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Formation of stable small cell number three-dimensional ovarian cancer spheroids using hanging drop arrays for preclinical drug sensitivity assays

Shreya Raghavan^a, Maria R. Ward^a, Katelyn R. Rowley^b, Rachel M. Wold^b, Shuichi Takayama^{b,c}, Ronald J. Buckanovich^{d,e}, and Geeta Mehta^{a,b,c,*}

^aDepartment of Materials Science Engineering, University of Michigan, Ann Arbor, USA

^bDepartment of Biomedical Engineering, University of Michigan, Ann Arbor, USA

^cMacromolecular Science and Engineering, University of Michigan, Ann Arbor, USA

^dDepartment of Obstetrics and Gynecology – Gynecologic Oncology, University of Michigan, Ann Arbor, USA

^eDepartment of Internal Medicine – Hematology/Oncology, University of Michigan, Ann Arbor, USA

Abstract

Background—Ovarian cancer grows and metastasizes from multicellular spheroidal aggregates within the ascites fluid. Multicellular tumor spheroids are therefore physiologically significant 3D in vitro models for ovarian cancer research. Conventional hanging drop cultures require high starting cell numbers, and are tedious for long-term maintenance. In this study, we generate stable, uniform multicellular spheroids using very small number of ovarian cancer cells in a novel 384 well hanging drop array platform.

Methods—We used novel tumor spheroid platform and two ovarian cancer cell lines (A2780 and OVCAR3) to demonstrate the stable incorporation of as few as 10 cells into a single spheroid.

Results—Spheroids had uniform geometry, with projected areas ($42.60 \times 10^3 \mu\text{m}^2$ – $475.22 \times 10^3 \mu\text{m}^2$ for A2780 spheroids and $37.24 \times 10^3 \mu\text{m}^2$ – $281.01 \times 10^3 \mu\text{m}^2$ for OVCAR3 spheroids) that varied as a function of the initial cell seeding density. Phalloidin and nuclear stains indicated cells formed tightly packed spheroids with demarcated boundaries and cell–cell interaction within spheroids. Cells within spheroids demonstrated over 85% viability. 3D tumor spheroids demonstrated greater resistance (70–80% viability) to cisplatin chemotherapy compared to 2D cultures (30–50% viability).

Conclusions—Ovarian cancer spheroids can be generated from limited cell numbers in high throughput 384 well plates with high viability. Spheroids demonstrate therapeutic resistance

*Corresponding author at: 2300 Hayward St. H.H. Dow Building Department of Materials Science and Engineering University of Michigan, Ann Arbor MI 48109, USA. mehtagee@umich.edu (G. Mehta).

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relative to cells in traditional 2D culture. Stable incorporation of low cell numbers is advantageous when translating this research to rare patient-derived cells. This system can be used to understand ovarian cancer spheroid biology, as well as carry out preclinical drug sensitivity assays.

Keywords

Ovarian cancer; High throughput; Multicellular tumor spheroids; 3D culture; Preclinical drug testing

1. Introduction

Ovarian cancer is the leading cause of gynecological mortality. It is associated with a rapid acquisition of chemoresistance to chemotherapies. Due to chemotherapy resistance, patients who relapse will ultimately die of their disease [1–3]. While numerous compounds have shown pre-clinical promise as new ovarian cancer therapeutics, no new compounds have significantly improved the survival of patients with ovarian cancer for the past 30 years [4–6]. This indicates a need for better preclinical in vitro models.

Traditionally, drug screens have been performed on conventional 2D monolayer cultures of cells. However 3D cultures may be the more physiologically relevant. Over the past two decades, multicellular 3D tumor spheroids have been established as in vitro tumor models. Given that ovarian cancers often grow as spheroids in patient ascites, spheroids are particularly relevant for ovarian cancer [7, 8]. Indeed, cells within spheroids have a lower proliferation rate, similar to that observed in tumors in vivo, compared to the cells grown in 2D monolayer cultures [9, 10]. As such, it is reported that multicellular tumor spheroids can improve preclinical drug screening [11].

Several methods have been utilized thus far to generate tumor spheroids in vitro. Rotary vessel bioreactors and spinner flask methods, have been used for spheroid generation, however these have not been broadly applied, as they require specialized equipment, and complicated protocols. Non-adherent surfaces have also been utilized to promote spheroid formation, however uniformity in spheroid size and number of cells incorporated into a spheroid remain an ongoing challenge. Conventional hanging drop cultures eliminate the need for specialized equipment, and rely instead on surface tension to promote cellular aggregation. However, liquid handling in conventional hanging drop cultures is difficult and long-term maintenance of these cultures is challenging, with significant evaporation issues, as well as, difficulty in harvesting spheroids to pursue further analysis [12, 13].

Hanging drop array plates combine the advantage of conventional hanging drop cultures of promoting cell–cell interaction and aggregation with amenability to high throughput liquid handling systems [14]. These plates have been used to generate human epithelial carcinoma spheroids, prostate cancer spheroids as well as non-cancer primary stem- and progenitor cell spheroids [14, 15]. Recently, Leung et al. demonstrated that the spheroids generated using hanging drop array platforms demonstrate excellent circularity and compactness [16].

