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Multi-Layered, Hyaluronic Acid-Based Hydrogel Formulations Suitable for Automated 3D High Throughput Drug Screening of Cancer-Stromal Cell Co-Cultures

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2D cell culture models are simple, low cost, well suited for automated high-throughput drug screening, and have been successfully used to discover many clinically relevant anti-cancer compounds.^[1] However, due to its rigidity and lack of 3D architecture, tissue culture polystyrene poorly recapitulates *in vivo* tumor characteristics,^[2] and most candidate chemotherapy drugs identified from 2D culture screens fail clinical trials.^[3] Recently, 3D culture systems that better recapitulate the *in vivo* tumor microenvironment have become a

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

priority. In several models across tissue types, changes in gene expression, signal transduction and drug susceptibility from 3D culture better resemble *in vivo* results than does 2D culture.^[4–11] Many publications equate 3D cell culture with HTS of cancer spheroids without ECM, spheroids cultured on Matrigel™ or spheroids encapsulated in alginate.^[7,12,13] Low adhesion plates exclude cell-matrix adhesion, and instead force cells to form floating multicellular spheroids. Such systems inherently prevent the study of extracellular matrix (ECM) influence on tumor phenotype and suffer from difficult visualization of untethered clusters. Matrigel™ provides the most complex environment of ECM and growth factors,^[18] but lot-to-lot variation and low-temperature requirements for solubility complicate high-throughput dispensing. Alginate matrices offer 3D cell encapsulation, but produce non-uniform, non-transparent hydrogels that also hinder automated microscopy. Furthermore, alginates lack the cellular attachment sites that adherent cells require for survival.^[14] Similar biologically-derived hydrogel matrices, such as chitosan, offer comparable properties.^[15] Synthetic hydrogel models are available, but vary in their applicability to 3D cell culture and dispensing. Poly(ethylene glycol) (PEG)-based systems are a classic example, but the most common incarnation, utilizing UV-based photocrosslinking, can be harmful to cells.^[16] However, beyond that caveat, PEG is a relatively inert matrix which resists protein adsorption and can be functionalized with more biocompatible crosslinking chemistry, such as the commercially available Qgel or click chemistry-based hyaluronic acid (HA)-furan/PEG-maleimide gels.^[17] A clear need remains for a biocompatible, controlled, bioengineered matrix that simulates native 3D organization, and facilitates high-throughput dispensing and screening.

HA is a ubiquitous component of the ECM, and thus has broad applicability for 3D cell culture. More specifically, HA is a highly expressed ECM component in bone, the typical metastatic target tissue of prostate cancer (PCa),^[19] is associated with prostate tumor growth and lymph node metastasis,^[20,21] and is implicated in the progression of endometrial cancer (ECa).^[22] HA interacts with CD44 and hyaluronan mediated motility receptors (RHAMM/CD168) on the cell surface, triggering cell signaling pathways that drive tumor progression, including phosphatidylinositol 3 kinase (PI3K) and transforming growth factor beta receptors.^[23,24] Bacterial-produced HA polymer is well-defined and can be chemically modified to enable the formation of mechanically stable hydrogels through multiple crosslinking strategies.^[25–27] We implemented HA-based hydrogels using aqueous solutions of thiolated hyaluronan and thiol-reactive polyethylene(glycol) diacrylate (PEGDA),^[28] which provides predictable physical parameters (e.g. viscosity), defined gelation times of 20–30 minutes,^[29] remains optically clear, stable, and porous without burdensome temperature restrictions (e.g. in Matrigel™). This biocompatible thiol-acrylate chemistry also allows bottom-up modularity, enabling the covalent incorporation of ECM components (e.g. thiolated denatured collagen) to further mimic the native tumor microenvironment. This modularity enabled our orthogonal culture of both cancer and stroma in different ECM environments. Furthermore, full encapsulation of multiple cancer spheroids within a biologically-relevant ECM better replicates the *in vivo* condition (e.g. diffusion limitations, invasion potential, etc.). This HA chemistry is compatible with existing high throughput dispensing technology, allowing for rapid adoption with minimal adaptation.

We present a novel, HA-based multi-layer system for both mono- and co-culture of cancer cells validated for a 384-well plate format. Our multi-layer system enables paracrine interaction between cell types while maintaining distinct localization for facile visualization. This system provides highly reproducible cultures utilizing current high-throughput screening infrastructure. By incorporating well-characterized ECa and PCa cell lines, we tested our 3D model with a panel of chemotherapeutic compounds, focusing on drugs in clinical use and those that previously failed clinical trials.

