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Characterization of Aldehyde Dehydrogenase 1 High Ovarian Cancer Cells: Towards Targeted Stem Cell Therapy

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Abstract

Objective—The cancer stem cell (CSC) paradigm hypothesizes that successful clinical eradication of CSCs may lead to durable remission for patients with ovarian cancer. Despite mounting evidence in support of ovarian CSCs, their phenotype and clinical relevance remain unclear. We and others have found high aldehyde dehydrogenase 1 (ALDH^{high}) expression in a variety of normal and malignant stem cells, and sought to better characterize ALDH^{high} cells in ovarian cancer.

Methods—We compared ALDH^{high} to ALDH^{low} cells in two ovarian cancer models representing distinct subtypes: FNAR-C1 cells, derived from a spontaneous rat endometrioid carcinoma, and the human SKOV3 cell line (described as both serous and clear cell subtypes). We assessed these populations for stem cell features then analyzed expression by microarray and qPCR.

Results—ALDH^{high} cells displayed CSC properties, including: smaller size, quiescence, regenerating the phenotypic diversity of the cell lines *in vitro*, lack of contact inhibition, nonadherent growth, multi-drug resistance, and *in vivo* tumorigenicity. Microarray and qPCR analysis of the expression of markers reported by others to enrich for ovarian CSCs revealed that ALDH^{high} cells of both models showed downregulation of CD24, but inconsistent expression of

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Conflict of Interest

R.J.J. holds the patent for the stem cell marker Aldefluor and, under a licensing agreement between Aldagen and the Johns Hopkins University, is entitled to a share of royalties received by the University. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies. The remaining authors declare no competing financial interests.

CD44, KIT and CD133. However, the following drugable targets were consistently expressed in the ALDH^{high} cells from both models: mTOR signaling, her-2/neu, CD47 and FGF18 / FGFR3.

Conclusions—Based on functional characterization, ALDH^{high} ovarian cancer cells represent an ovarian CSC population. Differential gene expression identified drugable targets that have the potential for therapeutic efficacy against ovarian CSCs from multiple subtypes.

Keywords

ovarian cancer; cancer stem cells; aldehyde dehydrogenase 1; stem cell-targeted therapy

Introduction

Eighty percent of patients with advanced ovarian cancer show initial clinical responses to therapy, but almost all eventually relapse [1]. This transient clinical response is consistent with the cancer stem cell (CSC) hypothesis, which posits that the initial response would be attributed to the eradication of the bulk, differentiated cells [2, 3]. The persistence of a drug resistant subpopulation of cancer cells exhibiting a stem cell phenotype is further theorized to be responsible for relapse. A substantial body of recent laboratory evidence supports the existence of cell populations in ovarian cancer with stem cell features [4–8]. However, controversy exists regarding the phenotype of such so-called ovarian CSCs, as well as their clinical relevance [9–11].

Various “stem cell” markers have been proposed to enrich for ovarian CSCs, including CD44⁺, CD133⁺, CD24⁺, KIT⁺ (CD117) and aldehyde dehydrogenase 1 (ALDH1) [4–8, 12, 13], although conflicting data exist for each of these markers [9–11]. Work in our laboratory and others has identified ALDH1 as a marker of CSCs in many hematologic malignancies and solid tumors [14–21]. Furthermore, recent reports indicate that high ALDH1 expression (ALDH^{high}) may serve as a superior ovarian CSC marker [12, 13]. Accordingly, we analyzed ALDH^{high} ovarian cancer cells for CSC properties. Two ovarian cancer cell lines were tested: FNAR-C1 [22] and SKOV3 [28,29]. FNAR-C1 developed spontaneously in a female Lewis rat and displayed striking morphologic similarities to the human endometrial subtype of ovarian carcinoma, expressing estrogen receptor α , progesterone receptor, androgen receptor, her-2/neu, epithelial cell adhesion molecule, CA125, and nuclear b-catenin [22]. It can be carried as a cell line or passaged in the peritoneal cavity of immunocompetent female Lewis rats, a potential advantage over models requiring immunocompromised mice [22]. SKOV3 is a human ovarian cancer cell line, the subtype of which has been described as both clear cell and serous [28,29]. We find that ALDH^{high} cells from both models display phenotypic, biologic, and functional stem cell properties. Expression analysis identified genes and pathways consistently expressed in ALDH^{high} cells that may serve as therapeutic targets for the eradication of ovarian CSCs.

Materials and Methods

Cell Lines and Culture

FNAR-C1 rat ovarian cancer cells were derived as previously described [22]. The human ovarian cancer cell line SKOV3 and Taxol-resistant subclone were the kind gifts of Drs.

Alexander Stoeck, Tian-Li Wang and Ie-Ming Shih [23]. Cells were maintained in either standard medium [DMEM (Life Technologies, Grand Island, NY) for FNAR-C1 or RPMI (Life Technologies, Grand Island, NY) for SKOV3 + 10% FBS (Sigma, St. Louis, MO)] or KnockOut Medium [KnockOut DMEM (Life Technologies, Grand Island, NY) + 10% KnockOut Serum Replacement (Life Technologies, Grand Island, NY)]. Both media were supplemented with 2 mM L-glutamine (Life Technologies, Grand Island, NY), 100 U/mL penicillin (Life Technologies, Grand Island, NY) and 100 µg/mL streptomycin (Life Technologies, Grand Island, NY). Taxol-resistant SKOV3 cells were maintained in 33.3 nM paclitaxel (Taxol; Sigma-Aldrich, St. Louis, MO). All experiments were conducted on cells passaged less than 30 times within our lab.