Here, we characterize the stable formation of multicellular ovarian cancer spheroids using hanging drop array plates. Unlike prior hanging drop culture studies that utilized high

starting cell numbers, we describe the stable formation of uniform sized and spherical shaped spheroids with as few as 10 cells per spheroid. Compared to 2D culture, these spheroids demonstrated slower growth and greater chemotherapy resistance. Thus multicellular tumor spheroids can provide reliable methods for preclinical drug screening of novel chemotherapy drugs. The ability to generate spheroids from small cell numbers is particularly relevant when dealing with rare patient-derived cells such as cancer stem-like cells that may make up less than 1% of the total cellular population [17]. Therefore, this platform can provide a unique opportunity to study the biology of rare cancer cell populations.

2. Materials and methods

2.1. Materials

All tissue culture reagents were purchased from Life Technologies (Carlsbad, CA) unless specified otherwise. Growth medium was RPMI 1640 supplemented with 10% fetal bovine serum and 1.5× Antibiotics/Antimycotics. Ovarian cancer cell lines A2780 and OVCAR3 were purchased from ATCC (Manassas, VA). Hanging drop array plates were purchased from XCentric Mold and Engineering (Clinton Twp, MI).

2.2. Formation of stable ovarian cancer spheroids in hanging drop cultures

Ovarian cancer cell lines were cultured in growth medium till ~70% confluency, trypsinized per regular passage and counted on a hemocytometer. All cells utilized were between passage numbers 30 and 45. Cells were resuspended in complete growth medium, and 20 µl of cell suspension was added to each well of the hanging drop array plate [15]. Initial cell seeding densities were varied as 10 cells, 20 cells, 50 cells and 100 cells per 20 µl volume of the hanging drop. Each hanging drop array plate consisted of all four chosen cell densities (10, 20, 50 and 100 cells/drop) with 30 replicates of each condition. 3–5 hanging drop array plates were generated for each cell line, in order to consistently observe stable spheroid formation. The water reservoir on the hanging drop array plates was filled with 2.5 ml of sterile deionized water. Hanging drop array plates were then placed on top of a 6 well plate containing sterile deionized water, and the two plates were wrapped in Parafilm (Neenah, WI) and placed in a humidified 37 °C carbon dioxide incubator. 2–5 µl of fresh growth medium was added to the hanging drops every alternate day, to maintain a 20 µl drop volume.

2.3. Observation of spheroid formation and morphometry

Hanging drop plates were removed periodically for imaging. Live cell microscopy was used to monitor the formation of spheroids within each hanging drop. A spheroid was considered formed when a majority of the cells in a well were aggregated into a tight structure. Two days following initial plating, each well of the hanging drop array was examined to determine how many wells had integrated into multicellular aggregates. This data was recorded for the formation of spheroids from all cell lines. 3–5 representative images were obtained for each cell seeding density using a calibrated phase contrast microscope (Olympus IX81, Japan equipped with ORCA R2 Cooled CCD camera and CellSens software). Overall, 3–5 individual hanging drop array plates were imaged for each cell line

to obtain morphometric data. Calibrated 2D images were used to measure perimeter and area in Image J (National Institutes of Health). The polygon tool was used to measure perimeter, area and circularity in Image J. A projected sphere volume was calculated based on the measured perimeter obtained in Image J.

2.4. Proliferation and metabolic activity in hanging drop ovarian cancer spheroids

Alamarblue dye (Life Technologies, Carlsbad CA) was added in a 1/10 dilution to 10-, 20-, 50- and 100-cells/drop spheroids on the day of plating. Following 12-24 h of alamarblue addition and incubation, the 384 hanging drop array was placed in a fluorescence plate reader (Synergy HT, BioTek Instruments, Winooski, VT). Alamarblue fluorescence readings were obtained at 530 nm excitation and 590 nm emission. A baseline alamarblue fluorescence reading was obtained at Day 1 for each cell density. To quantify proliferation within spheroids, alamarblue readings were also obtained at Day 7, and compared to the baseline readings at Day 1. Proliferation was then expressed as a fold-increase at Day 7, compared to Day 1. Alamarblue fluorescence was also used in 2D monolayer cultures of A2780 and OVCAR3 cells in tissue culture treated 6-well plates. Fluorescence intensities were obtained at Day 1 and Day 7, to determine proliferation in 2D monolayer cultures for both A2780 and OVCAR3 cell lines.

