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Characterization of aldehyde dehydrogenase 1 high ovarian cancer cells: Towards targeted stem cell therapy

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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.

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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 CD44, KIT and CD133. However, the following druggable 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 druggable targets that have the potential for therapeutic efficacy against ovarian CSCs from multiple subtypes.

1. 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,

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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 β-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.

2. Materials and methods

2.1. 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.

2.2. 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 using the manufacturer's protocol (STEMCELL Technologies Inc., Vancouver, BC, Canada) to assess ALDH1 activity, then stained with 0.5–1 μg/mL propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) for 5 min. 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).

2.3. 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 20× phase contrast objective, Nikon DS-Qi1Mc CCD camera, and Nikon NIS Elements 3 software (Nikon Instruments Inc., Melville, NY).

2.4. 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 h, 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). R^2 was calculated using Prism 6 for Mac (GraphPad Software, La Jolla, CA). Graphs were generated using Excel 2008 for Mac (Microsoft, Redmond, WA).

2.5. Microarray and pathway analysis

Cells were sorted into RNAprotect 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 of 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).

Microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE82305 (http://www.ncbi.nlm.nih.gov/geo/query/acc.