Published in final edited form as:

Clin Cancer Res. 2017 November 15; 23(22): 6934–6945. doi:10.1158/1078-0432.CCR-17-0133.

Personalized Medicine Based Approach to Model Patterns of Chemoresistance, and Tumor Recurrence Using Ovarian Cancer Stem Cell Spheroids

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Abstract

Purpose: Chemoresistant ovarian cancers grow in suspension within the ascites fluid. In order to screen the effect of chemotherapeutics and biologics on resistant ovarian cancers with a personalized basis, we developed a 3D hanging drop spheroid platform.

Experimental Design: We initiated spheroids with primary ALDH⁺ CD133⁺ ovarian cancer stem cells (OvCSCs) from different patient samples, and demonstrated that stem cell progeny from harvested spheroids was similar to the primary tumor. OvCSC spheroids were utilized to initiate tumors in immune-deficient mice. Drug responses to cisplatin and ALDH targeting compound or JAK2 inhibitor determined if the OvCSC population within the spheroids could be targeted. Cells that escaped therapy were isolated and used to initiate new spheroids and model tumor re-emergence in a personalized manner.

Results: OvCSC spheroids from different patients exhibited varying, and personalized responses to chemotherapeutics. Xenografts were established from OvCSC spheroids, even with one single spheroid. Distinct responses to therapy were observed in distinct primary tumor xenografts similar to those observed in spheroids. Spheroids resistant to Cisplatin/ALDH inhibitor therapy had persistent, albeit lower ALDH expression and complete loss of CD133 expression while those resistant to cisplatin/JAK2 inhibitor therapy were enriched for ALDH⁺ cells.

Conclusions: Our 3D hanging drop suspension platform can be used to propagate primary OvCSCs that represent individual patient tumors effectively by differentiating *in vitro*, and

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Conflict of Interest Statement: The authors declare no potential conflicts of interest.

initiating tumors in mice. Therefore, our platform can be used to study cancer stem cell biology, and model tumor re-emergence to identify new targeted therapeutics from an effective personalized medicine standpoint.

Keywords

Ovarian cancer; Malignant Ascites; Cancer Stem Cells; Spheroids; Personalized Medicine

1. Introduction

Ovarian cancer is the most lethal gynecologic malignancy, with a projected 5 year mortality rate >70%(1). Cytoreductive surgery and adjuvant chemotherapy can yield a clinical response even in stage III-IV disease. Unfortunately, relapse and chemoresistance is observed in a majority of patients(2). In the era of personalized medicine, and development of targeted therapeutics based on patients' genetic status, it is important to develop *in vitro* platforms that can reliably test and predict patient response to novel therapeutics(3,4).

Particular to ovarian cancer, malignant ascites is a therapeutic challenge that is difficult to target even with the advent of intra-peritoneal chemotherapy(5–7). Malignant ascites is enriched in ovarian cancer stem-like cells (OvCSCs) that can contribute to therapeutic resistance(8–11). Previous studies have indicated that presence of CD133⁺ and ALDH⁺ cells in ovarian cancer patient samples, is a prognosis for reduced progression free survival and poor patient outcome(12–21). Moreover, the CSCs were found to be present in the tumors after treatment with carboplatin and paclitaxel, and these cells maintained their tumor-initiating properties(22). When CSCs between matched primary and recurrent ovarian tumor patient samples were compared, CSCs were found at higher percentages after recurrence(23). Therefore, clinical evidence supports the role of ovarian CSCs in therapy response and tumor recurrence.

Currently, preclinical drug screening *in vitro* utilizes monolayer adherent cultures that are convenient and economical to perform, but do not recapitulate the non-adherent ascites environment. This non-physiological screening approach often results in poor predictive power for drug efficacy in patients(24). *In vitro* studies that involve monolayer cultures of OvCSCs also result in a loss of the cancer stem cell-like phenotype and a loss of physiological relevance while studying their role in tumor biology(25–27). Studies that observe and establish chemoresistance and invasive properties of OvCSCs are traditionally performed on murine xenograft models, through serially passaging to maintain and demonstrate tumorigenicity(13,16,27,28). While these studies afford the opportunity to investigate therapeutic resistance with a cancer stem cell (CSC) context, murine xenograft assays are often expensive and labor intensive to perform.

Spheroids are highly valuable tools to recapitulate the ascites microenvironment *in vitro*, and study both chemoresistance patterns, as well as, tumorigenicity of OvCSCs. Particular to ovarian cancer, spheroids also mimic the cellular aggregates often found in suspension within malignant ascites, thereby serving as a highly physiologically relevant *in vitro* model. 3D culture has also been documented to maintain higher levels of cancer stem-like traits, compared to monolayer cultures(20,25,26). We have previously demonstrated the use of a

high throughput easy to use 384-well hanging drop array platform that can stably incorporate 10–100 ovarian cancer cells to form uniform size and shape spheroids for analysis of drug response(29,30). The ability to use small cell numbers to initiate spheroids is highly relevant in the context of OvCSCs, since they make up 0.1–0.5% of the cells found in primary ovarian tumors(8). Given this limitation in source material, platforms such as ours(29,30), that can promote homogenous replicates from a rare and scarce number of patient cells to test their responses to various combinations of dose and drug, are highly relevant and useful for therapeutic screening, especially for personalized medicine applications.