2.5. Cellular viability quantification in hanging drop ovarian cancer spheroids

Cellular viability was quantified using the Live/Dead viability kit (Life Technologies, Carlsbad CA). Cellular viability was quantified at Day 7 following the plating of cells in hanging drop arrays. Calcein-AM was added to final concentration of 2 μ M, and Ethidium homodimer-1 was added to a final concentration of 4 μ M to each hanging drop. Following a 45-minute incubation at 37 °C, hanging drops were harvested on to a pre-cleaned glass microscope slide, and imaged on an inverted confocal microscope (Olympus IX81, Japan equipped with a Yokogawa CSU-X1 confocal scanning laser, Andor iXon x3 CCD camera and MetaMorph 7.8 software). Fluorescence images were obtained at every z-axis encompassing the spheroids, at 488 nm for calcein-AM (live cells; green) and 561 nm for ethidium homodimer-1 (dead cells; red). A composite z-stack image was obtained using Metamorph for live and dead cells within the spheroid. Image J (National Institutes of Health) was used to quantify the percentage of live and dead cells within the spheroid from the z-projection images. Number of live and dead cells was expressed as a percentage of the total number of cells within each spheroid.

2.6. Demonstration of three-dimensional morphology of hanging drop ovarian cancer spheroids

At Day 7, spheroids were harvested on to a soft bed of 2% agarose. Spheroids were fixed in 4% neutral buffered formalin, and actin cytoskeletons were stained with fluorescently labeled phalloidin (AlexaFluor488, Life Technologies, Carlsbad CA). Nuclei were counterstained with DAPI (Life Technologies, Carlsbad CA). Inverted confocal microscopy was used to obtain fluorescence images over a range of z-heights spanning the height of the spheroids. Composite z-stack images were encoded in Metamorph, and presented as a merged image obtained at all the z-heights, demonstrating 3D morphology in ovarian cancer spheroids.

2.7. Drug response studies in hanging drop ovarian cancer spheroids

Hanging drop array plates were plated with A2780 cells or OVCAR3 cells at the following cell seeding densities: 10, 20, 50 and 100 cells/drop. Formation of spheroids was monitored at Day 2 in the hanging drop array plates. Cisplatin (SICOR Pharmaceuticals Inc.), a commercially available conventional chemotherapy drug, was added to the hanging drops at Day 2, following spheroid formation. A control condition where spheroids received no drug treatment was included in the hanging drop plates. Final drug concentrations in the hanging drop ranged as 1 μ M, 10 μ M and 50 μ M. Cells grown in two dimensional 96 well plates were also used as a control, for drug studies performed conventionally. 1000 cells were plated into each well of conventional flat bottomed tissue culture treated 96 well plates, and received either no drug or the same final concentrations of drug as the spheroids on the hanging drop arrays. Alamar blue was used to measure viability three days after cisplatin treatment.

Hanging drop array plates were imaged before addition of cisplatin, and subsequently three days after addition of cisplatin. Multiple images were obtained for the control and cisplatin-treated spheroids. Morphometric analysis for spheroid projected area was carried out using NIH Image J. Reduction in spheroid area was calculated by comparing the area of cisplatin treated spheroids to the measured area of untreated control spheroids at the same time point (three days after addition of cisplatin).

2.8. Data analysis

All experiments were repeated with 3–7 biological replicates with $n = 30$, in order to carry out statistics. Statistical data was analyzed using GraphPad Prism 5.0 (www.graphpad.com, San Diego, CA). All data is represented as mean $\pm$ standard error of the mean. Where appropriate, one-way or two-way ANOVAs were performed to assess statistical significance between means, with post-hoc Tukey tests for comparison between means. A $p < 0.05$ was considered significant. Levels of statistical significance are indicated in graphs, where appropriate with asterisks.

3. Results

3.1. A2780 form small cell number spheroids in the high throughput 384 hanging drop plates within 2 days

We first tested the ability of A2780 cells to form spheroids in a 384 well hanging drop plate array. In order to assess the utility of this assay for rare cell populations, we tested spheroid-forming capacity of 10, 20, 50 and 100 cells. Each well of a hanging drop array plate contained 30 replicates of 10-, 20-, 50- and 100-cell spheroids, and was examined microscopically every day up to Day 7. At least three different hanging drop array plates were imaged to record a percentage of the number of wells that consistently formed spheroids in all cell-seeding densities. Supplemental Table 1 summarizes the number of wells that formed multicellular aggregates at Day 2. Between 82.5 and 96% of the plated wells had formed aggregates at Day 2 (Supplemental Table 1).

Fig. 1A shows representative phase contrast micrographs obtained at Days 1 and 7. At Day 1, cells had aggregated however, phase contrast microscopy indicated that by Day 7 A2780 cells had formed spheroids with a tight, ideal shape (Fig. 1A) with clear boundaries being established. By Day 7 (Fig. 1A, Day 7), 100% of the wells in every initial cell seeding condition had formed spheroids, with tight defined boundaries.

In order to characterize the A2780 spheroids generated on the hanging drop array platform, multiple phase contrast images were used to measure projected 2D area and circularity. Over time there was an increase in projected area of A2780 spheroids (Fig. 1B). The size of the spheroids primarily varied as a function of the initial cell-seeding density. 10 cell spheroids started at $12.30 \pm 0.49 \times 10^3 \mu\text{m}^2$, and grew to $42.60 \pm 1.96 \times 10^3 \mu\text{m}^2$ by Day 7.