To measure thicknesses of the multi-layer system, fluorescent beads were deposited as a landmark of layer interfaces and imaged by confocal microscopy (Figure 1a). Hydrogel layers of 1–24 μL were dispensed with alternating 1 μL bead boundary layers (Figure S1, Supporting Information). Dispensed volumes of 12 μL for the cushion layer (mean thickness: $120.8 \pm 26.7 \mu\text{m}$, $n = 8$), 14 μL for the cancer layer ($460.7 \pm 136.9 \mu\text{m}$, $n = 8$) and 5 μL for the stromal layer ($115 \pm 28.5 \mu\text{m}$, $n = 5$), were determined to be optimal for generating a consistent cushion layer thick enough to prevent cellular sensing of the rigid plate,^[31] a cancer layer thick enough to allow multiple imaging locations, and a relatively flat stromal layer that could fit within a thin focal plane. While the volumes are similar, the cancer gel layer is substantially thicker than the cushion gel layer. This is due to wicking of the cushion layer into the corners and edges upon addition into an empty well. The addition of the cancer cell layer is less affected by this wicking and therefore results in a thicker gel layer.

To measure thickness variability across manufacturing lots of hydrogel, all layers were dispensed with a benchtop high throughput dispensing system. Three plates for each of 5 lots were dispensed with bead boundaries, and imaged with confocal microscopy to calculate layer thicknesses across all 384 wells (Table S1, Supporting Information). Analysis of inter-lot layer thickness showed mean $\pm$ standard deviation of the cushion layer was $105 \pm 86 \mu\text{m}$, the cancer layer was $526 \pm 132 \mu\text{m}$ and the stromal layer was $415 \pm 118 \mu\text{m}$. The thicker than expected stromal layer is due to reduced viscosity of HA-collagen solution compared to HA alone, resulting in a larger volume dispensed via peristaltic action as compared to hand pipetting. However, this difference did not negatively impact the function of the culture systems (data not shown). There was no clear bias in layer thicknesses within individual plates indicating that there was no systemic error in the dispensing of hydrogel layers (data not shown). Cancer layers were found between 200 μm and 425 μm from the plate bottom for over 90% of wells, providing reliable parameters for automated imaging.

Cancer cell survival in HA hydrogels was measured by imaging three z-slices per well. There were no clear differences in cancer cell spheroid morphology or size at different locations within the gel (Figure S2, Supporting Information). In 3D culture, it is difficult to accurately count individual cells stained with live/dead stain. Instead, two ratiometric methods for quantitating viability of cells grown in 3D spheroids, metabolic survival index (MetaSI) and nuclear survival index (NucSI), were employed as described in the Experimental Section. To understand the bias represented by the survival indices, a comparison was made of the cross-sectional area of both C4-2B and Ishikawa cells after 1 day of growth (Figure S3, Supporting Information). Median stained areas for calcein AM, Hoechst 33342 and ethidium dimer-2 were 374, 332, and 139 (C4-2B) or 317, 337, and 111

(Ishikawa), respectively. The similarity of the calcein and Hoechst areas indicate that each metric represents comparable bias. To ensure accurate comparisons between culture models, MetaSI and NucSI were calculated for all culture conditions, including 2D and 2.5D (NucSI reported in Supporting Information).

Hydrogels were tested for optimal growth and morphology of cancer and stromal cells. Single cell seeding was used as a direct adaptation of typical high throughput dispensing methodology. It is possible to pre-form multicellular spheroids prior to encapsulation, providing a larger ($>100\ \mu\text{m}$) population of spheroids for testing.^[32] However, use of small diameter tubing necessary for low volume culture could become clogged when used to dispense larger spheroids. Both Ishikawa and C4-2B formed viable multicellular spheroids in 3D mono-culture in HA hydrogels (see Figure 2). Both ESS-1 and HS27a had high viability and a spreading morphology in 2.5D culture only on HA-collagen hydrogels (Figure S4, Supporting Information). Thus, the multilayer culture system consisted of 3 layers: an acellular cushion layer to prevent cancer cell interaction with the plate bottom, a second layer for encapsulation of cancer cells, and a collagen-rich layer upon which adherent stromal cells were seeded (2.5D culture). This enabled a multi-layer hydrogel co-culture system with cell types grown in distinct locations (Figure 1b, left), facilitating imaging and analysis of individual cell types (Figure 1b, right), while providing a matrix suitable for stromal cell adherence and growth. Next, cell density was optimized for highest viability and spheroid size in mono-culture. Optimal densities for both cancer cell types were 5,000 cells per 14 μL hydrogel per well (Figure S5, Supporting Information). The optimal number of stromal cells to avoid confluence before the end of culture was 2,500 cells per well (data not shown).

