best Doctors in America”, 2010-present
Reproductive Scientist Development Program Phase II Scholar, 20010-2012
John R. Durant Award for Excellence in Cancer Research, UAB CCC, 2009
Ovarian Cancer Academy Award Scholar, Department of Defense, 2010-2015
CCTS Scientific Symposium award, 1st place, UAB CCTS, 2010
Listing in Cambridge Who’s Who among Executives and Professions in Research, Medicine, and Healthcare, 2011

EXPERIENCE/SERVICE

PROFESSIONAL SOCIETY MEMBERSHIPS
American College of Obstetricians and Gynecologists – Fellow
Society of Gynecologic Oncology – Full Member
American Association for Cancer Research – Full Member
American Society of Clinical Oncology – Full Member
Gynecologic Oncology Group – Member
The Gynecology and Obstetrics Society, Medical University of South Carolina, 2002-present
The Felix Rutledge Society, MD Anderson Cancer Center, 2007-present
UAB Griffin Society, University of Alabama at Birmingham, 2009-present
COMMITTEES

National
Marketing and Publications Committee, Society of Gynecologic Oncologists (SGO), 2009-2011
Annual Meeting Program Committee, Society of Gynecologic Oncologists (SGO), 2010-2011
Awards Committee, Gynecologic Cancer Foundation (GCF), 2010-2013
Education Committee, Society of Gynecologic Oncologists (SGO), 2011-2013
Website Development Task Force, Gynecologic Cancer Foundation (GCF), 2011
Board of Directors, Gynecologic Cancer Foundation (GCF), 2011-2014

Regional / Institutional
Clinical Ethics Committee, MUSC, 1998-2003
Academic Program coordinator, MUSC, 2001-02
Fellowship Admissions Committee, MDACC, Dept of Gynecologic Oncology, 2005-2006
Fellowship Planning Committee, MDACC, Dept of Gynecologic Oncology, 2005-2006
Steering Committee Member, Comprehensive Cancer Center School of Medicine Strategic Plan, UAB, 2011
Biorepository Development Committee, UAB School of Medicine, 2011
Endowed chair in Pathology Search Committee, 2011

GRANT REVIEWER SERVICE

National
CDMRP DOD Ovarian Cancer Research Program, Pathobiology Panel, Scientist Reviewer, 2009-2011
Gynecologic Cancer Foundation Research Grants/Awards Committee, Grant Reviewer, 2010-2011

Regional / Institutional
Ontario Institute for Cancer Research, Cancer Research Fund Translational Panel, Scientist Reviewer, 2009-2011
Ovarian Cancer Pilot Grant, University of Alabama at Birmingham Comprehensive Cancer Center, 2010

JOURNAL SERVICE

Editorial Board
Gynecologic Oncology Case Report

Ad-hoc Reviewer
Journal of Clinical Oncology
Cancer Research
Clinical Cancer Research
Oncogene
Cancer

Gynecologic Oncology
American Journal of Obstetrics and Gynecology
Expert Opinion on Therapeutic Targets
Tumor Biology

TRAINING COURSES TAKEN

Ethics Consult Service, Medical University of South Carolina, 1998-2003
AACR Molecular Biology in Clinical Oncology Workshop, Aspen, Colorado, 7/2004
Clinical and Translational Science Training Program, UAB CTSA, 2011

TEACHING/MENTORSHIP

Courses/Programs
Gross Anatomy Teacher’s Assistant, UNC School of Medicine, 1994-1995
Small group preceptor, Medical Ethics course, MUSC, 2002-2003
Angiogenesis section, Cancer Biology course, UAB Graduate School, 2010-present
Director of Resident Research, Dept of OB/GYN, UAB, 2010-present
Graduate Student Mentorship


Zachary Dobbins, MD/PhD candidate. *Development and validation of a primary xenograft model in ovarian cancer.* **Primary advisor,** UAB, 2011-present.

Graduate Student Advisor Committee Member


Postdoctoral mentorship

Adam Steg, PhD. *The role of Jagged1 and Sonic Hedgehog in ovarian cancer growth and chemoresistance.* UAB, 12/2009-current.

Resident and Fellow Mentorship


Medical Student Mentorship


Mata Burke, MS1. *Combined Hedgehog and Notch targeting in ovarian cancer.* UAB, 2011.

Technician Direct Supervision

Guillermo Armaiz-Pena, PhD. *Mediators of Stress-Induced Cancer Progression.* MDACC, 2004-2005.


Ashwini Katre, MS. *Chemoresistance mechanisms of ALDH1 in ovarian cancer.* UAB, 2010-present.
RESEARCH FUNDING

Active

Principle Investigator, Targeting Jagged in Ovarian Tumor Initiating Cells, Research Scientist Development Program Phase II (through the Ovarian Cancer Research Fund), 7/1/2009-6/30/2012, over 3 years.