Concordant with the increase in area of spheroids, alamarblue fluorescence also indicated a 9.80 ± 1.24 -fold increase in cell number, representing robust proliferation, and subsequently an increase in cell number within spheroids (Fig. 1C). The rate of growth, as assessed by both spheroid projected area and alamarblue stain was similar for all cell seeding densities. Interestingly, proliferation in spheroids was significantly slower, compared to the proliferation in 2D tissue culture dishes, where the fold increase was 30.38 ± 2.29 in alamarblue fluorescence observed over 7 days.

3.2. OVCAR3 successfully form small cell number spheroids in the high throughput 384 hanging drop plates

While A2780 has been widely used in the study of ovarian cancer, this cell line may not be representative of high-grade serous ovarian cancer [18]. Beaufort et al. demonstrated that the OVCAR3 cell line falls under the category of high-grade serous histotype, while the A2780 cell line was more representative of an endometrioid histotype [18]. We therefore replicated the small cell number spheroid studies with OVCAR3 cells. OVCAR3 cells typically took a day longer compared to A2780 cells for >70% of the cells to form single aggregates in the higher cell seeding densities (namely 50 and 100 cells/drop). Lower cell seeding densities (10 and 20 cells/drop) formed aggregates by Day 1, while only 40% of the wells in 50 and 100 cells/drop formed single aggregates (Fig. 2A, Day 1). By Day 2, 90–92% of the OVCAR3 hanging drops had formed multicellular aggregates (Supplemental Table 1). By Day 7, single, tightly-packed homogenous spheroids were formed in all wells, irrespective of initial cell-seeding densities (Fig. 2A). Spheroid size and cell number over time were once again assessed using phase contrast micrographs, and alamarblue fluorescence (Fig. 2B and C). The projected areas of OVCAR3 spheroids, similar to A2780 spheroids, were a function of the initial cell seeding densities, ranging from $37.24 \pm 7.61 \times 10^3 \mu\text{m}^2$ (10 cell spheroids), to $281.01 \pm 20.61 \times 10^3 \mu\text{m}^2$ (100 cell spheroids) on day 7. Areas of OVCAR3 spheroids were significantly different from each other, depending on initial cell seeding density ($*p < 0.05$, $n = 5$, one way ANOVA, Fig. 2B).

Similarly to A2780 cells, OVCAR3 cells in hanging drop cultures proliferated at a rate independent of initial cell seeding densities, proliferation rates, varied non-significantly between 10.23 ± 0.31 fold and 11.95 ± 1.28 fold (Fig. 2C). Again, proliferation within spheroids was lower than those observed in 2D monolayer cultures (30.51 ± 1.73 fold).

Similar results for spheroid formation were obtained with SKOV3 cells (Supplemental Fig. 10).

3.3. Multicellular ovarian cancer spheroids on the high throughput 384 hanging drop plates exhibit high viability

In order to establish the hanging drop platform as a stable method of generating multicellular tumor spheroids, we evaluated cellular viability within spheroids. At Day 7 robust calcein-AM (green) live cell stain was observed in all spheroids (Fig. 3). Few scattered dead cells were detected with ethidium homodimer (red) stain. Quantification of live/dead cells in A2780 spheroids indicated that live cells comprised 85.21 ± 1.02 to $93.44 \pm 1.39\%$ of cells. Regardless of initial cell seeding densities, there was no significant difference (one way ANOVA, $n = 5$) in the percentage of live/dead cells within A2780 spheroids, demonstrating the maintenance of excellent viability in 3D hanging drop array cultures (Fig. 3E).

OVCAR3 spheroids showed similar results. Initial cell seeding density had no significant effect on the percentage of live cells within OVCAR3 spheroids. Live/dead staining in OVCAR3 spheroids are shown in Fig. 3F–I. Viable live cells fluorescent for calcein-AM ranged from 91.56 ± 2.61 to $86.63 \pm 3.98\%$. Bar graphs demonstrating the summary of live and dead cells within OVCAR3 spheroids are shown in Fig. 3J. Individual channels for merged images are also shown in Supplemental Figs. 3 (A2780) and 4 (OVCAR3).

3.4. Multicellular ovarian cancer spheroids formed from small cell numbers demonstrate three-dimensional structure and cellular interactions

The hallmark of multicellular tumor spheroids is their three-dimensional presence. Nuclear arrangement and cortical actin staining of cells that constituted the spheroids allowed for the evaluation of the three-dimensional nature. Using inverted confocal microscopy, a range of z-heights was sampled to cover the heights of multicellular tumor spheroids. Spheroids harvested from hanging drop arrays on Day 7 were stained with Alexafluor-488 conjugated phalloidin (green) and counter stained with nuclear stain, DAPI (blue). Cortical actin staining using phalloidin is commonly used to demonstrate cell–cell interactions and cell aggregates in 3D culture systems, including spheroids [19–23]. Conjugated phalloidin stained the actin cytoskeletons of cells within spheroids. Even spheroids initiated with as few as 10 cells/spheroid initially (Fig. 4A, E) demonstrate a cortical staining with phalloidin, indicating cell–cell interaction and morphology consistent with substrate-free three-dimensional culture. Reconstructions of confocal z-stacks obtained for A2780 spheroids are illustrated in Fig. 4A–D, and OVCAR3 spheroids are depicted in Fig. 4E–H. Orthogonal projections with circularity, and projected volumes for spheroids are shown in Supplemental Figs. 1 (A2780) and 2 (OVCAR3). Individual channels for merged images are also shown in Supplemental Figs. 5 (A2780) and 6 (OVCAR3).

