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Comparative study of multicellular tumor spheroid formation methods and implications for drug screening

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ABSTRACT: Improved *in vitro* models are needed to better understand cancer progression and bridge the gap between *in vitro* proof-of-concept studies, *in vivo* validation, and clinical application. Multicellular tumor spheroids (MCTS) are a popular method for three-dimensional (3D) cell culture, since they capture some aspects of the dimensionality, cell-cell contact, and cell-matrix interactions seen *in vivo*. Many approaches exist to create MCTS from cell lines, and they have been used to study tumor cell invasion, growth, and how cells respond to drugs in physiologically-relevant 3D microenvironments. However, there are several discrepancies in the observations made of cell behaviors when comparing between MCTS formation methods. To resolve these inconsistencies, we created and compared the behavior of breast, prostate, and ovarian cancer cells across three MCTS formation methods: in polyNIPAAm gels, in microwells, or in suspension culture. These methods formed MCTS via proliferation from single cells or

passive aggregation, and therefore showed differential reliance on genes important for cell-cell or cell-matrix interactions. We also found that the MCTS formation method dictated drug sensitivity, where MCTS formed over longer periods of time via clonal growth were more resistant to treatment. Towards clinical application, we compared an ovarian cancer cell line MCTS formed in polyNIPAAm with cells from patient-derived malignant ascites. The method that relied on clonal growth (PolyNIPAAm gel) was more time and cost intensive, but yielded MCTS that were uniformly spherical, and exhibited the most reproducible drug responses. Conversely, MCTS methods that relied on aggregation were faster, but yielded MCTS with grape-like, lobular structures. These three MCTS formation methods differed in culture time requirements and complexity, and had distinct drug response profiles, suggesting the choice of MCTS formation method should be carefully chosen based on the application required.

KEYWORDS: *PolyNIPAAm, 3D, ovarian cancer, breast cancer, prostate cancer, mafosfamide.*

INTRODUCTION

Two-dimensional (2D) cell culture monolayers are traditionally used to study cancer biology, gain insight into mechanisms of cancer progression, and screen for novel anti-cancer treatments. However, traditional 2D cell culture platforms used in drug screening do not accurately model cancer tumors¹⁻³, as they lack cell heterogeneity, differentiated phenotypes, extracellular matrix (ECM) architecture, and drug resistance seen *in vivo*⁴⁻⁷. *In vitro* model systems that can better mimic the *in vivo* microenvironment could improve therapeutics discovery by reducing false positives that later fail in pre-clinical or clinical trials. Three-dimensional (3D) cell culture models can recapitulate some of the physiological behavior of *in vivo* tumors^{5,8}, including non-uniform distribution of oxygen and nutrients¹ and multicellular drug resistance⁹. Moreover, gene expression profiles of 3D cell culture models are often closer to gene expression profiles of tumors than 2D models^{4,10-11}. Therefore, 3D models can better reflect some of the complexity of tumors that impact biological responses, such as drug resistance, angiogenesis, cell migration, cell invasion, and metastasis^{5,12}.

The most common approach for 3D cell culture models is the multicellular tumor spheroids

(MCTS) model¹³⁻¹⁴, which can be applied to established cancer cell lines or samples isolated from patients¹⁵. MCTS are characterized by a 3D structure with strong cell-cell and cell-ECM interactions, which make them more similar to *in vivo* tumors over 2D cell monolayers^{2,7}. Unlike 2D models, MCTS models can also recapitulate the oxygen and nutrient gradients seen *in vivo*¹⁶⁻¹⁸. Because of these features, MCTS have been used to study tumor biology, and for high-throughput drug screening^{9,12,15,19}. MCTS are used in development and testing several therapeutic approaches like chemotherapy, radiotherapy, and immunotherapy¹⁹⁻²¹. Additionally, many researchers have found increased resistance in MCTS compared to cells on tissue culture polystyrene (TCPS)²²⁻²⁷. Despite these attributes, several factors limit the use of MCTS in drug discovery and development, including the need to 1) produce high quantities of uniform MCTS²⁸⁻²⁹, 2) predictably control MCTS size for consistent results¹⁶, and 3) technical adaptation of existing screening assays to 3D MCTS models²⁸⁻²⁹. Addressing these limitations could make 3D high-throughput drug screening methods more suitable to commercial development.