Principle Investigator, Characterization and Targeting of the Aldehyde Dehydrogenase Subpopulation in Ovarian Cancer, OC093443, Department of Defense Ovarian Academy Award, 7/1/2010 – 6/30/2015, over 5 years.

Prior


Principle Investigator, Characterization and therapeutic targeting of ovarian cancer stem cells, 5P50 CA083639, Career Development Award, Ovarian Cancer SPORE at MDACC, 9/1/2007 – 8/31/2009, over 2 years.


Patents Granted and Pending

Delivery of siRNA by neutral lipid compositions, MDACC, United States, 60/671,641, 4/15/2005, Filed.

PUBLICATIONS


**INVITED ARTICLES**


**ABSTRACT PRESENTATIONS**


* Equal contribution † Selected for Cover Art and/or Issue Highlights


81. Landen CN, Goodman B, Nick AM, Stone RL, Miller LD, Mejia PV, Jennings NB, Gershenson DM, Bast RC, 
    Coleman RL, Lopez-Berestein G, and Sood AK. Targeted therapy against aldehyde dehydrogenase in ovarian 

    ovarian cancer stem cells to recurrence. *Center for Clinical and Translational Science Annual Scientific 
    Symposium*, 2010. §

    taxane resistance in ovarian cancer. *Proceedings of the 42nd Annual Society of Gynecologic Oncologists Meeting*, 
    2011.

    Examination of matched primary and recurrent ovarian cancer specimens supports the cancer stem cell 

85. Zsebik G, Kim K, Straughn JM, Landen CN. Management of Complex Pelvic Masses Using the OVA1 Test: A 

    ubiquitin ligase EDD mediates platinum resistance and is a target for therapy in epithelial ovarian cancer. 

    2011.

§ Selected for Meeting Award

BOOK CHAPTERS

1. Landen CN and Holmes MA. Dysmenorrhea, in Conn’s Current Therapy 2002, R. Rakel and E. Bope, Editors, 


3. Landen CN and Lopez-Berestein G. Therapeutic Agents and Approaches, in *Targeted Therapy in the Age of 
    Personalized Cancer Care*, under construction.

    under construction.

CONFERENCES AND SYMPOSIA

Presentations at National or International Conferences

Invited

Differing mechanisms of inhibition of calcium rises in human uterine myocytes by indomethacin and nimesulide. 
The Donald F. Richardson Prize Paper Award Presentation. 50th Annual Clinical Meeting of the American 

Neuroendocrine modulation of STAT3 in ovarian cancer. Western Association of Gynecologic Oncologic 

Genomic Instability is Associated with Lack of Telomerase Activation in Ovarian Cancer. 6th International 
Conference on Ovarian Cancer, MD Anderson Cancer Center, 12/2005.

Therapeutic silencing of EphA2 by in vivo liposomal siRNA delivery. American Association of Cancer Research 

Targeting the αvβ3 integrin with a fully humanized antibody in ovarian cancer. 37th Annual Society of 

Page 14 Revised 8/1/2011

SiRNA Therapeutics in Ovarian Cancer. 7th Annual International Conference on Ovarian Cancer, Houston, TX, 11/2006.


“Nanoparticle Delivery Systems for siRNA Therapy.” 3rd Annual Symposium on Ovarian Cancer Research, Medical University of South Carolina, 5/2009.


Discussant


“KLF6-SV1 is a Novel Uterine Leiomyosarcoma Gene: From Transgenic Mouse Model to Human Disease.” 42nd Annual Meeting of the Society of Gynecologic Oncologists, Orlando, FL, 2011.

Moderator


Invited Seminars from Other Institutions


“In search of: Ovarian Cancer Stem Cells.” Lineberger Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, 10/2008.


“In search of: Ovarian Cancer Stem Cells.” Helen Diller Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, 10/2008.

“Update on screening and genetic susceptibility in gynecologic cancers.” The Gynecologic and Obstetrics Society, Medical University of South Carolina, 5/2011.

“Cancer Stem Cells: Clinically significant or an experimental phenomenon?” Felix Rutledge Society, MD Anderson Cancer Center, 5/2011.

Presentations at Local Conferences


“Therapeutic targeting of EphA2 in ovarian cancer.” Dept of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX, 4/2005.


“Cancer Stem Cells in Epithelial Ovarian Cancer.” Dept of Cancer Biology, MD Anderson Cancer Center, 4/2008.


“In search of... Ovarian Cancer Stem Cells.” Program in Experimental Therapeutics, University of Alabama at Birmingham, 1/2010.


**Personal Interests**

Family activities
Sports / Crosstraining
Hiking / camping
History
Philosophy / religion