Cell Sorting and Flow Cytometry

Adherent cells were released from flasks with 0.05% trypsin-EDTA (Life Technologies, Grand Island, NY) and pipetting. Cells were stained with Aldefluor reagent (STEMCELL Technologies Inc., Vancouver, BC, Canada) to assess ALDH1 activity, using the manufacturer's protocol then stained with 0.5–1 µg/mL propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) for 5 minutes. FNAR-C1 cells grown in KnockOut medium showed sensitivity to the mechanical forces of cell sorting and therefore were sorted using a MoFlo cell sorter (Beckman Coulter, Miami, FL), which uses a lower pressure than the FACSaria II. Because of substantial death after sorting, these cells were allowed to recover for three to four days in KnockOut medium before use in the following experiments: doubling time, cell cycle analysis and drug resistance. All other cells were sorted using a FACSaria II cell sorter (BD Biosciences, San Jose, CA).

To measure cell size, unsorted cells were trypsinized then stained with Aldefluor and PI as described above. The relative forward scatter, or size, of gated ALDH^{high} and ALDH^{low} cells was then compared. For cell cycle analysis, sorted cells were allowed to recover overnight after sorting in their corresponding medium. Cells were released from flasks as described above. No greater than 2.5×10^5 cells underwent methanol fixation (Fisher, Waltham, MA) followed by rehydration in PBS (Life Technologies, Grand Island, NY) and staining with 50 µg/ml PI and 10 µg/ml RNase (Roche, Indianapolis, IN). A minimum of 5000 singlet events were collected. To assay phenotypic diversity after culture, sorted cells were plated into vented T25 tissue culture flasks with either KnockOut or standard medium. After four days, cells were trypsinized and stained with Aldefluor and PI as described above. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). All flow cytometry plots and calculations were generated using FlowJo 8 (Tree Star, Inc, Ashland, OR).

Photomicrographs

Sorted cells were plated into 6-well tissue culture plates. When cells were at the desired level of confluency, photomicrographs were generated using a Nikon Eclipse TE2000E with a 20x phase contrast objective, Nikon DS-Qi1Mc CCD camera, and Nikon NIS Elements 3 software (Nikon Instruments Inc., Melville, NY).

Doubling Time

Sorted cells were plated into vented T25 tissue culture flasks in the medium they were cultured in prior to sorting. At 24-hour intervals ranging from 24 to 120 hours, cells were released from flasks with trypsin and pipetting as described above. Aggregates formed by ALDH^{high} FNAR-C1 cells were disrupted using Accumax and pipetting (EMD Millipore, Billerica, MA). Viable cells were identified by trypan blue exclusion (Life Technologies, Grand Island, NY), and counted using a hemacytometer (Hausser Scientific, Horsham, PA). Time points in exponential growth were used to calculate doubling times (Roth V. 2006 <http://www.doubling-time.com/compute.php>). R² was calculated using Prism 6 for Mac (GraphPad Software, La Jolla, CA). Graphs were generated using Excel 2008 for Mac (Microsoft, Redmond, WA).

Microarray and Pathway Analysis

Cells were sorted into RNeasy Protect Cell Reagent (Qiagen, Valencia, CA). Total RNA was isolated with the RNeasy Mini Kit with QIAshredder columns (Qiagen, Valencia, CA). Quality assessment and microarray analysis was performed at The Sidney Kimmel Cancer Center Microarray Core Facility. RNA quality was determined with a NanoDrop ND-1000 spectrometer (Thermo Scientific, Waltham, MA) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Rat samples were analyzed in triplicate using the Rat Gene Expression Microarray 4x44K v3 (Agilent Technologies, Santa Clara, CA). RNA spike-in controls (Agilent Technologies, Santa Clara, CA) were added, then samples were amplified and labeled using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Santa Clara, CA) and purified with the RNeasy Mini Kit. 0.825 µg of each Cy3-labeled sample was used for hybridization according to manufacturers protocol. Microarrays were scanned using an Agilent G2565AA Scanner with Agilent Scan Control 7.0 software (Agilent Technologies, Santa Clara, CA). Data were extracted with Agilent Feature Extraction 9.5.3.1 software (Agilent Technologies, Santa Clara, CA).

Samples from human cell lines were analyzed in triplicate using the Human HT-12 v4 bead chip (Illumina, San Diego, CA). RNA was amplified and labeled using the Illumina Total Prep RNA Amplification Kit (Ambion, Austin, TX). 750 ng biotin-labeled cRNA was hybridized and stained with streptavidin-Cy3. Arrays were scanned with the iScan System (Illumina, San Diego, CA). Data were extracted with the Gene Expression Module in GenomeStudio Software (Illumina, San Diego, CA). (GEO accession numbers will be provided during review.)

GeneSpring GX software version 11 (Agilent Technologies, Inc, Santa Clara, CA) was used for data analysis, and differentially expressed genes were calculated based on moderated ttest and fold-change. Pathway analysis was performed using Ingenuity Pathway Analysis Tool (Ingenuity® Systems, www.ingenuity.com, Redwood City, CA).

PCR Validation of Microarray

PCR validation of microarray data was performed at The Sidney Kimmel Cancer Center Microarray Core Facility using custom 96-well TaqMan array plates (Life Technologies, Grand Island, NY). 20 ng of cDNA was added to each well along with TaqMan Universal PCR Master Mix (Life Technologies, Grand Island, NY) in a final volume of 20 μ l. Each sample was assayed in triplicate. Data was collected using the ABI 7500 Real-Time PCR System (Life Technologies, Grand Island, NY) and analyzed with 7500 Fast System SDS software version 1.4 (Life Technologies, Grand Island, NY). The following endogenous controls were used: CXCL1 for FNAR-C1 and EEF1A1 for SKOV3.

In vivo Tumorigenicity

All animals were handled in accordance with the Johns Hopkins University Animal Care and Use guidelines (protocol numbers RA08M154 and RA11M116). Female Lewis rats received intraperitoneal injections with graded numbers of cells. As the FNAR-C1 cell line originated in a normal Lewis rat, the cells can be readily propagated in immunocompetent Lewis rats. Rats were monitored weekly and euthanized when becoming moribund or when tumors were detected by abdominal palpation. Two years after the injection date, remaining rats underwent necropsy. This time point was chosen as the endpoint for the study in order to maximize the time for our injected cells to form tumors yet minimize the chances of spontaneous tumors due to old age. All tumors underwent histological evaluation in a blinded manner to confirm tumor origin.