Ishikawa cells showed high ($>90\%$) MetaSI at all days and grew to a maximum median size of $19.4\ \mu\text{m}$ at day 7 (Figure 2a). Similarly, C4-2B cells had high survival and a maximal median size of $16.1\ \mu\text{m}$ at day 7, with little change in size after day 3 (Figure 2b). Spheroids comprised an average of 36 cells in $25\ \mu\text{m}$ diameter Ishikawa clusters ($n=4$) and 19 cells in $18\text{--}20\ \mu\text{m}$ diameter C4-2B clusters ($n=3$). Cancer cell clusters contained multiple layers of cells, which may result in a diffusion effect of drug treatment, more closely resembling the challenges of treating human tumor masses. Our cluster diameters parallel previously reported data for PCa cells in similar hyaluronate systems used in cytotoxicity tests.^[33] Analysis of ESS-1 (Figure 2c) and HS27a (Figure 2d) 2.5D mono-cultures showed the expected fibroblast morphology and high viability up to day 3, with a slight decrease in viability at day 5 from monolayer confluence. Blue staining at the periphery of the stromal cell images was due to the meniscus of the gel layer. Near the edges of the image, the confocal slice passes through the bottom of the cell, resulting in detectable signal from the nuclear stain and not from the cytoplasmic stain. Adjusting the focus shows that these cells are viable in a similar ratio to cells at the bottom of the meniscus (data not shown).

To facilitate growth of multiple cell types in a single well, media compatibility was assessed. As both cancer and stromal cell media were suitable for survival ($>90\%$ MetaSI) of both cell types, cancer cell media was chosen for all co-culture models (Figure S6, Supporting Information). Ishikawa or C4-2B cells were encapsulated in 3D HA and incubated for 2 days, then seeded with either ESS-1 or HS27a cells, respectively. After 5 days of co-culture,

Ishikawa and C4-2B showed typical spheroid formation, whereas ESS-1 and HS27a showed a distinct morphological change, forming cell-cell aggregates rather than more typical fibroblast morphology observed in mono-culture (Figure 2e). Stromal cell clusters in co-culture did not reach confluence (data not shown). Median diameters for Ishikawa and C4-2B spheroids in co-culture were 25.6 μm and 29.1 μm , respectively (Figure 2f) a statistically significant ($p < 0.001$) increase in spheroid size compared to mono-culture. Metabolic survival indices were high ($>90\%$) for all cells (Figure 2g). Increased cancer spheroid size and stromal cell aggregation indicate that this system facilitates paracrine communication. Incorporation of basic fibroblast growth factor^[34] and vascular endothelial growth factor^[35] into similar hydrogel matrices results in their slow release, demonstrating the capacity for small protein diffusion. Furthermore, we have modeled cancer-stroma crosstalk using HA hydrogel particles decorated with heparin as point sources to release heparin-binding epidermal growth factor. In that system, LNCaP spheroids were significantly larger after co-culture with synthetic stromal mimics than when cultured alone.^[36]

To automate the robust assembly of this cell culture system, mono- and co-culture systems were prepared using a high throughput benchtop dispensing system. After 7 days of growth (5 days for stromal cells in co-culture), cancer spheroids were stained for viability and imaged with an automated high throughput confocal microscope. Ishikawa spheroids grew to median diameter of 23.9 μm in mono-culture and 38.6 μm in co-culture with ESS-1. C4-2B spheroids grew to median diameters of 28.7 μm in mono-culture and 37.7 μm in co-culture with HS27a (Figure 2h). These values are significantly higher ($p < 0.001$) than the corresponding values from hand-pipetted cultures above. In addition, co-culture increased median spheroid diameter by 61% in Ishikawa and 31% in C4-2B cells. Assuming that spheroids are spherical, this corresponds with a volumetric increase of 421% for Ishikawa and 226% for C4-2B. Analysis of the MetaSI showed a slight, but significant ($p < 0.001$) reduction in both mono- and co-culture for Ishikawa (88.6% and 80.1%, respectively) and C4-2B (93.5% and 84%, respectively) from automated dispensing compared to manual dispensing (Figure 2i). NucSI for all mono- and co-cultures can be found in Figure S7, Supporting Information.