Current MCTS formation techniques include stationary or rotating culture incubation systems. Stationary formation systems include the liquid-overlay technique, the hanging drop method, and the suspension method in non-adherent plates^{1,30}. The advantages of these systems are low cost, easy operation, and production of reproducible MCTS³¹. However, they yield very few MCTS, which limits their adaptation to large scale studies. Rotating formation systems include the spinner flask method, the gyratory rotation system, and the roller tube system^{1,30}. The advantages of these techniques include massive production and control of culture conditions³¹, while the biggest limitations are the infrastructure, and high quantities of medium and drugs required³⁰. Although these methods each have their advantages, the existence of multiple MCTS formation methods introduces variability in MCTS size, shape, and formation ability^{14,32-33}. To the best of our knowledge, this is the first work that compares MCTS formation methods in terms of the implications in gene expression and drug response, which may improve future studies across many applications, such as high-throughput drug screening.

In this study, we explored how three MCTS formation methods changed gene expression and drug response in breast, prostate, and ovarian cancer cells. These methods required either aggregation followed by compaction or clonal proliferation, probably leading to different subpopulations of cells in the resulting MCTS. Although each method produced similar size MCTS, different methods lead to different

expression profiles of cell-cell and cell-matrix interaction genes. Moreover, the MCTS formation methods used dictated drug sensitivity, suggesting significant implications to improve future drug screening studies.

MATERIALS AND METHODS

Cell culture. All cell lines were cultured at 37°C and with 5% CO₂ unless otherwise noted. Cell culture supplies were purchased from Thermo Fisher Scientific (Waltham, MA) with the exception of bovine insulin (Sigma-Aldrich, St. Louis, MO). The cell lines AU565, BT549, BT474, HCC 1395, HCC 1419, HCC 1428, HCC 1806, HCC 1954, HCC 202, HCC 38, LNCaPcol, HCC 70, PC-3, SKOV-3, ZR-75-1 were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep). OVCAR-3 cells were cultured in RPMI with 20% FBS, 1% Pen/Strep, and 0.01 mg/ml bovine insulin. Hs578T, MCF7, MDA-MB-468, MDA-MB-231, MDA-MB-231 BoM (bone tropic), MDA-MB-231 BrM2a (brain tropic), MDA-MB-231 LM2 (lung tropic), SkBr3 were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 1% Pen/Strep. MDA-MB-175 was cultured in Leibovitz's L-15 medium with 10% FBS and 1% Pen/Strep without supplemental CO₂, and MDA-MB-134 and MDA-MB-361 were cultured in Leibovitz's L-15 medium with 20% FBS and 1% Pen/Strep without supplemental CO₂. The media described for their respective cell lines will be referred to as "routine culture medium" and any modifications will be noted where applicable. Highly metastatic MDA-MB-231 variants isolated from *in vivo* selection (BoM, BrM2a, and LM2) were kindly provided by Joan Massagué³⁴⁻³⁶, MDA-MB-231 by Sallie Smith Schneider, BT549, MCF7, and SkBr3 by Shannon Hughes, PC3 by Evan Keller, and LncAPcol derived through serial passage on collagen type I from LNCaP by Michael Long³⁷. SKOV-3 and OVCAR-3 were purchased from ATCC, and all others were kindly provided by Mario Niepel.

PolyNIPAAM MCTS. Single cells were suspended at 100 cells/μL in polyNIPAAM (Cosmo Bio USA, Carlsbad, CA) on ice and gelled as 150 μL volumes at 37°C for 5 minutes. Routine culture medium or routine culture medium + 100 ng/mL epidermal growth factor (EGF, R&D Systems, Minneapolis, MN) was added and medium was changed every 2-3 days until MCTS were collected at Day 14. MCTS were recovered from polyNIPAAM by replacing the cell culture medium with serum free medium at 4°C and

placing them on ice for 5 minutes. The dissolved gel was diluted in additional serum free medium and put in a conical tube to concentrate the MCTS via gravity sedimentation on ice for 30 minutes. The MCTS pellet was lysed for RT-PCR, or used for encapsulation in 3D hydrogels. MCTS were handled using cut pipette tips to minimize shear stress.

Microwells MCTS. Square pyramidal micro-wells (400 μm side-wall dimension) were fabricated as described previously³⁸⁻³⁹ or purchased (AggreWell, Stem Cell Technologies, Canada). For fabrication, master molds containing square-pyramidal pits were generated by anisotropic etching of 100 crystalline silicon in potassium hydroxide (KOH). Microwells surfaces for tissue culture were then generated from poly(dimethylsiloxane) (PDMS) using a two-stage replica molding process off the master mold as described previously³⁸⁻³⁹. Microwells were arranged in a square array with no space between adjacent wells and placed in 6 or 12-well plates. To prepare microwells for cell seeding, microwells surfaces were UV sterilized and pretreated with 5% Pluronic F-127 (Sigma Aldrich) for 30 minutes at room temperature and then washed twice with sterile water. Cells were distributed over microwells surfaces at concentrations of 1.03×10^5 cells/cm² or 1.00×10^4 cells/cm². After 24 hours, MCTS were collected by shaking the plate gently to dislodge most of them, and gently aspirating medium and MCTS. MCTS solution was spun down at 400 rpm for 5 minutes. Medium was removed, and the MCTS pellet was lysed for RT-PCR, or encapsulated in 3D hydrogels. MCTS were handled using cut pipette tips to minimize shear stress.