In Vitro Drug Resistance

Sorted cells were plated in sextuplicate at a density of 100 cells per well into 24-well tissue culture plates containing standard medium. Cells were exposed to Taxol and gemcitabine continuously and carboplatin for 72 hours. Data are presented as the percentage of wells that grew relative to no drug control (FNAR-C1: weeks 2–4, SKOV3: weeks 2–3). The number of biological replicates for each concentration is presented as “n” below. FNAR-C1 cells were exposed to the following concentrations of Taxol: 10 nM, 20 nM, 40 nM, and 60 nM (ALDH^{high}: n=4; ALDH^{low}: n=5). Taxol-resistant SKOV3 cells were exposed to the following concentrations of Taxol: 100 nM (n=6), 200 nM (n=7), 400 nM (n=8), 600 nM (n=7), and 800 nM (n=3); carboplatin (Sigma-Aldrich, St. Louis, MO): 250 ng/ml (n=1), 500 ng/ml (n=5), 750 ng/ml (n=5), 1 μ g/ml (n=5), and 1.25 μ g/ml (n=4); and gemcitabine (Gemzar; Eli Lilly and Company, Indianapolis, IN): 2 nM (n=3), 3 nM (n=3), 4 nM (n=5), 5 nM (n=5), 6 nM (n=2), and 7 nM (n=2). Excel 2008 was used to generate graphs. The Kruskal-Wallis test was used to assess statistical significance.

Results

Aldefluor Defines Distinctive Populations

Initial experiments with the FNAR-C1 rat model of ovarian cancer showed a small ALDH^{high} population, as measured with the Aldefluor reagent. However, continued culture in standard medium containing serum quickly resulted in the loss of ALDH^{high} cells (Fig. 1A; mean = 2.05%, range = 0.23–9.3%). Culturing FNAR-C1 cells in Knockout medium,

which is a proprietary, serum-free medium designed for the maintenance of embryonic stem cells in an undifferentiated state, maintained a large ALDH^{high} population, representing approximately half of the cells (Fig. 1B; mean = 56.88%, range = 15.7 – 86.5%).

Like FNAR-C1 cells, SKOV3 cells cultured in standard serum-containing medium showed few ALDH^{high} cells (Fig. 1C; mean = 2.12%, range = 0.18 – 6.28%). In contrast to the FNAR-C1 cells, culture of SKOV3 cells in KnockOut medium did not substantially change the percentage of ALDH^{high} cells (data not shown). However, when SKOV3 cells developed Taxolresistance, a distinct ALDH^{high} population emerged in standard medium (Fig. 1D; mean = 3.25%, range = 1.18 – 12.1%). Furthermore, culturing Taxol-resistant SKOV3 cells in KnockOut medium expanded the ALDH^{high} population (Fig. 1E; mean = 20.22, range = 10.0% to 42.2%).

ALDH^{high} Cells Exhibit Stem Cell Morphologic and Growth Characteristics

Commonly accepted properties of stem cells include non-adherent growth and the absence of contact inhibition. FNAR-C1 cells grow adherently on plastic. Upon reaching confluency, ALDH^{low} cells showed substantial death, which resulted in large areas devoid of cells (data not shown). ALDH^{high} FNAR-C1 cells failed to undergo growth arrest upon achieving confluency and instead formed large three-dimensional clusters of cells, which we refer to as nodules (Fig. S2A). In addition to forming nodules, a portion of ALDH^{high} FNAR-C1 cells grew as nonadherent clusters, or spheroids (Fig. S2B). These spheroids formed regardless of the confluency of the culture and contained viable cells based on trypan blue exclusion. ALDH^{low} FNAR-C1 cultures produced no nodules, and supernatant from the cell cultures lacked viable cells (data not shown). ALDH^{high} Taxol-resistant SKOV3 cells also displayed nonadherent growth through the formation of spheroids (Fig. S2C), which were not observed in ALDH^{low} SKOV3 cells (data not shown).

Stem cells are typically smaller in size than their differentiated counterparts; this is generally attributed to their relative quiescence. Accordingly, ALDH^{high} cells from both FNARC1 and Taxol-resistant SKOV3 cells were smaller than their ALDH^{low} counterparts (Fig. S3). ALDH^{high} cells had substantially longer doubling times than ALDH^{low} cells, revealing a slower growth rate (Fig. 2A, B; ALDH^{low} FNAR-C1: 15.58 hours, ALDH^{high} FNAR-C1: 32.21 hours, ALDH^{low} SKOV3: 27.04 hours, ALDH^{high} Taxol-resistant SKOV3: 39.62 hours). Cell cycle analysis showed that the slower growth rate was a result of a greater percentage of ALDH^{high} cells in the G1/G0 phase of the cell cycle compared to ALDH^{low} cells (Fig. 2C–F). Furthermore, the decreased proliferation of ALDH^{high} cells was associated with downregulation of genes important for progression from G1 to S phase relative to ALDH^{low} cells (Figs. S4 and S5). In the FNAR-C1 model, these genes included c-myc, neuregulin 1 and cyclin D1. ALDH^{high} Taxolresistant SKOV3 cells exhibited downregulation of cyclin dependent kinase 6, transcription factor DP-1, and ABL1 with concurrent upregulation of cyclin-dependent kinase inhibitor 1A.