In this study, we demonstrate the use of a spheroid-based platform to generate spheroids from primary ovarian cancer stem cells derived from patient ascites(12). We further demonstrate the predictive potency of spheroid-based therapeutic assays compared to those conducted *in vivo* in murine xenograft models established from OvCSC spheroids. We perform drug sensitivity assays in the context of OvCSCs, by using the conventional chemotherapeutic drug, cisplatin, and combining it with a novel ALDH targeting compound, in development, compound 673A(31), as well as, a prominent JAK2 inhibitor, ruxolitinib(32). Platinum based agents are the core chemotherapeutic for treatment of ovarian cancer(33,34). In addition to cisplatin, we chose two compounds, in order to study if the OvCSC population within spheroids could be targeted owing to their established elevated aldehyde dehydrogenase activity(13), as well as, increased contribution of the JAK2/STAT3 pathway in the maintenance of the cancer stem cell pool(35,36). Lastly, by identifying populations that escape drug treatment, we demonstrate the capability of this platform to model the re-emergence of tumors using therapy-escaping resistant cells. The establishment of this model will allow for the study of resistant cell populations and their biological contribution to the emergence of therapeutic resistance in ovarian cancer, including from a strong personalized medicine standpoint.

2. Materials and Methods

2.1 Materials

All tissue culture reagents, and media supplements were purchased from Life Technologies (Carlsbad, CA) unless specified otherwise. Primary ascites were collected from patients with informed signed consent under an institutionally approved IRB protocol. Cisplatin was purchased from Sigma Aldrich (St. Louis, MO), and the ALDH inhibitor compound 673A was a gift from Dr. Ronald Buckanovich's lab and ruxolitinib was a gift from Dr. Karen McLean's lab. The ALDEFLUOR Assay Kit was purchased from StemCell Technologies (Vancouver BC).

2.2 Isolation of OvCSCs from primary patient ascites

Cells were recovered from primary patient ascites following protocols established previously(12). Briefly, primary patient malignant ascites infusions were centrifuged to recover a cell pellet, and red blood cells were lysed following manufacturer's protocol of a commercial ACK lysis buffer (Life Technologies). Cells were obtained from xenografted tumors using the tumor cell dissociation kit (Miltenyi Biotech, San Diego CA) following

manufacturer's protocols. After a 40µm filtration to obtain single cell suspensions, cells were recovered by centrifugation and resuspended in a flow cytometry appropriate buffer (FACS Buffer; phosphate buffered saline supplemented with 2% fetal bovine serum). OvCSCs were isolated based on concurrent elevated aldehyde dehydrogenase (ALDH) activity and expression of CD133, following protocols established previously(12). Cells were counted using a hemocytometer, and adjusted to 1×10^6 cells/ml. The ALDEFLUOR Assay was carried out as per manufacturer's protocol, using diethylaminobenzaldehyde (DEAB) as a negative control for aldehyde dehydrogenase (ALDH) staining. Simultaneously, cells were incubated with activated ALDEFLUOR reagent, or allophycocyanin(APC) conjugated CD133 antibody (Milentyi Biotech, San Diego CA) to isolate CD133⁺ cells based on surface antigen expression and ALDH⁺ cells on the basis on ALDH activity. A negative control for CD133-APC staining was established using an isotype matched APC-conjugated antibody, to identify background staining. Cells were incubated in staining solutions for 30–45 minutes at 37°C, washed with PBS, and resuspended in FACS Buffer with 300µM 4',6-diamidino-2-phenylindole (DAPI). Cells were sorted on a flow cytometer, and discrete single cell viable populations were identified. Appropriate gates were set for ALDH and CD133-APC using DEAB stained tubes, and APC-isotype antibody tubes. OvCSCs were isolated as the small fraction of cells positive for both ALDH activity and the expression of CD133-APC (Supplementary Figure 1). For this study, OvCSCs were FACS sorted and obtained from three different patient samples: Pt259 (stage IV progressive primary peritoneal carcinoma), Pt224 (stage IV, platinum resistant, ovarian adenocarcinoma), and Pt152 (stage IIIC, recurrent platinum-resistant high grade ovarian adenocarcinoma).

2.3 Hanging drop culture for spheroid formation from OvCSCs

FACS sorted OvCSCs were harvested, and concentrated using high-speed centrifugation. Cells were resuspended in serum-free growth media (SFM; DMEM/F12 supplemented with 5ng/ml basic fibroblast growth factor, 5ng/ml epidermal growth factor, 1X B27 supplement, 1X insulin-transferrin-selenium supplement, 1X non-essential amino acids and 1X antibiotics and antimycotics). Cell counts were obtained using a hemocytometer, and adjusted such that a 20µl volume contained 10 OvCSCs. Spheroids were initiated using 10 OvCSCs per spheroid, following protocols established previously(29). Spheroids were maintained in SFM for a period of 7 days and imaged using live phase contrast microscopy to follow spheroid formation. Proliferation within spheroids was monitored using the alamarblue assay, as established previously(29,30). For spheroids initiated from cells isolated from xenografted tumors, spheroids were initiated using 50 cells/drop.

2.4 Flow cytometric analysis for OvCSC progeny within spheroids

At Day 7, OvCSC spheroids were harvested and resuspended in FACS buffer. Spheroids were mechanically dissociated into single cell suspensions and filtered through a 40µm filter. Cell counts were adjusted to a concentration of 100,000 cells/ml. Cells were recovered via centrifugation and resuspended in ALDEFLUOR Buffer, provided within the ALDEFLUOR assay kit. ALDEFLUOR assay was performed following manufacturer's protocols, using DEAB as an established negative control, and activated ALDEFLUOR reagent for positive staining of ALDH activity. Simultaneously, an isotype matched APC antibody was used for

a negative control to identify positive CD133 expression with an APC-conjugated CD133 antibody. Cells were divided into equal volumes and concentrations across tubes, with at least 10,000 cells in each tube. Stained cells were incubated in the 37°C incubator for 30–45 minutes, recovered by centrifugation, and resuspended in FACS buffer with 300µM DAPI to assess viability. Cells were processed through a flow cytometer, and forward and side scatter were used to isolate single cells, that were viable and DAPI negative. Gates were established using DEAB (less than 0.2% false positive) and APC-isotype (less than 0.2% false positive), and stained tubes were used to estimate the percentage of cells that were: ALDH⁺ CD133⁻, ALDH⁻ CD133⁺, ALDH⁺ CD133⁺ and ALDH⁻ CD133⁻ (Supplementary Figure 2. At least 10,000 cells were counted for each flow analysis.