Confocal imaging also indicated a three-dimensional presence for the spheroids, owing to the range of Z-heights observed during imaging. Z-heights varied as $72.10 \pm 13.59 \mu\text{m}$ (10 cells/drop) to $90.16 \pm 12.18 \mu\text{m}$ (20 cells/drop) to $276.80 \pm 29.84 \mu\text{m}$ (50 cells/drop) and

404.80 ± 13.59 μm (100 cells/drop), indicating the three-dimensional nature of ovarian cancer spheroids generated in hanging drop arrays.

Nuclear counterstaining demonstrated the tight packaging of cells within the three-dimensional spheroid space. Taken together, the assembly of the cellular structures (discernable from cortical actin staining) and the counterstained nuclei demonstrate the three-dimensional nature of self-assembled ovarian cancer spheroids formed on hanging drop array plates.

3.5. Chemoresistance observed in small cell number ovarian cancer spheroids on the high throughput 384 hanging drop plates

As noted above, an important advantage of 3D culture may be that it more accurately reflects in vivo biology. In order to test the ability of hanging drop array platforms to more accurately predict resistance to therapeutics, A2780 and OVCAR3 cells were allowed to form spheroids in hanging drop arrays. Both of these cell lines are chemosensitive in vitro yet resistant to therapy in vivo. Spheroids were then treated with cisplatin at Day 2 in concentrations that ranged from 1 μM to 50 μM. In parallel, similar numbers of A2780 and OVCAR3 cells were treated in 2D culture with the same concentrations of cisplatin. Following cisplatin treatment, live cell microscopy was used to monitor the spheroids within hanging drop arrays. Representative images are demonstrated for control untreated spheroids and cisplatin-treated spheroids following 3 days of drug treatment (Figs. 5 and 6). Morphometric analysis was carried out on calibrated images to determine a change in spheroid sizes upon cisplatin treatment.

3.5.1. Cisplatin treatment on A2780 spheroids—In general, A2780 tumor spheroids were highly resistant to cisplatin except at the highest dose. With 50 μM cisplatin spheroids initiated with 10 or 20 cells demonstrated reduction in size, number, and viability (Fig. 5). Spheres initiated with 50 or 100 cells were resistant to 50 μM therapy. In contrast, A2780 cells grown in 2D culture demonstrated significant reduction in growth.

Morphometric measurements indicated that the projected 2D areas of cisplatin treated A2780 spheroids were reduced, when compared to control untreated spheroids. Three days following cisplatin treatment, A2780 spheroids with 10 cells/drop demonstrated the most significant decrease in area of 30.94 ± 3.46% (Fig. 5A, *** $p < 0.001$, one-way ANOVA) in response to the 50 μM dose of cisplatin. No significant decrease in area was obtained with lower doses of cisplatin (1 μM and 10 μM). A significant drop in area was also observed with the 50 μM cisplatin dose in 20 cells/drop spheroids averaging at 22.27 ± 3.88% (Fig. 5A, *** $p < 0.001$, one-way ANOVA). With increasing initial cell seeding densities, and subsequently larger spheroids, the drop in projected spheroid area was less drastic (Fig. 5A), indicating chemoresistance in the three dimensional spheroids. Reduction in areas ranged from 5.96 ± 2.22% in 100 cell spheroids, to 10.56 ± 2.01% in 50 cell spheroids. Representative spheroid images of control untreated and 50 μM cisplatin treated spheroids are shown in Fig. 5C.

Alamarblue fluorescence was utilized to monitor metabolic activity in A2780 spheroids in hanging drop array cultures. 2D cultures in 96 well plates were utilized as a control for

cisplatin treatment. In response to cisplatin treatment, cells within the 2D cultures demonstrated a lower alamarblue fluorescence activity with increasing concentration of cisplatin, reaching $34.33 \pm 3.71\%$ at the $50 \mu\text{M}$ dose. In contrast, A2780 spheroids in hanging drop cultures demonstrated significant chemoresistance to the $50 \mu\text{M}$ cisplatin dose ($***p < 0.001$, two-way ANOVA, Fig. 5B). Metabolic activity assessed by alamarblue fluorescence in A2780 spheroids remained high in response to cisplatin treatment, ranging from $80.69 \pm 3.544\%$ (10 cell spheroids) to $91.18 \pm 1.701\%$ (100 cell spheroids). A2780 tumor spheroids undergo varying degrees of apoptosis and necrosis in response to cisplatin treatment of 3 days, analyzed by flow cytometry for the apoptotic marker Annexin-V and the necrotic marker Propidium Iodide (Supplemental Fig. 7).