ALDH^{high} Cells Are Capable of Regenerating the Phenotypic Diversity of the Cell Line and Are Tumorigenic *In Vivo*

ALDH^{low} and ALDH^{high} cells were isolated from both the FNAR-C1 and SKOV3 lines by cell sorting, then cultured in standard and KnockOut medium. ALDH^{low} cells isolated from both cell lines generated only ALDH^{low} cells *in vitro*, even when cultured in KnockOut medium that optimally supports the growth of ALDH^{high} cells (Figs. 3A, B, E and F). Conversely, ALDH^{high} cells remained ALDH^{high} when cultured in KnockOut medium but rapidly generated ALDH^{low} cells in standard medium, indicating their ability to regenerate the phenotypic diversity of the cell line (Figs. 3C, D, G and H).M

The gold standard assay for cancer stem cells is increased *in vivo* tumorigenicity. In order to test the tumorigenicity of ALDH^{high} and ALDH^{low} cells, graded numbers of sorted FNAR-C1 cells were injected intraperitoneally into immunocompetent Lewis rats. The only rats to develop tumors were those receiving ALDH^{high} cells. Three out of 16 rats receiving 10⁵ ALDH^{high} cells and one out of 12 rats receiving 10³ ALDH^{high} cells formed tumors (tumors became palpable at days 148, 177 and 222 for 10⁵ cells and day 222 for 10³ cells). None of the rats injected with ALDH^{low} cells developed tumors (10⁵ cells, n=8; 10³ cells, n=9). This provides *in vivo* support for the conclusion that ALDH^{high} cells represent the tumorigenic population within FNAR-C1 cells.

ALDH^{high} Cells Display Multi-Drug Resistance

ALDH^{high} FNAR-C1 cells showed a statistically significant increased resistance to Taxol (Fig. 4A). When examining the sensitivities of SKOV3 cells, it was inappropriate to compare Taxol-resistant cells to Taxol-sensitive cells, and so ALDH^{high} Taxol-resistant SKOV3 cells grown in KnockOut medium were compared to ALDH^{low} Taxol-resistant SKOV3 cells grown in standard medium. ALDH^{high} Taxol-resistant SKOV3 cells showed a statistically significant increased resistance to Taxol and gemcitabine (Fig. 4B, C) and a trend towards resistance to carboplatin (Fig. 4D). Therefore, ALDH^{high} cells displayed resistance to chemotherapy drugs with different mechanisms of action and resistance.

Expression of ALDH1 family members in ALDH^{high} Cells

In order to correlate the Aldefluor staining patterns in isolated cells with ALDH1 expression, the expression levels of ALDH1 family members were examined. In ALDH^{high} FNAR-C1 cells, there was 86.522-fold upregulation (325.761-fold by qPCR) of ALDH1A2 relative to ALDH^{low} FNAR-C1 cells, but no differences in ALDH1A1 or ALDH1A3 were detected. ALDH^{high} Taxol-resistant SKOV3 cells expressed ALDH1A1 at a 23.699-fold greater level (44.494-fold by qPCR) than ALDH^{low} SKOV3 cells. ALDH1A2 and ALDH1A3 were expressed at comparables levels in both SKOV3 cell populations.

Inconsistent Expression of Putative Ovarian Cancer Stem Cell Markers in ALDH^{high} Cells

While CD24 has been proposed as an ovarian cancer stem cell marker, disagreements exist concerning whether ovarian CSCs express it to a greater or lesser degree than differentiated cells [6, 10]. ALDH^{high} Taxol-resistant SKOV3 cells exhibited substantial downregulation of CD24 relative to ALDH^{low} SKOV3 cells (Table 1). ALDH^{high} FNAR-C1 cells also showed downregulation of CD24 compared to ALDH^{low} cells, but to a lesser degree than in SKOV3

cells (Table 1). However, all populations expressed considerable levels of CD24, potentially limiting its usefulness as a marker for isolation.

High expression of CD44 has been reported to enrich for ovarian CSCs [24]. However, ALDH^{high} FNAR-C1 cells exhibited substantial downregulation of CD44 relative to ALDH^{low} FNAR-C1 cells (Table 1). Moreover, there was no difference in CD44 expression between ALDH^{high} Taxol-resistant SKOV3 cells and ALDH^{low} SKOV3 cells, with both populations showing high levels of expression (Table 1).

Published reports of putative ovarian CSCs demonstrate stark variability in KIT expression, which is reflected in our findings [4, 7]. In FNAR-C1 cells, KIT expression was too low for differences to be reliably detected by microarray, however qPCR showed that KIT expression was largely confined to the ALDH^{high} population (Table 1). ALDH^{high} Taxol-resistant SKOV3 cells exhibited the opposite pattern with expression of KIT only reliably detected in ALDH^{low} SKOV3 cells (Table 1).

Putative ovarian CSCs have also been isolated based on CD133 positivity [5]. CD133 expression was too low to be reliably detected in FNAR-C1 cells using microarray analysis. By qPCR analysis, CD133 was undetectable in the ALDH^{high} population but appeared to be expressed at very low levels in the ALDH^{low} fraction (Table 1). In ALDH^{high} Taxol-resistant SKOV3 and ALDH^{low} SKOV3 cells, CD133 expression was too low for reliable detection by either microarray or qPCR (Table 1).

Potential Therapeutic Targets to Eliminate ALDH^{high} Cells

In seeking potential therapeutic targets, we hypothesized that prognostic indicators in ovarian cancer would be differentially expressed in ovarian CSCs. However, steroid hormone receptors, gonadotropin receptors and CA125 were not consistently differentially expressed in the ALDH^{high} cells of both models (Table S1). Similarly, ABC transporters and developmental pathways have been implicated in ovarian CSCs. Again, any differential expression in ALDH^{high} cells was not consistent between the two cell lines (data not shown). As a result of this, a systematic survey of the microarray data was conducted to identify those genes and pathways expressed in the ALDH^{high} cells of both models. This data was then examined for readily drugable targets. These genes and pathways are described as potential therapeutic targets that may be effective in eradicating ovarian CSCs.