2.5 Drug treatment on OvCSC spheroids in hanging drop array culture

OvCSC spheroids were initiated in 384-well hanging drop arrays using 10 OvCSCs/spheroid. Spheroids were allowed to aggregate and form a 3D microtissue over a period of 7 days, at which point flow cytometry-defined populations of OvCSC progeny were established. For drug treatment, a 10X stock of drug was prepared independently and 2µl of drug was added to the 20µl hanging drop containing spheroids to result in a final concentration of 1X. Several drug-dosing regimens were carried out including: cisplatin 50µM, the ALDH inhibitor compound 673A 50µM, cisplatin 50µM + 673A 50µM, the JAK1/2 inhibitor, ruxolitinib 500nM, and/or cisplatin 50µM + ruxolitinib 500nM. The concentrations for drug dosing were arrived at following dose response curves, where the effect of the drug was maximal for the different patient samples, while simultaneously demonstrating platinum resistance in OvCSC spheroids. The effect of drug was assayed on spheroids at 72hrs, using the alamarblue assay to determine viability after drug treatment (Supplementary Figure 3). Control untreated spheroids were maintained for the same duration in culture. At least 20 spheroids (technical replicates) were assayed per experiment, with 3–5 biological replicates. Drug treated spheroids were imaged using phase contrast microscopy to observe morphological differences in spheroids exposed to drug compared to control untreated OvCSC spheroids. Flow cytometry was used to analyze OvCSC progeny following 72-hour drug treatment to identify if chemotherapy targeted specific populations of OvCSC progeny. Lastly, spheroids were either treated with 500nM ruxolitinib or left untreated, and collected and lysed in RIPA buffer (Invitrogen) with complete proteinase inhibitor and phosphatase inhibitor (Roche). Insoluble material was removed by centrifugation. Protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad). Lysates were separated by gel electrophoresis and transferred onto a nitrocellulose membrane. Primary antibodies for immunoblotting include anti-phospho-STAT3 (tyrosine705, #9131, Cell Signaling), anti-total-STAT3 (#9139, Cell Signaling Technology), and anti-GAPDH (Thermo Scientific). Following incubation with the appropriate secondary antibody, bands were visualized using the ECL Kit (Thermo Scientific).

2.6 Recovery of drug treated spheroids to model tumor re-emergence and serial passaging

Following 72 hours of maximal dose drug treatment (cisplatin/673A), OvCSC spheroids were harvested and suspended in FACS buffer with 300µM DAPI. Cells were flow sorted

based on DAPI negativity, therefore isolating cells that remained viable following drug treatment. Spheroids were re-initiated in hanging drop array plates with cells suspended in SFM media, and imaged with phase contrast microscopy to visualize spheroid formation. To preliminarily model tumor re-emergence in the most platinum sensitive (Pt259) and most platinum resistant (Pt152) ascites samples, spheroids were collected from these two patient samples, mechanically dissociated to generate single cell suspensions, and re-counted to initiate 100 cells/drop spheroids for the next passage. Spheroids were serially passaged for 7 cycles (P0 to P6) over a period of 7 weeks. Flow analysis was performed as outlined previously at the end of each cycle to characterize OvCSC markers (ALDH and CD133). This allowed us to identify OvCSC populations within serially passaged spheroids, while cells from the same population were allowed to re-form spheroids, serving as a preliminarily model tumor re-emergence *in vitro*.

2.7 Tumor initiation and drug treatment from OvCSC spheroids in NSG mice

OvCSC spheroids were generated from the Pt259 sample, using 10 cells per 20 μ l drop. Spheroids were harvested at Day 7, recovered using centrifugation, and resuspended gently in 200 μ l of 1:1 mixture of SFM and Matrigel, while maintaining spheroid integrity. The following groups were used to identify tumor initiation: 1 spheroid, 2 spheroids, 10 spheroids, 50 spheroids, and 500 spheroids. 5000 freshly isolated OvCSCs were used as a positive control for the tumor initiation assay. Spheroids or freshly isolated OvCSCs were injected subcutaneously into flank or axilla of non-obese diabetic/severe combined immunodeficient/IL2R $\gamma^{-/-}$ (NSG) mice. Several days following injection, the injection sites were palpated and measurements were obtained using a caliper to record tumor volumes. 4–8 bi-axial tumor injections were performed for each group, and the number of injections that formed tumors were noted and expressed as percentage initiating tumors. Tumor volumes were recorded, until tumors reached an end point of 1500–2000mm³, whereupon the mice were euthanized. Tumors were dissected, cleaned and placed in biopsy cassettes for processing for histology and subsequent Hematoxylin and Eosin staining. Additionally, cells were isolated from the tumors using a tumor cell dissociation kit (Miltenyi Biotec). Isolated cells were used to initiate spheroids in hanging drop array plates, with 10 cells in each drop.

For drug treatment *in vivo*, tumors were initiated routinely from 10 spheroids of either Pt259 or Pt152 OvCSC samples. Once palpable tumors were established (~40–50mm³), mice were divided into two groups – control untreated, and cisplatin/673A treated. Drug dosing began the day palpable tumors were obtained, and mice were injected with 1mg/kg cisplatin intraperitoneally once weekly, and 673A at 20mg/kg intraperitoneally everyday for 21 days or until tumor burden end-point. When end-point was reached, tumors were dissected and processed for histology. Routine Hematoxylin and eosin staining was performed on cross-sections of tumor xenografts, along with staining for the proliferative marker Ki67, and the apoptosis marker cleaved Caspase 3. Isolated cells were used for flow cytometry analysis to determine OvCSC progeny in control and drug treated tumors *in vivo*.