3.5.2. Cisplatin treatment on OVCAR3 spheroids—Similar to A2780 spheroids, OVCAR3 spheroids were also treated with the same doses of cisplatin. Morphometric analysis indicated that a drop in spheroid area was observed upon cisplatin treatment. Reduction in area was quantified compared to control untreated OVCAR3 spheroids analyzed at the same time points. OVCAR3 spheroids with 10, 20, and 50 cells/drop all demonstrated a significant reduction in area (Fig. 6A, $**p < 0.01$, one-way ANOVA) ranging from $34.3 \pm 2.68\%$ to $13.42 \pm 1.80\%$. 100 cells/drop spheroids had no significant reduction in area compared to control untreated spheroids, demonstrating a loss of only $7.26 \pm 0.59\%$.

Metabolic activity monitoring using alamarblue fluorescence indicated that 2D cultures of OVCAR3 cells treated with cisplatin demonstrated a rapid loss of viability, significant even at the lowest $1 \mu\text{M}$ dose of cisplatin ($88.97 \pm 0.72\%$, $***p < 0.001$, two-way ANOVA, Fig. 6B). At the $50 \mu\text{M}$ dose, viability was still further diminished to $56.86 \pm 1.09\%$. In contrast, OVCAR3 spheroids generated on hanging drop arrays had a significantly higher viability even at the $50 \mu\text{M}$ dose, ranging from $65.70 \pm 2.68\%$ in 10 cells/drop spheroids to $91.28 \pm 2.21\%$ in 100 cells/drop spheroids ($*p < 0.05$, two-way ANOVA, Fig. 6B). Representative micrographs of control untreated and $50 \mu\text{M}$ cisplatin treated OVCAR3 spheroids are demonstrated in Fig. 6C. Supplemental Fig. 8 demonstrates the OVCAR3 spheroids undergo apoptosis and necrosis upon 3 days of cisplatin treatment. OVCAR3 cells within spheroids remain more viable to cisplatin treatment compared to OVCAR3 cells grown on monolayer cultures. Similar results were obtained with SKOV3 cells (Supplemental Figs. 11, 12 and 13).

4. Discussion

Epithelial ovarian carcinomas have the highest mortality rate amongst all gynecologic malignancies, partly due to the fact that current chemotherapeutic regimens fail to achieve sustained remission [2, 3, 24]. Metastatic dissemination of ovarian carcinoma can occur through cells that shed from the primary ovarian tumor and float and aggregate as multicellular spheroids in the peritoneal cavity, and eventually invade the mesothelium [8, 25, 26]. Spheroid formation has been considered an important intermediate step that facilitates cell survival, displays chemoresistance and aids in metastasis [8, 26–28]. Therefore, in vitro multicellular tumor spheroids are good candidates, not only for assessing

preclinical drug and biological agent sensitivity, but also to study ovarian cancer spheroid biology.

Hanging drop array platforms offer a unique advantage over conventional hanging drop methods and liquid overlays on ultra low attachment plates that are currently utilized to generate ovarian cancer spheroids [29–33], often overcoming difficulties of liquid handling and long-term culture [9, 12, 13, 34]. Spheroids generated in this array platform can also incorporate a defined number of cells resulting in uniformly sized and shaped spheroids.

In this report, we have described the use of a new high-throughput amenable 384 well hanging drop array platform to form and evaluate ovarian tumor spheroids in vitro. Using commercially available ovarian cancer cell lines we demonstrate the stable incorporation of as few as 10 cells per hanging drop. The number of cells incorporated into the spheroid and the sizes of the resultant spheroids are uniform, and vary as a function of initial cell seeding densities.

We demonstrated that ovarian cancer spheroids could be generated with uniform volumes, high circularity, and a good three-dimensional presence. The formation of serous OVCAR3 spheroids, and non-serous A2780 spheroids and SKOV3 spheroids are demonstrated (Figs. 1, 2 and Supplemental Fig. 10). The slow rate of proliferation in 3D spheroids is more typical of in vivo growth conditions, and was independent of initial cell-seeding density in spheroids. Concomitant with the fold increase in alamarblue proliferation, spheroid volume increased in a range between 6-fold and 12-fold for most spheroids (Supplemental Figs. 1–2). 100 cells/drop A2780 spheroids alone demonstrated a fold increase of 16.29 in spheroid volume. This was because 100 cells/drop typically took a day longer for cells to aggregate into a single drop, and volume measurements from spheroidal aggregates obtained on Day 1 did not include all the cells in the well.

We performed viability assays on the spheroids in the hanging drop arrays that were visualized and quantified using conventional confocal microscopy. Though labor intensive, we correlated live/dead viability data with the high-throughput amenable alamarblue fluorescence readings to establish the hanging drop array platform as a relevant and effective drug-screening platform. Ovarian cancer cells within spheroids demonstrated high viability in the 10-, 20-, 50- and 100-cell drops. This indicated extensive cell–cell contact within the self-assembled spheroids that promoted cellular viability. Three-dimensional presence and cell–cell interaction was further confirmed using confocal microscopy upon cytoskeletal actin and nuclei staining. Cortical actin staining using phalloidin is commonly used to demonstrate cell aggregates in 3D culture systems, including spheroids [19–23]. The observance of cortical actin staining, as opposed to diffuse cytoplasmic staining and stress fiber staining of cells grown on monolayers, indicated the three-dimensional morphology of cells grown in attachment-free culture.