Suspension MCTS. Single cells were seeded at 1.05×10^4 cells/cm², 1.05×10^3 cells/cm², or 1.05×10^2 cells/cm² in a 6-well flat ultra-low attachment plate (Corning, Tewksbury, MA). After 3 days, MCTS were collected by aspiration of medium and MCTS. MCTS solution was spun down at 400 rpm for 5 minutes. Medium was removed, and the MCTS pellet was lysed for RT-PCR or encapsulated in 3D hydrogels. MCTS were handled using cut pipette tips to minimize shear stress.

Characterization of gene expression by RT-PCR. The expression of cell-cell adhesion molecules and ECM mRNA transcripts was measured by quantitative RT-PCR. Total RNA was isolated using the GenElute mammalian total RNA kit (Sigma-Aldrich) and 0.5 μg total RNA was reverse transcribed using the RevertAid reverse transcription system (Thermo). 10 ng cDNA was then amplified using 10 pmol integrin-specific primers (Table S1) and the Maxima SYBR green master mix (Thermo) on

a Rotor-Gene Q thermocycler (Qiagen, Valencia, CA) as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 45 cycles at 95°C for 10 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Both β -actin and ribosomal protein S13 were included as reference genes to permit gene expression analysis using the $2^{-\Delta\Delta C_t}$ method⁴⁰.

PEG-maleimide (PEG-MAL) hydrogels. 3D hydrogels were prepared with a 20 kDa 4-arm PEG-maleimide (PEG-MAL, Jenkem Technology, Plano, TX) at 10 wt% solution with 2 mM of cell adhesion peptide RGD (see supplemental information) and cross-linked at a 1:1 ratio with 1 kDa linear PEG-dithiol (Sigma Aldrich) in sterile 2 mM triethanolamine (pH 7.4). Volumes of the hydrogels were limited to 10 μ L to avoid oxygen and nutrient diffusion limitations. Gelation proceeded for 5 minutes at 37°C to ensure complete polymerization before the addition of culture medium. MCTS were transferred to 3D PEG-MAL hydrogels; using similar seeding densities were similar for the three MCTS formation methods. PolyNIPAAm MCTS were encapsulated at a ratio of one 150 μ L polyNIPAAm gel to nine 10 μ L PEG-MAL hydrogels, microwells MCTS at a ratio of 4 cm², 1 mL to nine 10 μ L PEG-MAL hydrogels, and suspension MCTS at a ratio of 9 cm², 3 mL to nine 10 μ L PEG-MAL hydrogels. MCTS created via either polyNIPAAm, microwells or suspension were transferred with cut pipette tips to minimize shear stress.

Primary ovarian cancer ascites culture. Ascites samples were received from patients undergoing paracentesis at UMass Medical School (Worcester, MA), were transported to UMass Amherst (Amherst, MA) on the day of collection, and used immediately upon receipt. Samples were de-identified and were IRB exempt. Pathology reports are provided in Table S2. Either single cells or ovarian carcinoma ascites spheroids (OCAS) were recovered from patient samples. For single cells, the ascites fluid was centrifuged at 1,000 rpm for 10 minutes at 4°C, and the supernatant was removed. Red blood cells were removed by resuspending the cell pellet in cold red blood cell lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, and 0.0037% EDTA)⁴¹. The tube was rotated at room temperature for 10 minutes, cells spun down at 1,000 rpm for 10 minutes at 4°C, and washed with PBS once prior to seeding on TCPS or in polyNIPAAm. For collection of OCAS directly from the patient sample, ascites fluid was filtered through a 40 μ m mesh cell strainer. The retained OCAS were collected by using a cut pipette tip on the inverted cell strainer and encapsulated directly into polyNIPAAm or 3D PEG-MAL hydrogels.

Supernatant from ascites pellets was stored at -80°C and filtered through 0.45 µm syringe filter prior to use as a culture medium without any additional supplements for single cell and OCAS polyNIPAAm culture.

