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

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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.

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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 (42.60×10^3 μm–475.22×10^3 μm^2 for A2780 spheroids and 37.24×10^3 μm^2–281.01×10^3 μm^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 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.

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

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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 1.5X 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 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 μM, and Ethidium homodimer-1 was added to a final concentration of 4 μM 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;

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