2.8 Data Analysis

Flow cytometry data was analyzed using Summit software (Beckman Coulter, Brea, CA). Percentage of cells determined positive by flow cytometry was expressed after analyzing at

least 10,000 events per experimental condition. 3–5 independent flow cytometry analyses were performed, to identify an average percentage of OvCSC populations. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc, San Diego CA). All data is expressed as mean $\pm$ standard error of the mean, and is an average of at least 3–8 independent experiments.

Drug viability data was determined and quantified using the alamarblue assay, as outlined previously(29,30). Briefly, normalized viability was calculated by comparing the alamarblue fluorescence of drug treated spheroids to control untreated spheroids, and expressed as a percentage. Statistical analysis was performed using one- or two-way ANOVAs where appropriate, and levels of statistical significance are indicated on figures. All data is expressed as mean $\pm$ standard error of the mean, and is an average of at least 3–8 independent experiments.

3. Results

3.1 Formation and characterization of OvCSC spheroids from primary malignant-ascites derived patient samples

Three primary patient ascites samples were chosen from the tumor bank with a confirmed ovarian cancer origin, Pax8 and p53 expression (Supplementary Figure 4). OvCSCs were isolated from the primary ascites samples based on their concurrent expression of CD133 and elevated ALDH activity. The different patient samples had unique profiles of expression of ALDH⁺, CD133⁺ and ALDH⁺ CD133⁺ populations, as indicated in Figure 1A, B and C. For example, the primary Pt224 sample contained 19.8 $\pm$ 3% ALDH⁺ populations, with only 1.2 $\pm$ 0.3% CD133⁺ populations (Figure 1B). In contrast, the primary Pt152 sample contained only 1.3 $\pm$ 0.2% ALDH⁺ populations while CD133⁺ was high, averaging 18.4 $\pm$ 3.1% positivity. OvCSCs (ALDH⁺ CD133⁺ cells) were isolated roughly between 0.13% and 0.45% of the total population of cells analyzed.

Isolated OvCSCs were plated in hanging drop arrays, with 10 cells per drop to generate spheroids. Cells aggregated in the hanging drop arrays at Day 1, and proliferated and compacted over 7 days to form tight compact OvCSC spheroids (Figure 1D, E, F). Fold proliferation was measured within spheroids using the alamarblue assay, and 6–8 fold proliferation was observed in spheroids from all three patient samples over 7 days in hanging drop culture.

Consistent with our previous studies(15), flow analysis of OvCSC spheroids 7 days after seeding (representative plots shown in Figure 1G, H, I), indicated that ALDH⁺CD133⁺ cells in suspension were capable of both self-renewal and differentiation; all four cell populations (ALDH⁺CD133⁺, ALDH⁺ CD133⁻, ALDH⁻CD133⁺, and ALDH⁻CD133⁻) were observed. Quantification and comparison of OvCSC populations between the primary malignant ascites patient sample and OvCSC spheroids indicated that a similar OvCSC progeny profile was maintained during hanging drop spheroid culture in our platform (Figure 1J, K, L).

3.2 Treatment of OvCSC spheroids with cisplatin and ALDH inhibitor

OvCSC spheroids from Pt259, Pt224 and Pt152 samples were allowed to form for 7 days and treated with 50 μ M cisplatin, 50 μ M 673A (an ALDH1A1, ALDH1A2, ALDH1A3 specific inhibitor—to be described in a separate publication), or a combination of both. Control spheroids were untreated, and were maintained for the same duration in hanging drop culture. The effect of drug was assayed at 72 hours following drug addition, using the alamarblue fluorescence viability assay. Visual microscopic examination indicated that Pt259 OvCSC spheroids were more sensitive to cisplatin, 673A and the combination treatment. Compared to Pt259 spheroids, Pt224 spheroids were more resistant to the drug treatments, with Pt152 being the most resistant, indicated by lack of cell death in the micrographs (Figure 2A).

Quantification of viability indicated statistically significant differences in the response of the three different patient samples to cisplatin/673A chemotherapy (Figure 2B). Control untreated spheroids from all three different patient samples were between 93–100% viable. However, in cisplatin treated spheroids, Pt259 was the most responsive sample, with only 35.4 $\pm$ 1.9% viability compared to Pt152, which was the most resistant sample with 69.6 $\pm$ 2.4% viability (* p <0.001, two-way ANOVA). Pt259 and Pt224 were most responsive to the ALDH targeting compound 673A with only 40–49% viability, while Pt152 spheroids were largely resistant with 70.1 $\pm$ 2.6% cells within spheroids still viable. For combination therapies that included both cisplatin/673A treatments, Pt259 and Pt224 spheroids were most sensitive (10–28% viability), while Pt152 spheroids were significantly more resistant with 44.1 $\pm$ 1.9% viability (* p <0.001, two-way ANOVA). All three patient samples were sensitive to combined cisplatin/673A, but to differing degrees (Figure 2B, * p <0.001, two-way ANOVA), and featured combination indices of less than 1, indicating synergy between the drugs.

Flow analysis determined OvCSC progeny populations affected by the drug treatment. Cisplatin/673A treatment uniformly reduced ALDH⁺ and ALDH⁺ CD133⁺ populations in all three patient samples, while additionally also heavily targeting CD133⁺ populations in Pt152 OvCSC spheroids (Figure 2C). This indicated that a combination of cisplatin/673A effectively targeted OvCSC populations and progeny (Figure 2D). Compared to control untreated spheroids, ALDH and CD133 values were reduced in all patient samples, and ALDH⁺ CD133⁺ populations were completely annihilated by cisplatin/673A treatment (see Supplementary Figure 5 for CSC populations after treatment with cisplatin and 673A alone).