Proliferation and drug screening. AU565, BT549, SKOV-3, and primary ovarian single cancer cells were seeded in RPMI with 5% FBS at 6,250 cell/cm². MCTS from the same cell lines formed in polyNIPAAm, microwells, and suspension were recovered and encapsulated in 3D PEG-MAL hydrogels. OCAS from primary ovarian cancer samples were collected and also encapsulated in 3D PEG-MAL hydrogels. Drugs were added after 24 hours, and cells were incubated with drugs for 48 hours. Cisplatin (Tocris Bioscience, United Kingdom), paclitaxel (MP Biomedicals, Santa Ana, CA), sorafenib (LC Laboratories, Woburn, MA), or mafosfamide (Niomech, Germany) were added in ten-fold serial dilutions at concentrations of 10⁻⁵-10² µM, and dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used as a vehicle control. Viability was assayed after 48 hours of incubation using the CellTiter Glo luminescent viability assay (Promega, Madison, WI). Luminescence values were read in a BioTek Synergy H1 plate reader (Winooski, VT), and GR₅₀⁴² was calculated using GraphPad Prism v6.0h (La Jolla, CA) for each cell line and drug. GR₅₀ and proliferation results for the three MCTS formation methods are presented and discussed as relative to TCPS (fold change from the TCPS results).

MCTS staining. MCTS in microwells plates at days 0 and 1, and from polyNIPAAm, microwells, and suspension encapsulated in 3D PEG-MAL hydrogels for 24 hours or 3 days, were assessed for viability with live/dead staining (L3224, Thermo) according to the manufacturer's instructions, and for proliferation via Ki67 immunofluorescence. For the Ki67 staining, samples were rinsed three times with PBS, fixed with 4% formaldehyde, permeabilized with Tris-buffered saline (TBS) containing 0.5% Triton X-100 (Promega), and blocked with AbDil (2 wt % bovine serum albumin (BSA) in TBS with 0.1% Triton X-100, TBS-T). Samples were incubated for 2 hours at room temperature with the primary antibody (ab16667, 1:200-Abcam, UK), washed, and incubated with goat anti-Rabbit IgG (H+L) secondary antibody for 2 hours (Alexa Fluor 647, 1:500, Promega). Cell nuclei were labeled with DAPI at 1:10000 (Thermo) for 5 minutes. Brightfield imaging was performed on a Zeiss Axio Observer Z1 (Carl Zeiss AG, Oberkochen, Germany), and fluorescence imaging on a Zeiss Spinning Disc Cell Observer SD (Zeiss).

Statistical analysis and correlations. Statistical analysis was performed with GraphPad Prism v6.0h. Data shown are the averages of the means from three separate biological replicates, and the error bars represent standard error (95% confidence level). Patient samples had only one biological replicate each. Statistical significance was evaluated by two-way analysis of variance (ANOVA), followed by Tukey's post-test for pairwise comparisons. Spearman rank correlation is reported as ρ with significance (p) determined by a two-tailed t-test. For both tests, $p < 0.05$ was considered statistically significant. $p < 0.05$ is marked with *, ≤ 0.01 with **, ≤ 0.001 with ***, and ≤ 0.0001 with ****, $p \geq 0.05$ was considered not significant ('ns').

Image processing. Image J (NIH, Bethesda, MD) was used for diameter and circularity measurements of MCTS and cells, as well as compiling individual staining images.

Information for "RGD synthesis" and "Gene Expression Clustering and PCA" can be found in Supplemental Materials and Methods.

RESULTS

PolyNIPAAm, microwells, and suspension methods generated diverse MCTS across cell lines. We screened the MCTS formation abilities of 23 breast, 2 ovarian, and 2 prostate cancer cell lines across three different MCTS methods: polyNIPAAm gels, microwells, and suspension in non-adherent plates (Figures 1b, S1). For all the methods, single cells were seeded, and after 14 days (polyNIPAAm), 3 days (suspension), or 1 day (microwells), and MCTS reached approximately 100 μm . Many cell lines formed uniformly-sized MCTS that had at least a 2-fold increase in mean diameter, with many reaching approximately 100 μm in diameter in 14 days (i.e., AU565, BT549, LNCaPcol, PC-3, SKOV-3, OVCAR-3, HCC 1419, HCC 1428, MCF7, MDA-MB-231, SkBr3, ZR-75-1, BT474). Some cell lines (i.e., HCC 1954, Hs578T) were incapable of growing into MCTS, and most of these cells died within the 14-day period (data not shown). Other cell lines formed few MCTS (i.e., HCC 1806, MDA-MB-468, MDA-MB-231 LM2, HCC 70), suggesting that only a small percentage of cells were capable of forming MCTS via this method.

We observed many different morphologies across these MCTS, and they were reminiscent of work reported from the Bissell group that described four classes of spheroids that formed in 3D laminin-rich ECM (3D IrECM): round, mass, grape-like, and stellate⁴³. In polyNIPAAM, BT474 and MCF7 formed very compact MCTS, while SkBr3 and MDA-MB-468 formed grape-like MCTS. As for the cell lines that were stellate in 3D IrECM, BT549 formed compact MCTS, MDA-MB-231 formed large, loose MCTS, while Hs578T did not form MCTS in polyNIPAAM. Invasion or cell spreading reminiscent of the stellate morphology were never observed because there were no cell-matrix adhesion sites in the polyNIPAAM gel, and it was not cell-degradable. We did not examine any cell lines that were categorized by Bissell as round.