Lastly, ovarian cancer spheroids generated on the hanging drop array plates demonstrated significant chemoresistance in response to cisplatin treatment. Conventional 2D cultures were used as controls, where the 50 μ M cisplatin dose reduced the number of surviving cells to ~34–56%. In contrast, even 10-cell spheroids presented significant chemoresistance,

losing only 20–35% of cells. Furthermore, dependent on the size of the spheroids, which in itself varied as a function of the initial cell seeding density, cisplatin treatment reduced projected areas of the spheroids. The drop in spheroid area was sharper for the smaller 10- and 20-cell spheroids compared to the bulkier 50- and 100-cell spheroids. This observed chemoresistance could be attributed to several factors including (i) the slower proliferation rate within spheroids, (ii) diffusional limitations in the three-dimensional spheroid structure compared to 2D, (iii) microenvironmental changes in 3D spheroids, and finally a (iv) possible change in cellular phenotype of ovarian cancer cells in three-dimensional culture compared to 2D [10, 36–40]. We also further demonstrated the treatment of SKOV3 spheroids with conventional chemotherapy drugs including doxil, cisplatin and gemcitabine (Supplemental Figs. 11, 12, and 13). The observed chemoresistance in the spheroids further validated the applicability of these low cell spheroids for predictive preclinical drug screening.

Generating spheroids using such low cell numbers is important when dealing with rare patient samples, such as isolated sorted cancer stem cells which make up <1% of the total cellular population [41, 42]. With the cancer stem cell hypothesis gaining more traction for its role in chemoresistance, recurrent disease and poor prognosis [7, 43], it is of prime importance to develop and test new therapies that target this cellular population. Since this research describes the stable incorporation of as few as 10 cells into a three-dimensional spheroid, the hanging drop platform is highly suited in dealing with rare patient-derived cell populations. Additionally, this platform is also highly amenable to addition of both extracellular matrix molecules, as well as, stromal cells to recapitulate the heterogeneity of the tumor microenvironment. The high-throughput format combined with the stable incorporation of low cell numbers will facilitate personalized medicine. Uniform high throughput production of spheroids from low cell numbers derived from malignant as cites will be an important intermediate step for preclinical drug screening for novel biological therapies.

5. Conclusion

In this paper, we describe the stable generation of ovarian cancer spheroids from two different ovarian cancer cell lines, serous OVCAR3 and non-serous A2780 cells. We utilized 384 well hanging drop arrays to generate multicellular tumor spheroids, derived from as few as 10 cells. Ovarian cancer spheroids were uniform in size depending on the initial cell seeding density. The use of the hanging drop array plate facilitates high throughput applications as well as multiplexed applications, including the use of liquid handling systems and automated plate readers. Stable incorporation of small cell numbers into multicellular spheroids holds promise to handle rare cellular populations like ovarian cancer stem cells derived from patient samples. Furthermore, spheroids generated from as few as 10 cells/spheroid demonstrate significant chemoresistance compared to conventional 2D drug treatment, holding promise for the applicability of this spheroid-generating platform for the development of personalized drug screens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We describe stable incorporation of low cell numbers into ovarian cancer spheroids.
- Spheroids have uniform geometry and three-dimensional presence.
- Spheroids contain viable cells that can be utilized for high throughput drug screens.
- Spheroids are chemoresistant to cisplatin compared to conventional monolayer cultures.
- Spheroids generated on hanging drop platforms are high throughput amenable.

Fig. 1.

Formation of small cell number A2780 spheroids on hanging drop array plates. (A) Representative phase contrast micrographs of A2780 spheroids on Day 1 and Day 7. Spheroids of A2780 cells were initiated with 10, 20, 50 and 100 cells per drop on hanging drop array plates. Spheroid formation was studied using live cell microscopy. Cells within hanging drops aggregated into a spheroid-like structure on Day 1. At Day 7, tight spheroids with clear boundaries were observed. Scale bar = 100 μm . (B) Projected area of A2780 spheroids. Calibrated images were used to obtain morphometric data at Day 1 and Day 7 to determine spheroid sizes. Areas of A2780 spheroids increased from Day 1 to Day 7 in hanging drop cultures, as a function of the initial cell seeding density. Projected 2D spheroid areas were significantly different ($*p < 0.05$, one-way ANOVA) on Day 7 between all cell densities. (C) Proliferation in A2780 spheroids. Proliferation within spheroids was assessed using a fluorescence-based alamarblue assay. A fold-increase in alamarblue fluorescence intensity was observed in all spheroids. Regardless of initial cell seeding densities, proliferation index varied non-significantly from 9 fold to 11 fold over 7 days in hanging drop culture.

Fig. 2.