3.3 Treatment of OvCSC spheroids with cisplatin and ruxolitinib

Similar to cisplatin/673A, OvCSC spheroids were treated with the JAK1/2 inhibitor ruxolitinib at 500nM, or in combination with cisplatin 50 μ M. Microscopic examination and viability quantification both indicated that all three patient samples were responsive to drug treatments (Figure 3A, B). Viabilities ranged from 20.6% (Pt259) to 33.0% (Pt152). Compared to control untreated spheroids, viability was significantly reduced (* p <0.001, two-way ANOVA; Figure 3B) in all drug treatments in all three patient samples. Synergy between both drugs was evident from the combination indices of less than 1 for all three patient samples. Flow analysis of OvCSC progeny targeted by cisplatin/ruxolitinib treatment

demonstrated that CD133⁺ populations were effectively targeted in all three patient samples, while ALDH⁺ populations increased at least in one sample Pt152 (Figure 3C, D, see Supplementary Figure 5 for CSC populations after treatment with cisplatin and ruxolitinib individually). Even the previously resistant Pt152 was sensitive to the synergistic cisplatin/ruxolitinib combination treatment, as demonstrated by the loss of phosphorylated STAT3 in ruxolitinib treated spheroids (Figure 3E), in line with reports that note improved sensitivity of ovarian cancer cells to conventional therapies following STAT3 inhibition(37,38). The sensitization observed in our samples under cisplatin/ruxolitinib treatment could be attributed to the targeting of the elevated IL6/STAT3 signaling axis reported in resistant ovarian cancer by the JAK1/2 intermediary inhibitor, ruxolitinib(32,36).

3.4 Modeling tumor re-emergence following cisplatin/673A treatment in vitro

Following cisplatin/673A treatment, live cells from spheroids were sorted using flow cytometry to isolate cells that escaped cisplatin/673A treatment. Spheroids were re-initiated with these cells to preliminarily model tumor re-emergence *in vitro* (Figure 4A). All three patient ascites samples reliably formed spheroids, indicative of the ability of residual viable cells to reform a 'tumor' following cisplatin/673A treatment (Figure 4A). Flow analysis indicated that ALDH⁺ OvCSC progeny had reliably repopulated within these spheroids (Figure 4B), despite their initial depletion after cisplatin/673A treatment (Figure 2C). Despite the differential response to therapy, the remnant resistant cells demonstrated similar proliferative capacity (Figure 4C). At the first re-passage, the extent of repopulation of ALDH⁺ progeny did not match the original fraction present in these samples (Figure 1C, G, K), except in Pt152 samples where the ALDH⁺ cells exceeded those in the original sample (5.1% in spheroids re-emerged following cisplatin/673A treatment versus 1.4% in spheroids originally initiated from the primary patient sample; Figure 4D). These spheroids were serially passaged over seven cycles, to demonstrate tumor re-emergence over a significant time scale of 7 weeks *in vitro* (Figure 4E, F). Flow analysis indicated that ALDH⁺ OvCSC progeny had reliably repopulated within these spheroids (Figure 4E, F), despite their initial depletion after cisplatin/673A treatment (Figure 2C). The extent of repopulation of ALDH⁺, CD133⁺ and ALDH⁺/CD133⁺ progeny approached and even surpassed the original fraction present in the patient samples (Figure 1J, K, L).

3.5 Tumor initiation of Pt259 OvCSC spheroids in an NSG mouse model

We next tested the ability of OvCSC derived spheroids to initiate tumors in mice. Subcutaneous tumors were initiated with Pt259 OvCSC spheroids in NSG mice. As a positive control, 5000 freshly isolated OvCSC cells from the malignant ascites sample were used to initiate tumors (Figure 5A). Photographs of Hematoxylin and Eosin staining from primary patient samples and tumors xenografts derived from OvCSC spheroids demonstrate a similar histology for each sample (Figure 5B, Supplementary Figure 6). Ten or more spheroids were capable of 100% tumor initiation (Figure 5C). At six weeks, 2 spheroids had an 80% tumor initiation capacity and a single spheroid had initiated tumors in 16.7% of mice. 1 injected spheroid grew at a rate of 12.5mm³/week, while 2 spheroids grew at 36mm³/week. 10 spheroids grew at 94.3 mm³/week, while 50 spheroids grew at a rate of 181 mm³/week. In comparison, 5000 freshly isolated OvCSCs had a lower growth rate of 43.8 mm³/week (Figure 5D). When tumors reached ~1500mm³, tumors were harvested,

dispersed as single cells, and spheroids were re-initiated in hanging drop arrays. Spheroids were easily generated from the xenografted tumors (Figure 5E, F).

3.6 Drug treatment and characterization of OvCSC spheroid xenografts with cisplatin/673A in vivo

Tumors were initiated in NSG mice using 10 OvCSC spheroids from both Pt259 and Pt152 samples. Once palpable tumors were formed (approximately 40–50 mm³ in volume), drug treatment with cisplatin/673A was initiated for 21 days. Consistent with *in vitro* spheroid drug sensitivity, Pt259 initiated xenografts demonstrated an essentially complete response to therapy, where only 1 tumor (out of 8) was palpable and was able to be excised at the end of the time period of study (Figure 6A). Histology of resected tumors demonstrated significant ‘treatment effect’ with notable cell death and necrosis throughout (Figure 6B, C). Widespread expression of the apoptotic marker cleaved Caspase-3 further indicated the extent of cell death in drug treated Pt259 xenograft tumors (Figure 6C). FACS analysis of control and treated tumors demonstrated that ALDH⁺, CD133⁺ and ALDH⁺ CD133⁺ populations were significantly targeted/eliminated with drug treatment (Figure 6D).

In contrast to Pt259, consistent with the resistance observed in spheroids *in vitro*, Pt152 tumors were only minimally responsive to cisplatin/673A treatment (Figure 6E). Visually, no difference in proliferative index indicated by Ki67 was observed between Pt259 and Pt152 xenograft tumors (Figure 6B, F). Corresponding histology and sparse cleaved caspase-3 staining also indicated minimal cell death within Pt152 xenograft tumors (Figure 6F, G) compared to untreated control tumors. Concomitantly, FACS analysis also indicated that while the CD133⁺ cell population was effectively depleted with drug treatment compared to untreated controls, ALDH⁺ populations remained unchanged with cisplatin/673A treatment on Pt152 tumors (Figure 6H).