The polyNIPAAM method allowed growth of MCTS in more than half of the cell lines tested, independent of breast cancer clinical subtype (data not shown). Interestingly, a negative correlation was found between doubling times⁴⁴ and MCTS size (Figure S2a), indicating that faster growing cells produce bigger MCTS in polyNIPAAM. In addition, we found that most cell lines formed bigger MCTS when grown with supplemental EGF, although some did not change (i.e., HCC 1428, HCC 1806, BrM2a, BoM, MDA-MB-134, MDA-MB-361, HC 1954, and Hs578T) and a few decreased in size, such as AU565, MDA-MB-231, and MDA-MB-468, which already make large MCTS (Figure S3). The data showed that EGF caused only a small size increase for cell lines that formed small MCTS, while it caused larger size increases in the majority of the cell lines that made larger MCTS even without EGF.

All cell lines that formed MCTS in the polyNIPAAM method were collected after 14 days of culture, and encapsulated into 3D PEG-MAL hydrogels. This was done to demonstrate that the MCTS were easily handled, and remained intact when transferred to another system. Viability was assessed after 3 days, and the majority of the MCTS were viable (Figure S4). There was no evidence of a necrotic core, but likely this was because the MCTS diameters were relatively small.

We next selected a subset of the cell lines that had at least a 2-fold increase in diameter in the polyNIPAAM method (red text in Figure 1b) to compare MCTS formation in microwells (Figure 1c) and suspension (Figure 1d) methods. In these aggregation methods, MCTS size increased with seeding cell density (Figure S5), and densities of 1.00×10^4 cells/cm² (microwells) and 1.05×10^4 cells/cm² (suspension) were used to achieve MCTS sizes between 80-150 μ m (Figure S6). In the microwells, the

cells slowly coalesced into MCTS for the first ~10 hours, then the MCTS further compacted over the first day of culture (Figure S7 and Video S1). MCTS from microwells were both viable and proliferative (Figure S8). Among the six cells lines used for the microwells and suspension methods, there was no correlation between MCTS size and growth rate, likely because these methods are dependent upon cell aggregation rather than growth (Figure S2b and c). Since MCTS in microwells compacted over time, circularity was found to increase, which eventually yielded more uniform MCTS. In contrast, circularity was preserved in polyNIPAAM throughout the growth process, and decreased in suspension (Figure S9). It is likely that MCTS in suspension became less compact over time because there were no solid surfaces that cells could contact. By contrast, cells in polyNIPAAM are confined in the gel, and cells in the microwells are in contact with the microwell surfaces. Together, these results demonstrated that MCTS can be formed with three different methods. Furthermore, the differences in culture times suggest that in the polyNIPAAM method single cells grew into MCTS (Figure 1 and S2a), whereas in the microwells and suspension methods, MCTS formed as a result of aggregation, independent of proliferation (Figure S2b-c).

Fibronectin and claudin 4 expression depend on MCTS formation method. Gene expression was quantified in breast, prostate, and ovarian cancer cell lines in the three MCTS formation methods and compared to basal gene levels in 2D TCPS culture. To select the relevant genes for this study, RNA-Seq data was analyzed from breast cancer cell lines (MDA-MB-231 and SkBr3) that were grown on TCPS, in polyNIPAAM for 14 days, or grown in polyNIPAAM and then dissociated and plated back onto TCPS (Figure S10a; GSE93562). Using Gene Set Enrichment Analysis (GSEA), we observed that cell surface receptor-linked signal transduction genes, including several integrins, were enriched on TCPS when compared to MCTS formed in polyNIPAAM for 14 days, while cell-cell adhesion genes, such as claudin 4, were enriched in cells grown into MCTS in polyNIPAAM over 14 days compared to TCPS (Figure S10b-c). From this dataset, we selected a subset of cell adhesion genes including integrin subunits (α_2 , β_1 and β_4), cell-cell junction proteins (cadherins 3 and 5 and claudin 4), and the ECM protein, fibronectin, to examine in each MCTS formation method.