Differential gene expression of ALDH^{high} cells from both models indicates increased mTOR signaling (Figs. S7 and S8). The primary mechanism of increased mTOR signaling involved inhibition of the TSC complex, which inhibits mTOR activation through RHEB [25]. In FNAR-C1 cells, this inhibition was due to upregulation of TSC complex inhibitors (IRS1, PRKCH and RPS6KB2) and downregulation of a complex activator (PRKAG2). ALDH^{high} Taxol-resistant SKOV3 cells primarily suppress the activity of the TSC complex via upregulation of complex inhibitors (PIK3R1 and RPS6KB2). ALDH^{high} cells from both models also exhibited upregulation of the mTOR activator RRAGD (Table 2). Increased expression of many pathway components downstream of the TSC complex in ALDH^{high} cells of both cell lines further supported activation of mTOR signaling in these cells.

Ovarian cancer specimens commonly express her-2/neu (ERBB2) [26]. FNAR-C1 cells did not display differential expression of her-2/neu (Table 2). However, strong expression was observed in both populations, with her-2/neu in the upper 40% of all genes when ranked by signal strength. Quantitative PCR confirmed this high level of expression with her-2/neu being detected less than 3 cycles after the endogenous control. SKOV3 cells expressed her-2/neu at a sufficiently high level as to suggest overexpression, with a slight, but consistent, upregulation of her-2/neu in ALDH^{high} cells (Table 2). When ranked by signal strength, the microarray signal for ALDH^{high} Taxol-resistant SKOV3 cells was in the top 3%.

CD47 prevents phagocytosis by macrophages and is highly expressed in normal and malignant stem cells [27–29]. ALDH^{high} cells of both models expressed approximately 2-fold higher levels of CD47 than their ALDH^{low} counterparts as measured by microarray and qPCR (Table 2).

Both ALDH^{high} populations also exhibited upregulation of fibroblast growth factor 18 versus their ALDH^{low} counterparts (FGF18; Table 2). FGF18 primarily signals through fibroblast growth factor receptor 3 (FGFR3), which was expressed in all populations tested. As measured by microarray, FNAR-C1 cells expressed FGFR3 at levels similar to her-2/neu, and expression in SKOV3 cells ranked in the upper 20% of measured genes.

Discussion

One of the best-studied hematopoietic stem cell markers is ALDH, specifically the ALDH1 family of enzymes also known as retinaldehyde dehydrogenases. Additionally, ALDH1 is a marker for stem cells from most other tissues, and its expression decreases as stem cells differentiate [30]. Putative CSCs from most malignancies also express ALDH1. Although the precise function of ALDH1 enzymes in stem cell biology is unclear, they act as general aldehyde scavengers and are a critical component of retinoic acid biosynthesis. We studied two different model systems representing two distinct ovarian cancer subtypes, a spontaneously occurring rat ovarian cancer of the endometrioid subtype and the human ovarian cancer cell line SKOV3 of the serous or clear cell subtype. We hypothesized that potential biologic similarities between putative ovarian CSCs from different subtypes and two species would suggest fundamental features of ovarian CSCs. Accordingly, we found that high ALDH1 activity isolated cells that exhibited stem cell characteristics. The ALDH^{high} cells displayed an absence of contact inhibition, nonadherent growth, smaller size, quiescence, and multi-drug resistance. Moreover, the ability to regenerate the phenotypic diversity of the cell line *in vitro* and produce tumors *in vivo* was exclusive to the ALDH^{high} subpopulation. These data suggest that ovarian CSCs in both models are likely contained within the ALDH^{high} population.

CD44, KIT and CD133 have been reported to enrich for ovarian CSCs in both cell lines and patient material, but the data have been inconsistent. We also found them to be inconsistently expressed in the ALDH^{high} cells of the two models. Both ALDH^{high} populations did display decreased expression of CD24, although the difference in expression compared to the ALDH^{low} population may not be sufficient to permit isolation of the cells. A lack of

consensus exists in the published literature concerning expression of these markers in ovarian CSCs with some studies finding higher expression of CD24, CD44, CD133 and KIT, but others finding reduced expression in the putative ovarian CSC population [4–13]. The inconsistencies in expression identified here and elsewhere in the literature may be due to biological differences between distinct ovarian cancer subtypes or intertumoral heterogeneity, but more work is needed to ascertain the relationship between these different markers.

These data confirm the utility of ALDH1 as an ovarian CSC marker, and raise questions about the broad applicability of other proposed ovarian CSC markers: CD44, KIT, and CD133. Few studies to this point have proposed targets for ovarian CSCs, perhaps in part because of inconsistent definitions for these cells. Several clinically targetable genes and pathways were highly expressed in ALDH^{high} cells of both models, and thus may serve as ovarian CSC targets, including mTOR, her-2/neu, CD47, and FGF18 / FGFR3. Therapeutic interventions targeting the mTOR pathway or CD47 would likely preferentially target CSCs over their more differentiated counterparts. In contrast, targeting her-2/neu or FGF18 / FGFR3 would lack specificity, but would potentially affect CSCs in addition to more differentiated cell populations. Drugs targeting the mTOR pathway and her-2/neu are already in clinical use, and several FGFR3 inhibitors and antibodies targeting CD47 are beginning clinical trials. Future work will be necessary to identify the optimal targeted therapy and to validate these findings in primary human tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- ALDH^{high} ovarian cancer cells phenotypically resemble cancer stem cells (CSCs).
- Based on gene expression ALDH^{high} and ALDH^{low} cells are biologically distinct.
- Other reported ovarian CSC markers do not consistently identify ALDH^{high} cells.
- Potential therapeutic targets include: mTOR signaling, Her-2/neu, CD47 and FGFR3.