4. Discussion

Malignant ascites is a therapeutic challenge in ovarian cancer. Ascites contains tumor cells growing in suspension as spheroids, enriched in ovarian cancer stem-like cells (OvCSCs) (7,11). In the era of personalized medicine, development of diagnostic and therapeutic tools that can leverage individual differences between seemingly similar patient samples, and direct a more therapeutically relevant treatment option is of high importance. Our platform provides an approach to study and characterize individual patient samples with an OvCSC context, relevant to drug screening and the study of tumor biology.

Several hierarchical markers for OvCSC identification have been documented, including the dual expression of elevated aldehyde dehydrogenase (ALDH) and CD133, with a potential plasticity in the expression of these markers as cells switch from stem to progenitor to committed differentiation statuses (12,15,20,39). Operationally, OvCSCs are cells with both increased tumor initiation capacity and the ability to asymmetrically divide to generate non-self cells, recreating the heterogeneity of the primary patients’ malignancy (26).

Owing to the fact that CSCs are notoriously plastic, and maintaining them in an undifferentiated state to study their role in drug resistance has been difficult *in vitro* (26), we

developed a spheroid model where we could isolate OvCSCs (based on ALDH⁺ CD133⁺) from primary patient malignant ascites, and incorporate them into a 3D structure wherein OvCSC populations are similar to those observed in the primary patient sample, indicating a strong use for this platform in personalized medicine for drug screening with an OvCSC context.

We examined OvCSC progeny within our spheroids based on ALDH and CD133 alone, since their expressions have been previously reported to be partially rescued with spheroid and serum-free *in vitro* culture, compared to other OvCSC markers(20). In fact, spheroids initiated from specific OvCSC subsets (ALDH⁺ only, CD133⁺ only, ALDH⁻/CD133⁻) had different sensitivities when treated with cisplatin, as demonstrated in Supplementary Figure 7. The likelihood of other heterogeneous markers, including CD24, CD44, CD117, etc. present in ovarian tumors and their contributions to a phenotype cannot be ruled out(40,41).

Our spheroid model demonstrated that with the suspension culture, not only is the non-adherent ascites microenvironment partially recapitulated, but the populations of OvCSCs and their progeny are recapitulated to the same levels as observed in the primary patient ascites samples, thereby maintaining the heterogeneity of the tumor and making a subsequent drug study physiologically relevant, and customizable for individual patient samples.

Indeed, in line with previously published research, the use of cisplatin along with the ALDH targeting compound 673A resulted in a marked loss of ALDH⁺ OvCSC progeny within spheroids(31,42). Our platform was able to discern the sensitivity of the three different patient samples to the synergistic cisplatin/673A and cisplatin/ruxolitinib combination therapies, and this correlated with their clinical history for platinum response, as well as, the amounts of OvCSC populations contained within the primary and spheroid samples. Based on the initial clinical therapy response to platinum-based chemotherapy, ovarian cancer patients are classified into three categories: platinum refractory, platinum resistant, and platinum responsive. Platinum-refractory patients do not respond to platinum-based therapy and show progression during the course of the therapy. Meanwhile, platinum resistant tumors can recur within 6 months of cisplatin treatment, and although considered incurable, are still responsive to chemotherapy to a variable degree(34). Our data demonstrates differences in therapeutic response between patient samples, and also represent the variability in the clinical response to chemotherapy after recurrence. The implications of these findings strengthen the platform and its utility towards personalized medicine, wherein individual patient samples can be tested with a higher fidelity for drug response, compared to conventional monolayer drug screens (Supplementary Figure 8).

This is an important *in vitro* platform that can be developed to model and study tumor re-emergence following drug treatment. By sorting for live cells that escape chemotherapy, and reforming spheroids with them in a long-term *in vitro* culture setting, one can potentially study the phenotypes of these cells that make them inherently more chemoresistant than parent primary tumors. We were unable to reform spheroids from cells following cisplatin/ruxolitinib treatment, in line with studies that demonstrate that the re-passaging ability of

spheres is severely limited following JAK1/2 and subsequent pSTAT3 inhibition in CSCs(35,43), which we observe in ruxolitinib treated spheroids (Figure 3E).

Lastly, the hanging drop spheroid platform appears to be an excellent means to expand rare tumor cells *ex vivo* for primary tumor generation. Our xenograft model demonstrates that as few as 1 injected spheroid (initiated with 10 cells/spheroid) is capable of generating tumors in NSG mice with 16.7% success while 2 spheroids are capable of >80% tumor generation efficiency. With as few as 10 injected spheroids, we obtained 100% efficiency in initiating tumors. The tumor establishment times were also significantly shorter in our spheroid-xenograft model system, compared to latency reported previously for ovarian cancer PDX samples (~74 days)(44) or ovarian cancer tumor initiating cells (60–102 days)(41). Liao et al.(45) also reported reduced tumor latency times (31–54 days) when injecting cells derived from spheroids, compared to bulk parental cells. Our spheroid-xenograft model demonstrates similar or lower latency times, indicating that spheroid formation and maintenance magnifies the tumorigenicity of OvCSC cells. Taken together, these results suggest that this platform could be used to rapidly establish patient xenografts from limited specimens such as core biopsies.

Since we obtained dramatically different responses to *in vitro* cisplatin/673A treatment between Pt259 and Pt152 spheroids, we treated the xenografts initiated from these two patient samples *in vivo* with cisplatin/673A. Our data indicated that drug dose responses obtained *in vitro* in spheroids matched the response of xenografted tumors *in vivo* very closely. Flow analysis of drug treated tumors *in vivo* compared to control tumors also indicated that cisplatin/673A in combination was able to target ALDH+ populations in Pt259 effectively, and not as effectively in Pt152 xenografts, similar to *in vitro* data. This further strengthens the predictive potency of the *in vitro* hanging drop platform to assay drug responses in patient samples, with a clear OvCSC context.