We found that either claudin 4 or fibronectin were upregulated in all MCTS methods compared to TCPS (Figure 2a) with the few exceptions of OVCAR3 in polyNIPAAM, and BT549 and PC-3 in

microwells. Gene expression patterns varied with the time required for MCTS formation across the three methods. Fibronectin was downregulated for all the cell lines in microwells, while claudin 4 was upregulated in polyNIPAAm method for all the cell lines, except for AU565 and OVCAR-3. These gene expression changes were dependent on the cell line as well as the MCTS formation method. Variations in gene expression with MCTS formation method was confirmed by dendrogram clustering for all combinations of examined cell lines and methods, which revealed that TCPS and polyNIPAAm primarily clustered together, and the shorter methods of microwells and suspension also clustered together (Figure 2b). This was further confirmed by principal component analysis (PCA), which revealed that samples did not cluster by cell line or cancer type (data not shown), but rather that PC1 separated samples by method, with those that had the shortest and longest times of culture being the most distinct from one another (Figure 2c). Although all of these systems formed MCTS of similar size, the expression of cell contact genes varied across methods, which may have affected the compactness of MCTS. We thus hypothesized that MCTS that upregulated cell-cell contact genes and took longer times to form MCTS may be more resistant to first line therapies than 2D TCPS due to their compact morphology.

MCTS growth method dictated drug response. To test drug resistance, we selected two breast cancer cell lines, one triple negative (BT549) and one HER2-enriched (AU565) for drug screening experiments. These subtypes have poor prognosis and they are clinically treated with chemotherapeutic drugs⁴⁵. Breast cancer MCTS were created with the three methods, encapsulated in 3D PEG-MAL hydrogels, and treated with either the chemotherapeutic drugs cisplatin or paclitaxel, or the targeted drug sorafenib, which is a Raf kinase inhibitor⁴⁶ (Figure 3a). Transferring the MCTS to the 3D PEG-MAL hydrogels allowed us to test cell response to the drugs as a function of MCTS formation method, independent of the effects of the MCTS formation platform. The maximum MCTS diameter for these two cell lines was kept at 150 μm (Figure S8). In all cases, the MCTS were intact and viable 24 hours after encapsulation in 3D PEG-MAL (Figure S11).

MCTS proliferation was measured relative to TCPS as a baseline for cell growth across platforms. AU565 and BT549 cells proliferated less in all the three methods used to form MCTS compared to TCPS (Figure 3b). Next, drug response was determined by calculating the GR₅₀ for MCTS formed by all three methods, as well as for each cell line grown on TCPS. Drug responses were reported

as fold changes between GR_{50} values of each drug in individual 3D MCTS models and TCPS; a fold change of >1 means that the 3D MCTS model displayed greater drug resistance than TCPS. We found that the MCTS formation method dictated drug sensitivity. For example, AU565 cells were less sensitive to cisplatin in MCTS formed in polyNIPAAm compared to suspension and microwells (Figure 3b). However, the effect of formation method on drug response also varied between cell lines. For example, BT549 were more sensitive to cisplatin when they were formed in the polyNIPAAm method, and less sensitive when they were formed in the suspension method, while the opposite was true for AU565. We also found that drug response varied across drugs: AU565 MCTS cultured in microwells were significantly more sensitive to paclitaxel, while they were less sensitive to cisplatin and sorafenib. Interestingly, MCTS formed in all methods were slightly more sensitive to sorafenib than on TCPS, with the exception of AU565 MCTS formed in suspension. This may be because sorafenib works as a targeted agent rather than interfering with cell division. Overall, the microwells method sensitized MCTS to drugs, with the exception of the BT549 response to paclitaxel. However, MCTS created with the polyNIPAAm and suspension methods were more resistant to drug treatments, although the response was slightly more heterogeneous across conditions. We then hypothesized that MCTS formed in polyNIPAAm could show similar response to drugs as seen in *in vivo* tumors. To test this hypothesis, we compared drug responses of SKOV-3 MCTS and patient-derived ovarian carcinoma ascites.

SKOV-3 MCTS modeled primary ovarian cancer drug response. With the goal of discovering whether MCTS were able to recapitulate features of primary patient samples, we compared the drug responses of SKOV-3 MCTS with that of cells gathered from ascites of ovarian cancer patients. We seeded single cells and OCAS collected from patients in polyNIPAAm to determine if single cells could form MCTS and if OCAS seeding would increase in MCTS size. There was variation in growth for single cells between patients (P1, P2, and P3) and culture medium (Figure 4a). However, OCAS seeded in polyNIPAAm did not appreciably increase in size compared to single cells (Figure 4a). Because most samples were not able to form MCTS from single cells in our polyNIPAAm method, and ascites samples are often rich in OCAS, we collected these samples and encapsulated them directly into our 3D PEG-MAL hydrogel to capture native architecture, cell-cell contacts, and cell type heterogeneity for drug

screening. These samples were compared to SKOV-3 MCTS, which were formed in polyNIPAAm before being transferred to 3D PEG-MAL hydrogels.