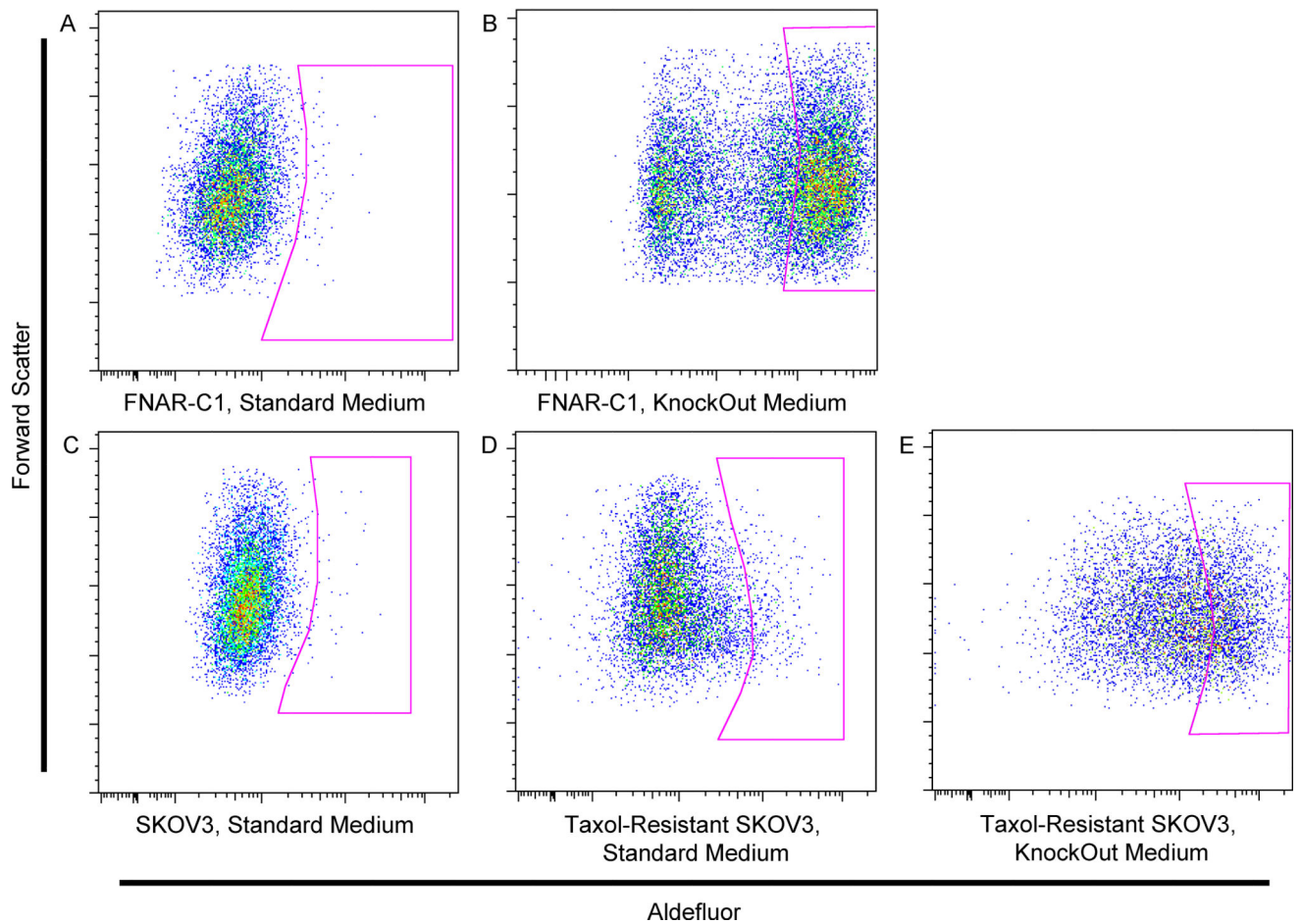


Figure 1. ALDH1 Activity of Ovarian Cancer Cell Lines

Representative Aldefluor staining pattern of (A) FNAR-C1 cells grown in standard medium containing serum, (B) FNAR-C1 cells grown in KnockOut medium, (C) SKOV3 cells grown in standard medium, (D) Taxol-resistant SKOV3 cells grown in standard medium, and (E) Taxol-resistant SKOV3 cells grown in KnockOut medium. DEAB was used as a negative control for ALDH1 activity (shown in Figure S1).