Formation of small cell number OVCAR3 spheroids on hanging drop array plates. (A) Representative phase contrast micrographs of OVCAR3 spheroids on Day 1 and Day 7. Spheroids of OVCAR3 cells were initiated with 10, 20, 50 and 100 cells on hanging drop arrays. Live cell microscopy was used to monitor spheroid formation, and calibrated images were used to obtain spheroid area measurements indicative of their size. Cells within hanging drops aggregated to form spheroid-like structures by Day 1, and continued to aggregate and form tightly packed spheroids with defined boundaries by Day 7. Scale bar = 100 μm . (B) Projected area of OVCAR3 spheroids. Area varied as a function of initial cell seeding density, and was significantly higher at Day 7 compared to Day 1 ($*p < 0.05$, one-way ANOVA). (C) Proliferation in OVCAR3 spheroids. Proliferation of cells within spheroids was assessed using a fluorescence-based alamarblue assay. Fold increase in alamarblue fluorescence varied non-significantly from 10 fold to 11 fold over 7 days in 3D culture.

Fig. 3.

Viability of cells within multicellular ovarian cancer spheroids. (A–D) Live/Dead staining on A2780 spheroids with varying cell densities, with minimal red/dead cell staining. Following 7 days in hanging drop array culture, A2780 or OVCAR3 spheroids were incubated with calcein-AM and ethidium homodimer. Live cells within spheroids were indicated by green fluorescence for calcein-AM, while dead cells were indicated by red fluorescence for ethidium homodimer. Confocal microscopy was used to image calcein and ethidium homodimer fluorescence through the height of the spheroids. (E) Quantification of Live/Dead staining in A2780 spheroids. A bar graph representation of the percentage of live and dead cells within the different spheroids is depicted. Excellent viability was observed, with <15% of cells staining red. (F–I) Live/dead staining on OVCAR3 spheroids with varying cell densities. (J) Quantification of Live/Dead staining in OVCAR3 spheroids. Bar graph representation of the percentage of live and dead cells within the OVCAR3 spheroids is depicted. On an average, <12% of the cells stained red for ethidium homodimer. Scale bar = 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Three-dimensional structure of multicellular ovarian cancer spheroids. (A–D) Three-dimensional structure of A2780 spheroids plated with 10, 20, 50 or 100 cells/drop. Actin cytoskeletons of cells within spheroids were stained at Day 7 by incubation with fluorescently conjugated phalloidin. Nuclei were counter stained with DAPI. Stained spheroids were observed on the confocal microscope, and merged images are presented. (E–H) Three-dimensional structure of OVCAR3 spheroids plated with 10, 20, 50 or 100 cells/drop. Three-dimensional morphology observed with cortical actin cytoskeleton within cells and spanning cell–cell interactions. DAPI immunostaining confirms the self-assembly into tight spheroid three-dimensional structure. Scale bar = 100 μ m.

Fig. 5.

Chemoresistance in A2780 spheroids in response to cisplatin treatment. (A) Decrease in projected area in cisplatin treated A2780 spheroids. A2780 spheroids on hanging drop arrays were treated with varying doses of cisplatin. Morphological changes in spheroids were observed using live cell microscopy, and morphometry was performed to note a change in spheroid area with cisplatin treatment. Spheroid areas were reduced in cisplatin treated spheroids compared to untreated control A2780 spheroids. At the 50 μM cisplatin dose, 10 cell spheroids and 20 cell spheroids dropped significantly in area by 30% and 22.27% respectively ($***p < 0.001$, one-way ANOVA) of the control untreated spheroids. (B) Viability and chemoresistance in A2780 spheroids. Alamarblue fluorescence was utilized to monitor viability in A2780 spheroids treated with cisplatin compared to untreated A2780 spheroids. Compared to the 2D conventional 96-well cultures, three-dimensional spheroids demonstrated higher viability ($***p < 0.001$, two-way ANOVA) at the 50 μM cisplatin dose. (C) Representative phase contrast micrographs of untreated control and 50 μM cisplatin treated spheroids within hanging drop arrays.

Fig. 6.

Chemoresistance in OVCAR3 spheroids in response to cisplatin treatment. (A) Decrease in projected area in cisplatin treated OVCAR3 spheroids. OVCAR3 spheroids on hanging drop arrays were treated with varying doses of cisplatin. Morphological changes in spheroids were observed using live cell microscopy, and morphometry was performed to note a change in spheroid area with cisplatin treatment. Spheroid areas were reduced in cisplatin treated spheroids compared to untreated control OVCAR3 spheroids. At the 50 μM cisplatin dose, 10-, 20-, and 50-cell spheroids dropped significantly in area by 15–35% (** $p < 0.01$, one-way ANOVA) of the control untreated spheroids. (B) Viability and chemoresistance in OVCAR3 spheroids. Alamarblue fluorescence was utilized to monitor viability in OVCAR3 spheroids treated with cisplatin compared to untreated OVCAR3 spheroids. At the 50 μM cisplatin dose, compared to the 2D conventional 96-well cultures, three-dimensional spheroids demonstrated higher viability (* $p < 0.05$, ** $p < 0.01$, two-way ANOVA). (C) Representative phase contrast micrographs of untreated control and 50 μM cisplatin treated spheroids within hanging drop arrays.