We chose to examine the response to cisplatin and paclitaxel, which are first line clinical therapeutics for ovarian cancer, and mafosfamide, a drug that has never been in clinical trials for ovarian cancer, but had promising results on TCPS⁴⁷⁻⁴⁸. First, we found that the SKOV-3 MCTS grown in polyNIPAAm had similar responses to cisplatin and paclitaxel compared to the patient-derived OCAS (Figure 4b). To corroborate that patient-derived OCAS response was the same as what was seen in the actual clinical response, we compared our results with pathology reports of the patient samples (Table S2). Interestingly, OCAS collected from patients P1, P3 and P6, previously treated with platinum-based therapy (cisplatin or carboplatin), predicted cisplatin resistance where TCPS models did not (Figure 4b). The exception was high resistance to paclitaxel observed for P6, who had been previously treated with this drug, which potentially explains the observed resistance (Table S2). This suggests that OCAS from patients transferred directly into 3D PEG-MAL hydrogels may model drug response more accurately than TCPS. Surprisingly, mafosfamide, a drug that has never been used to treat ovarian cancer, was more effective relative to TCPS against P1 and P3 OCAS (Figure 4b). These data demonstrate that MCTS in polyNIPAAm could be a good model for the discovery of new drugs for the treatment of primary ovarian cancer.

DISCUSSION

To the best of our knowledge, a comparative analysis between MCTS formation methods has never been reported in a single study. Traditional models generate very large MCTS, generally created through the hanging drop method or suspension in non-adherent plates^{22,25,49}. These techniques often use just one large MCTS within a well of a 96-well plate, and are limited by the presence of a necrotic core⁵⁰, which affects drug response and assay readout. A system that can generate many smaller MCTS (to limit diffusion and hypoxia effects and allow encapsulation in many hydrogels), would be an improvement over current 3D drug screening models. The methods described here can generate a large number of small MCTS (between 50-150 μm) (Figure S8), which were easily recovered and transferred to other materials while maintaining high viability (Figure S11). Additionally, these MCTS provided many

features of tumors, such as oxygen and nutrient diffusion limitations (but not to the extent that a necrotic core is present).

MCTS have previously been used to study tumor biology and drug resistance. However, MCTS size, shape and even the ability to form MCTS changes with the method and conditions. Piggott et al. formed MDA-MB-231 MCTS with ultra-low adherence plates, while Iglesias et al. failed to do so with the same method³²⁻³³, likely due to differences in seeding density. Casey et al. found that SKOV-3 cells did not form MCTS with the liquid overlay technique⁵¹, while in this work we present SKOV-3 MCTS formation with all three methods (Figure 1). SkBr3s did not form MCTS when seeded onto soft agar⁵², but formed MCTS in our polyNIPAAM method. Moreover, SkBr3s, MDA-MB-231s, and MDA-MB-468s did not form MCTS when seeded in suspension⁵³, but formed large, loose MCTS in our polyNIPAAM method (Figure 1b). Our polyNIPAAM results are in good agreement with work from the Bissell group using IrECM. For example, both works observe a mass phenotype in BT474 and MCF7, a grape-like appearance in AU565, SkBr3, and MDA-MB-468.⁴³ These results stress the need to compare the behavior of cell lines across multiple MCTS formation methods.

Our data suggested that cells employ one of two possible mechanisms for MCTS formation: they either secrete their own local matrix to provide binding sites and structure, or they rely on cell-cell contacts. MCTS formed in the microwells were more likely to increase claudin 4 expression, while those formed via confined growth in the polyNIPAAM method or in suspension had higher expression of claudin 4 and fibronectin (Figure 2a). Immunofluorescence staining has shown that in free MCTS (a MCTS configuration with significant spacing between cells) fibronectin is distributed within the intercell space throughout the spheroid, while in compact MCTS fibronectin is found on the outer edge of the MCTS⁵⁴. This can be compared with our suspension and polyNIPAAM MCTS, respectively. On the other hand, knocking down claudin 4 reduces *in vitro* MCTS formation⁵⁵ because it is essential for tight junctions. Interesting, claudin 4 is expressed in the majority of ovarian cancers⁵⁶, and also in our SKOV-3 and OVCAR-3 MCTS. Additionally, breast cancer cells grown into MCTS using the overlay method, similar to our suspension method, upregulated claudin 4 and several other cell-cell adhesion genes⁵⁷. These results are largely cell-line dependent, without any correlation to cancer type.