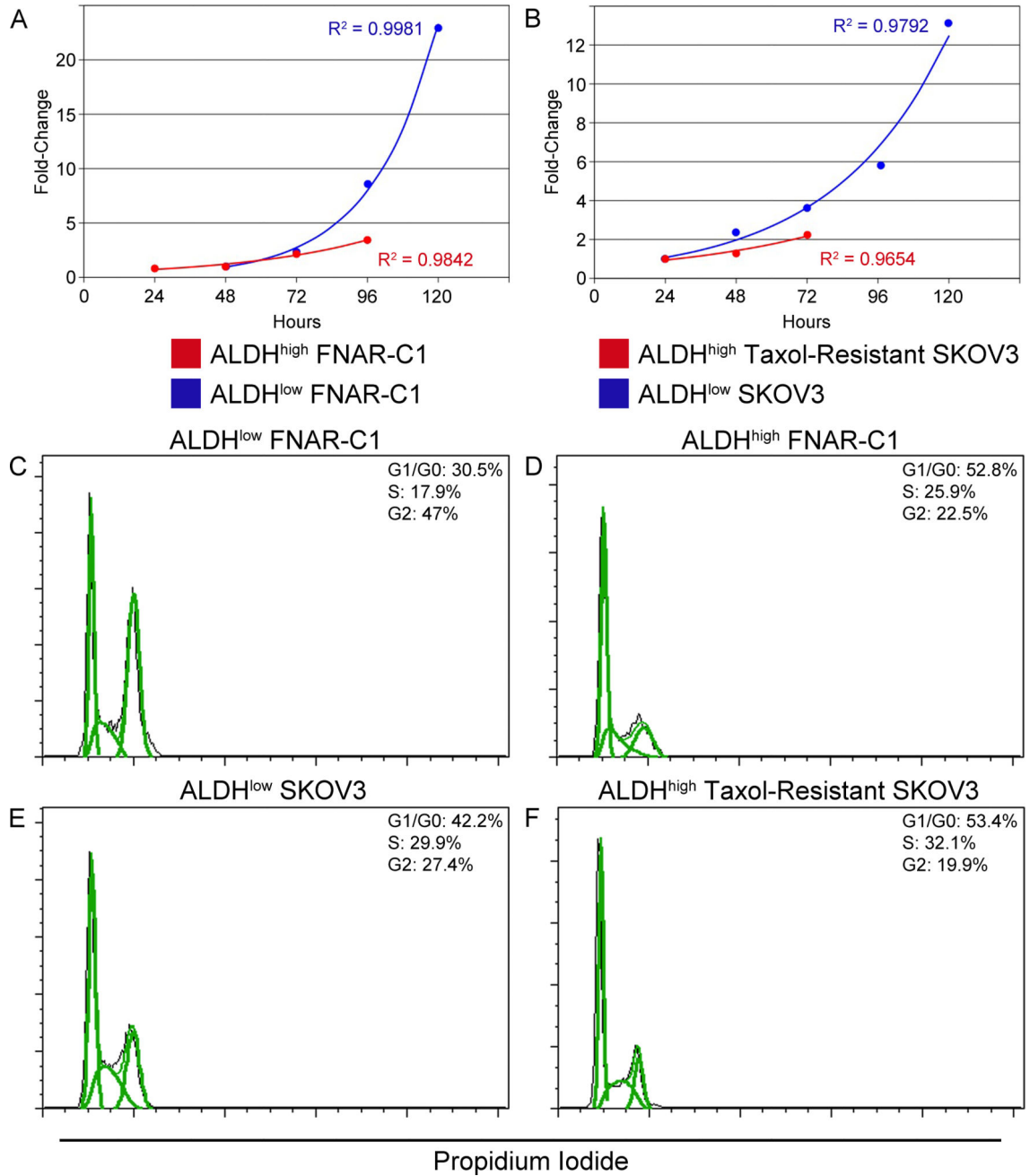


Figure 2. Proliferative Status of Cells Based on ALDH activity
 Growth curves of sorted: (A) FNAR-C1 cells and (B) SKOV3 cells. (C-F) Representative cell cycle analyses using propidium iodide: (C) ALDH^{low} FNAR-C1 cells, (D) ALDH^{high} FNAR-C1 cells, (E) ALDH^{low} SKOV3 cells, and (F) ALDH^{high} Taxol-resistant SKOV3 cells.

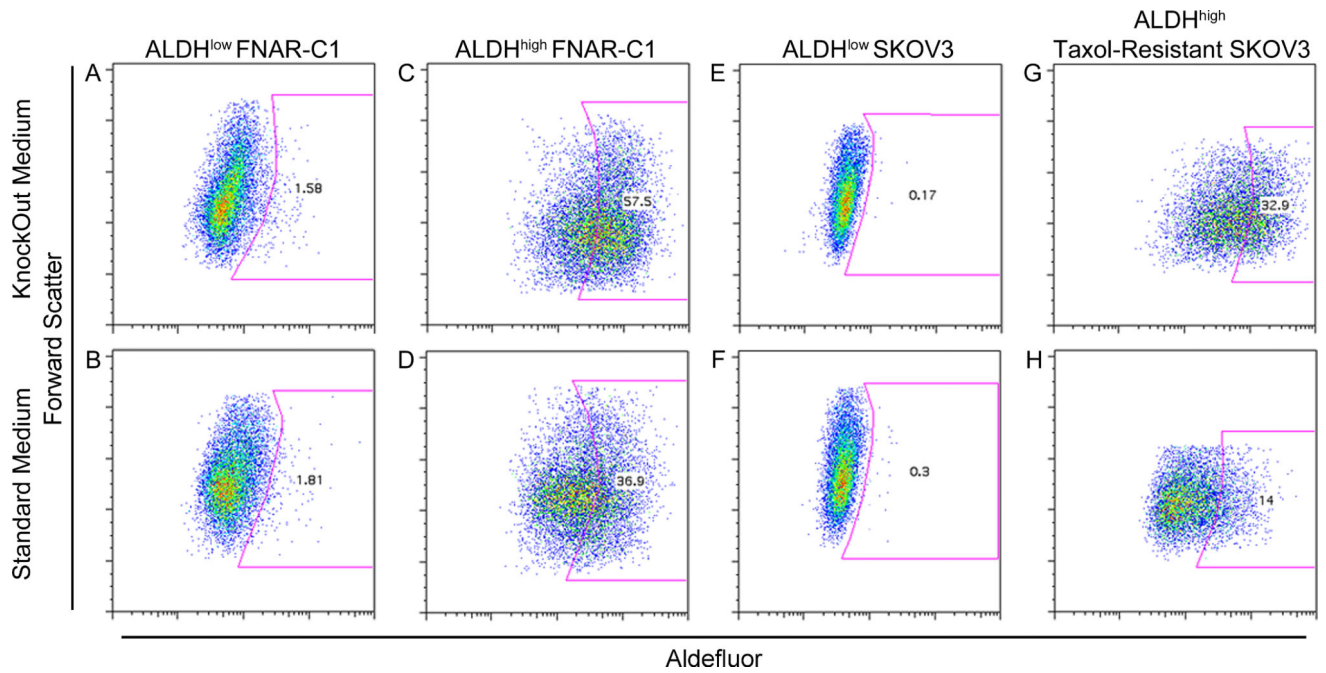


Figure 3. Regeneration of Phenotypic Diversity

Sorted ALDH^{low} FNAR-C1 cells (A, B), ALDH^{high} FNAR-C1 cells (C, D), ALDH^{low} SKOV3 cells (E, F), and ALDH^{high} Taxol-resistant SKOV3 cells (G, H) were plated in either KnockOut medium (A, C, E, G) or standard medium (B, D, F, H) for four days and then stained for Aldefluor using DEAB as a negative control (shown in Figure S6).