In conclusion, we have demonstrated a robust *in vitro* platform that incorporates primary patient malignant ascites-derived OvCSCs into spheroids. These spheroids recapitulate primary tumor specimens, suggesting a strong application for this platform in personalized medicine and spheroid biology studies. Our platform possesses the ability to investigate biological properties of malignant OvCSCs, while retaining their tumorigenic potential upon transplantation. Responses to varying drug treatments (including cisplatin, 673A and ruxolitinib) are different in the patient samples investigated, and correlate with *in vivo* drug studies in murine xenografts, indicating that the spheroid platform has a high predictive potency for drug dose responses. The spheroid intermediary for OvCSCs also dramatically reduces the latency times of tumor establishment. Lastly, our platform can also be used to study and model tumor biology from a re-emergence of tumor after drug treatment perspective, to allow for closer investigation of the phenotype of cells that escape primary chemotherapy. Since the role of aberrant somatic mutations in serous ovarian cancer pathophysiology is still emerging(46,47), this platform can play a strong role in stratifying seemingly similar patient samples, and leveraging individual differences in tumor growth rates, biology, OvCSC composition, and therapeutic resistance to direct more precise therapies in a personalized standpoint.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the DOD OCRP Early Career Investigator Awards W81XWH-13-1-0134 (GM) and W81XWH-15-0194 (KM). Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under award number P30CA046592. The authors would like to thank Dr. Ilana Chefetz-Menaker and Dr. Kun Yang for generous contribution of reagents. The authors acknowledge the efforts from Martin White and Mark Savary at the University of Michigan Flow Cytometry Core. The authors would also like to acknowledge Caymen Novak, Sarah Snyder and Eric Horst for performing histology.

Funding source: This work was supported by the DOD OCRP Early Career Investigator Awards W81XWH-13-1-0134 (GM), W81XWH-15-0194 (KM) and the National Cancer Institute of the National Institutes of Health under award number P30CA046592.

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STATEMENT OF TRANSLATIONAL RELEVANCE

Ovarian cancer is the most lethal gynecologic malignancy with nearly 70% relapse rate within 24 months. In order to improve cure rates for ovarian cancers, we developed a patient derived 3D platform to personalize the screening of chemotherapeutics and biologics for resistant ovarian cancers. Our hanging drop spheroid platform uses small number of patient derived ovarian cancer stem cells, and allows for quick, high throughput, and inexpensive drug assays which produce comparable results to xenograft models in shorter time. This work demonstrates the utility of our platform in personalized drug assays, which could be used to rapidly inform clinicians of optimal treatment routes for each individual patient, thereby potentially improving clinical outcomes and preventing suboptimal initial treatments. Additionally, our platform can be utilized in fundamental studies in cancer stem cell biology, as well as, in modeling tumor re-emergence, in order to identify new targeted therapeutics from a personalized medicine standpoint.

Figure 1: Spheroids generated from malignant ascites-derived OvCSC maintain OvCSC heterogeneity

(A, B, C): OvCSCs were isolated using fluorescence-activated cell sorting from malignant Pt259, Pt224, and Pt152 ascites based on the concurrent elevated activity of aldehyde dehydrogenase (ALDH) and CD133 expression.

(D, E, F): Spheroids were initiated in hanging drop arrays using 10 Pt259 OvCSCs, and observed using phase contrast microscopy at Day 7 (scale bar = 100 μ m).

(G, H, I): Following spheroid culture for 7 days, harvested spheroids were processed for flow analysis to identify OvCSC populations in Pt259 OvCSC spheroids – several populations were identified, ALDH⁺, CD133⁺, ALDH⁺/CD133⁻ and ALDH⁺/CD133⁺. OvCSC populations within spheroids mimic the primary patient profile.

(J, K, L): Quantification of flow analyses indicated that spheroid culture maintained or enhanced OvCSC populations to similar extents as observed in the primary samples.

Figure 2: Patient derived OvCSC spheroids exhibit differential response to cisplatin/673A drug treatment

(A): Spheroids were initiated from 10 OvCSCs/drop in hanging drop arrays, and allowed to form over 7 days in serum-free culture. On Day 7, spheroids were treated with a single or combination dose of cisplatin 50µM/compound673A 50µM. Control spheroids were untreated, but maintained for the same period in culture. Phase contrast micrographs at 72 hours following drug treatment demonstrate that control untreated spheroids remain compact, while drug treated spheroids are either progressively smaller and/or appear to have necrotic foci within them (scale bar = 100µm).

(B) Alamarblue fluorescence was used to assess viability of drug treated spheroids, indicating that while all OvCSC spheroids were responsive to cisplatin and/or 673A, their chemosensitivity varied from patient to patient. Pt259 OvCSC spheroids were most sensitive to drug treatment, and Pt152 was most resistant to drug treatment; * $p < 0.05$ two-way ANOVA, on all drug treated samples compared to control untreated spheroids.

(C): Representative flow cytometry plots on CSC populations within cisplatin/673A treated spheroids from all three patient samples.

(D): Summary of flow analysis data indicated that cisplatin/673A treatment targeted ALDH⁺ populations routinely in Pt259 and Pt224, and also additionally targeted CD133⁺ populations in Pt152; *** $p < 0.001$, one-way ANOVA, between control untreated spheroids and cisplatin/673A treated spheroids.

Figure 3: Patient derived OvCSC spheroids exhibit differential response to cisplatin/ruxolitinib drug treatment

(A) Phase contrast micrographs of control untreated and cisplatin 50µM/ ruxolitinib 500nM treated spheroids from three different patient samples demonstrate that drug treated spheroids have reduced spheroid sizes and cell death can be visually observed (scale bar = 100µm).