We examined how drug response changed with MCTS formation methods. Changes in drug

resistance compared to 2D TCPS were greater in MCTS formed in polyNIPAAM or suspension than the microwells method (Figure 3b). Decreased sensitivity of cancer MCTS to drug treatment in a 3D model versus 2D screening agrees with other studies that have looked at the effect of dimensionality on drug response²²⁻²⁷. Both breast cancer cell lines tested were slightly more sensitive to sorafenib in all the three methods compared to TCPS, with the exception of AU565 MCTS grown in suspension. This may be because sorafenib works as a targeted agent rather than interfering with cell divisions, as chemotherapeutic drugs do. We believe that the drug response observed in 3D drug screening is a result of the properties of the MCTS culture method. For example, MCTS formed in polyNIPAAM were more resistant to first line therapies due to their high proliferation and more compact morphology.

Finally, we compared our polyNIPAAM MCTS to patient ovarian cancer ascites, because drug approval for ovarian cancer therapy has begun to stagnate. Only two ovarian cancer drugs have been approved by the U.S Food and Drug Administration (FDA) in the past 10 years⁵⁸. Therefore, there is a pressing need to more accurately model ovarian cancer *in vitro* to better identify effective treatments. In this study, OCAS from patient-derived samples were compared to an established ovarian cancer cell line formed in MCTS in polyNIPAAM. In most cases, the patient-derived and cancer cell line MCTS grown in polyNIPAAM responded similarly (Figure 4b). Our results suggest that the 3D methods presented here may show drug efficacy and drug resistance for an individual patient more accurately than TCPS. Interestingly, patient samples were more sensitive to mafosfamide compared to TCPS, a drug that has never been in clinical trials for ovarian cancer. This shows that selection of an MCTS method that mimics an *in vivo* environment can facilitate future drug discovery.

In ovarian cancer, the presence of ascites indicates disease progression and poor prognosis⁵⁹. The disseminated cells and OCAS in the peritoneum are those that become metastatic, and contribute to drug resistance and recurrence⁶⁰⁻⁶¹. Ascites fluid is enriched in cancer stem cells, which can form tumors *in vivo*⁶². Therefore, patient-specific drug screening of the cells isolated from ascites would be extremely beneficial to make treatment decisions that could result in better patient outcomes. The clinical application of drug screening to ascites would be relatively easy because the fluid is in great excess in many patients. However, only few research groups have performed drug screening in ascites samples^{50,63}, and they have demonstrated that ascites responses mimic cell line data. Sensitivity to carboplatin and

paclitaxel in ascites-derived cells treated *in vitro* mimicked the clinical chemosensitive or chemoresistant phenotype in each patient⁶³. Therefore, MCTS formation methods that can grow MCTS from patient derived ascites, can be useful clinical tools.

Selection of a suitable 3D MCTS model is not straightforward. Drug response in 3D MCTS models depends on the cell line and drug of interest, as well as the MCTS formation method. The most accurate 3D MCTS model would produce MCTS similar to the *in vivo* MCTS, as well as exhibit similar gene expression. The choice of MCTS formation method may also be based on whether a clonal growth method (polyNIPAAm) or an aggregation method (microwells, suspension) is desired. Finally, the time requirements of each method are different, which is a practical factor to be considered for drug screening assays.

CONCLUSIONS

We applied three different MCTS formation methods: polyNIPAAm gels, microwells, and suspension culture across several cell lines to investigate the implications of these methods on gene expression and drug response. To form MCTS with these methods, cells rely on either the production of ECM (fibronectin) or the robust expression of cell-cell contact genes (claudin 4). MCTS that were formed with the three methods were then used for drug screening in a 3D hydrogel platform. We found that drug sensitivity was dependent on MCTS formation technique. To address the need for improved patient treatments, we compared the drug response of MCTS of an ovarian cancer cell line grown in polyNIPAAm to OCAS obtained directly from patients and we demonstrated that our 3D drug testing platform is a good model for patient-derived samples.

SUPPORTING INFORMATION

Supplemental Materials and Methods: RGD Synthesis; Gene Expression Clustering and PCA

Table S1: Characterization of gene expression by RT-PCR

Table S2: Pathology report of patient samples

Figure S1: MCTS size distribution in polyNIPAAm.

Figure S2: PolyNIPAAAM MCTS size negatively correlated with TCPS doubling time

Figure S3: EGF affected MCTS size in polyNIPAAAM.

Figure S4: Many breast cancer cell lines formed MCTS in polyNIPAAAM and could be transferred to 3D PEG-MAL hydrogel

Figure S5: MCTS size increased with seeding cell density in microwells and suspension

Figure S6: MCTS reached sizes around 50-150 μm

Figure S7: MCTS became compact over time in microwells.

Figure S8: MCTS proliferated in microwells

Figure S9: Circularity was preserved in polyNIPAAAM, increased in microwells, and decreased in suspension

Figure S10: RNA-Seq on breast cancer cells revealed enrichment of surface receptor linked signal transduction

Figure S11: MCTS formed in polyNIPAAAM, microwells, and suspension could be encapsulated in PEG-MAL.

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Notes

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