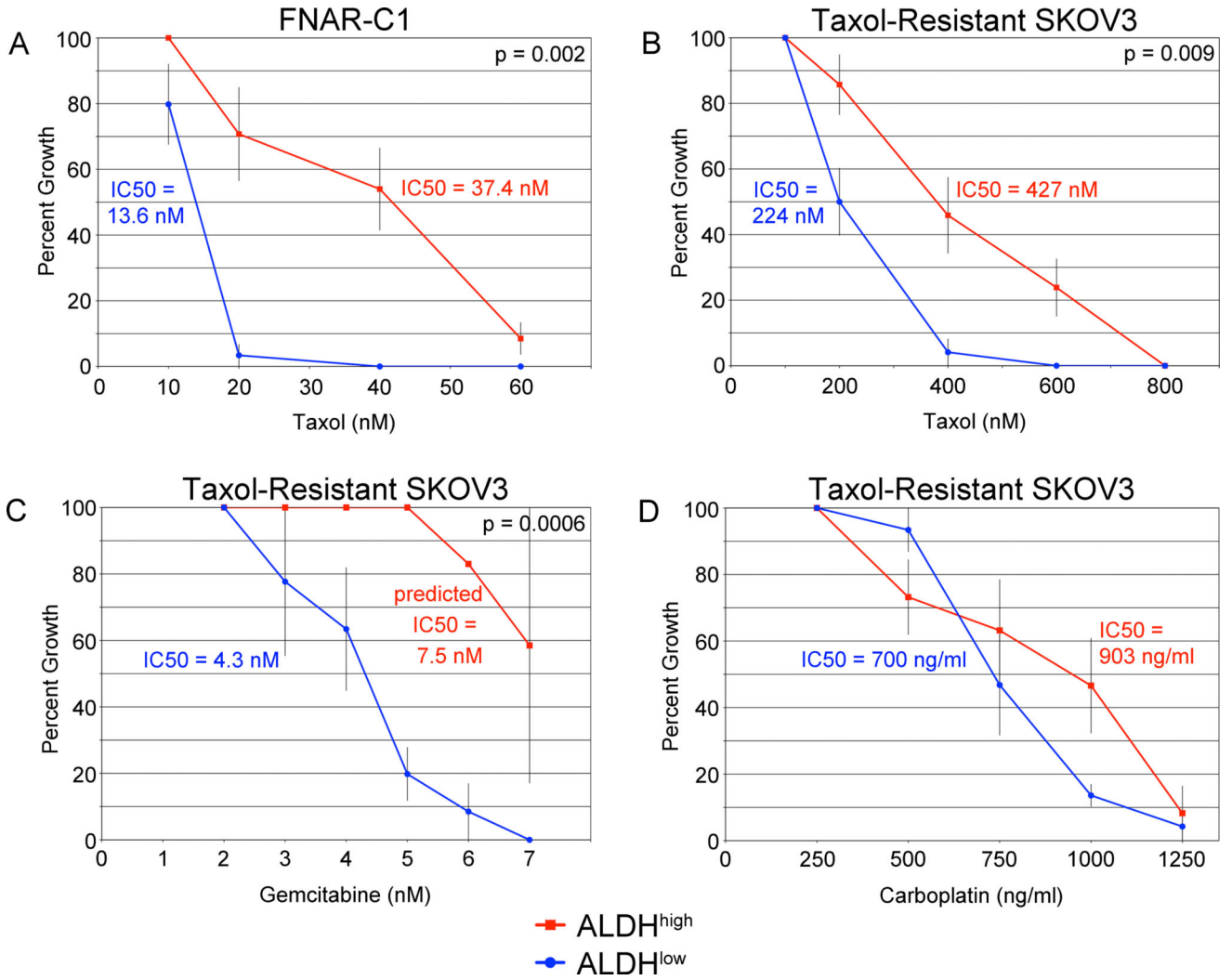


Figure 4. Relative Drug Resistance of ALDH^{high} Cells
 Sorted cells were assayed for their ability to grow in increasing concentrations of drugs. Error bars show the standard error. Numbers of replicates are detailed in Materials and Methods. (A) FNAR-C1 cells cultured with Taxol. (B–D) Taxol-resistant SKOV3 cells cultured in: (B) Taxol, (C) gemcitabine, and (D) carboplatin.

Table 1
Differential Expression of Previously Reported Ovarian Cancer Stem Cell Markers

Fold-change values from microarray and qPCR are presented

| Gene | FNAR-C1 Microarray | FNAR-C1 PCR | SKOV3 Microarray | SKOV3 PCR |
|------------------|-----------------------|----------------|---------------------|--------------|
| CD24 | -1.763 | -1.560 | -9.458 | -41.667 |
| CD44 | -21.481 | -22.727 | -1.188 | -1.003 |
| KIT | ** | 10.644 | -1.968 | -333.333 |
| CD133 (PROM1) | ** | ↓ | ** | ** |

FNAR-C1 data are presented as ALDH^{high} FNAR-C1 cells versus ALDH^{low} FNAR-C1 cells. SKOV3 data are presented as ALDH^{high} Taxol-resistant SKOV3 cells versus ALDH^{low} SKOV3 cells.

↓ signifies that the gene was detectable in ALDH^{low} cells but not in ALDH^{high} cells.

** Signifies that the gene was not reliably detectable in either ALDH^{high} or ALDH^{low} cells.

Table 2
Differential Expression of Potential Therapeutic Targets

Fold-change values from microarray and qPCR are presented

| Gene | FNAR-C1 Microarray | FNAR-C1 PCR | SKOV3 Microarray | SKOV3 PCR |
|----------------------|-----------------------|----------------|---------------------|--------------|
| RRAGD | 3.467 | | 2.309 | |
| ERBB2 (her-2/neu) | -1.040 | -1.147 | 1.320 | 1.308 |
| CD47 | 2.088 | 1.961 | 1.827 | 2.079 |
| FGF18 | 2.912 | 3.793 | 1.778 | 3.775 |

FNAR-C1 data are presented as ALDH^{high} FNAR-C1 cells versus ALDH^{low} FNAR-C1 cells. SKOV3 data are presented as ALDH^{high} Taxol-resistant SKOV3 cells versus ALDH^{low} SKOV3 cells. Empty cells signify that the gene was not tested with that assay.