(B): Viability measurements using alamarblue fluorescence indicated that while all OvCSC spheroids were responsive to cisplatin and/or ruxolitinib, their chemosensitivity varied between patient samples; * $p < 0.05$, two-way ANOVA, on drug treated spheroids compared to control untreated spheroids.

(C): Representative flow cytometry plots demonstrate CSC populations remnant in drug treated spheroids.

(D): Graphical summary of flow analysis of OvCSC populations in cisplatin/ruxolitinib treated versus control untreated spheroids, indicating that cisplatin/ruxolitinib treatment effectively targeted CD133⁺ populations routinely in all patient samples; * $p < 0.05$, *** $p < 0.001$, one-way ANOVA, between control untreated spheroids and cisplatin/ruxolitinib treated spheroids.

(E): Immunoblot analysis of phosphorylated STAT3 (pSTAT3), compared to total STAT3 (tSTAT3) in control untreated (No Tx) or ruxolitinib 500nM treated (Rux) OvCSC spheroids. Against a GAPDH loading control, STAT3 phosphorylation is inhibited with ruxolitinib treatment in Pt259 and Pt152 OvCSC spheroids.

Figure 4: Remnant viable cells cause re-emergence of ovarian tumor and CSC populations in cisplatin/673A treated OvCSC spheroids

(A) Phase contrast micrographs of spheroids generated from viable cells surviving cisplatin/673A treatment. Insets show spheroids at Day 1 following cell seeding (scale bar = 100μm).

(B): Representative flow cytometry plots of OvCSC populations in the re-emerging spheroids at Day 7, following cisplatin/673A treatment.

(C): Quantification of proliferation within spheroids over 7 days, formed from viable cells after cisplatin/673 treatment, indicating robust proliferation and recovery following cisplatin/673A therapy. No statistical significance was observed in the proliferation rates between patient samples.

(D): Graphical quantification of OvCSC populations derived from flow analysis, indicating the presence of ALDH⁺ populations, minimal to no recovery of ALDH⁺/CD133⁺ or CD133⁺ populations.

(E): Flow analysis of serially passaged recovered Pt259 OvCSC spheroids indicates an increasing expression of ALDH⁺, CD133⁺ and CD133⁺/ALDH⁺ populations, over seven generations. OvCSC populations approach and even seem to surpass original populations.

(F): Flow analysis of serially passaged recovered Pt152 OvCSC spheroids demonstrates an increase in ALDH⁺, CD133⁺ and CD133⁺/ALDH⁺ OvCSC progeny. CD133⁺ progeny do not approach original levels in Pt152 spheroids even after seven generations.

Figure 5: Patient malignant ascites-derived OvCSC spheroids initiate tumors in xenografts

(A): Macroscopic pictures of tumors observed in NSG mice with subcutaneous injections of 5000 OvCSCs or 10 injected spheroids.

(B): Photomicrographs of hematoxylin and eosin stained 6 μ M sections, obtained from primary patient debulking specimens and OvCSC derived tumor xenograft from the sample patient (Primary patient scale bar = 200 μ m; OvCSC derived tumor xenograft scale bar = 100 μ m).

(C): Tumor initiation for the varying experimental conditions, indicating that 500 injected spheroids initiated tumors in less than a week of injection, while 50 injected spheroids, and 5000 OvCSCs initiated tumors within 7 days.

(D): Comparison of the growth rate and tumor volumes of tumors initiated using 1–500 spheroids or 5000 freshly isolated OvCSCs. All groups contained 4–8 injected tumors. (E): Phase contrast micrographs of spheroids initiated from tumor cells isolated from xenografted tumors, using 50 cells/drop (scale bar = 100 μ m).

(F): Follow up phase contrast micrographs of spheroids at Day 7, indicating that spheroid forming abilities were retained even upon xenograft (scale bar = 100 μ m).

Figure 6: Drug response of OvCSC xenografts match drug response of patient malignant ascites-derived OvCSC spheroids treated with cisplatin/673A

(A): Tumors were initiated from Pt259 and Pt152 spheroids using 10 injected spheroids. Once palpable tumors were obtained, control groups received a sham saline injection, while the experimental drug group received cisplatin/673A. Tumor volumes of control (black trace) and drug treated (red trace) Pt259 xenografts are plotted, indicating a decrease in volume of drug treated Pt259 xenografts compared to control. Shaded gray area indicates the window for tumor burden ($1500\text{--}2000\text{mm}^3$) upon which mice were sacrificed.

(B): Hematoxylin and eosin and proliferative index marker Ki67 staining of control Pt259 xenograft tumor cross-sections (scale bar = $100\mu\text{m}$).

(C): H&E staining and cleaved caspase 3 staining of drug treated Pt259 xenografts indicating cell death in the cisplatin/673A treated tumors (scale bar = $100\mu\text{m}$).

(D): Representative flow analysis plots for control untreated and drug treated tumors, where drug treated tumors demonstrate reduced OvCSC populations.

(E) Tumor volumes of control (black trace) and drug treated (red trace) Pt152 tumors, where there is minimal difference in tumor volumes between the two experimental conditions. Shaded gray area indicates the window for tumor burden ($1500\text{--}2000\text{mm}^3$) upon which mice were sacrificed.

(F) H&E and Ki67 staining of Pt152 xenografts visually demonstrate no difference in proliferative index when compared to Pt259 xenografts (scale bar = $100\mu\text{m}$).

(G): Hematoxylin and eosin and cleaved caspase-3 staining of Pt152 xenograft tumor cross sections indicate minimal cell death in cisplatin/673A treated tumors compared to control untreated tumors (scale bar = $100\mu\text{m}$).

(H): Representative flow analysis plots for drug treated and control untreated tumors, where marked losses of CD133^+ and $\text{ALDH}^+ \text{CD133}^+$ populations are observed.