Phenotypic markers for PCa and ECa cells and associated stromal counterparts were validated in 2D for each cell type (Figure 3) and employed for staining cells within HA hydrogels. In 3D mono-culture, Ishikawa cells expressed MUC1 and EIG121 (Figure 3a), and C4-2B cells expressed PSA and EGFR (Figure 3b), as expected. ESS-1 cells expressed CD10 and HDAC2 (Figure 3c) when cultured in 2.5D on HA-collagen. HS27a expressed the ubiquitous mesenchymal cell markers, CD105 and vimentin (Figure 3d). Cells were co-cultured to identify changes in phenotypic marker expression. Both Ishikawa and C4-2B retained expression patterns of phenotypic markers, with no significant changes due to co-culture (Figure 3a and 3b). ESS-1 and HS27a cells when co-cultured, however, demonstrated a distinct morphological change, forming small aggregates in accordance with live/dead staining. ESS-1 expression of HDAC2 was both cytoplasmic and nuclear in Ishikawa co-cultures, instead of the primarily nuclear localization observed in mono-culture (Figure 3c). Cytoplasmic sequestration of HDAC2 is associated with terminal differentiation and inhibition with senescence.^[37,38] It is possible that co-culture of ESS-1 cells with Ishikawa

cells may trigger differentiation and senescence of ESS-1 cells. This is in agreement with the observation that the stroma tends to be lost in in endometrial tumor microenvironment.^[39] In contrast, no clear change in staining of phenotypic markers in HS27a cells was seen (Figure 3d). The poor adherence of stromal cells in co-culture with cancer cells may be a result of increased perlecan expression, which is associated with poor cellular adhesion.^[40,41] In vivo, this would be seen as a reactive stromal condensation, reported to be anti-inflammatory,^[42] and has been reported to be part of both reaction to cancer and decidualization reactions in the uterus,^[43] hence a common stromal phenomenon.

To demonstrate culture system compatibility with existing high-throughput robotics, and assess differences in cell sensitivities in 3D culture compared to 2D culture, cancer cells were treated with a panel of 232 unique chemotherapeutic compounds for 3 days at 10 μ M. Drugs that induced survival indices statistically significantly below the DMSO control were considered cytotoxic (Table S2 and S3, Supporting Information). Drugs were categorized by mechanism of action, and a comparison of drug cytotoxicity was made between 2D and 3D culture. All drug categories that contained at least one drug with differential cytotoxicity between 2D and 3D culture are shown in Table 1. Ishikawa cells in 3D culture were more sensitive to two drugs and more resistant to 25 drugs, and tended to be more resistant to signaling pathway inhibitors. C4-2B cells in 3D culture were more sensitive to 30 drugs and more resistant to 5 drugs, and tended to be more sensitive to cell cycle and DNA replication and repair inhibitors. In each case, differing resistance from 3D culture was highly drug- and cell line-dependent. Given the reported^[33] diffusion limitation of spheroids, increased drug resistance would be expected. Tendency toward increased susceptibility in 3D HA C4-2B culture contrasted with increased resistance in 3D HA Ishikawa culture, implying that cell-specific molecular changes are more important than morphological changes associated with 3D culture. In a 3D ovarian tumor model, cells in 3D reconstituted basement membrane and cells in 2D upregulated a number of cell survival pathways and therefore were resistant to PI3K inhibitors. Furthermore, the authors attribute the resistance to cell-matrix interactions.^[44] In HA culture, there are cell-matrix interactions that are not activated (*e.g.* integrin binding), the lack of which may explain the increased resistance in 2D in some cases. In other studies, LNCaP cells in 3D HA culture showed increased doxycycline resistance, associated with higher expression of multidrug-resistance associated efflux proteins MRP and LRP1,^[45] further corroborating the importance of molecular changes. In addition, comparison of JIMT1 breast cancer cells grown in 2D, on low adhesion plates, 2.5D culture on Matrigel™ or mouse xenograft showed Matrigel™ cultures most closely resembled the xenograft. Furthermore, the formation of spheroids from growth on low-adhesion plates did not resemble the xenograft.^[7] In addition, control of the concentrations and combinations of ECM components is important for understanding cellular behavior, as shown for cell fate decisions of mouse embryonic stem cells.^[46] These data indicate that cell-ECM interactions are required for maintaining an *in vivo*-like microenvironment and cellular phenotype.

To assess accuracy of these 3D tumor models, clinical drug efficacy from published human clinical trials for PCa or ECa were compared with our *in vitro* cytotoxicity data. An extensive literature search was performed to identify compounds that are in clinical use as an accepted therapy (passed clinical trials), or were shown to be ineffective for either ECa

(Table S4, Supporting Information) or PCa (Table S5, Supporting Information). Clinical trial results were pooled and estimated clinical efficacy was calculated by the number of patients with a tumor response (partial or complete) or a PSA response (>50% reduction, PCa only) divided by the total number of patients tested.

Cytotoxicity in 3D HA-based mono- and co-culture systems was compared with 2D and 3D-alginate models (Figure 4a) for drug concentrations from 0–10 μ M. Representative dose response curves are presented in Figure 4b and 4c. Dose response curves for all tested compounds and both survival indexes can be found in Figures S8–S11, Supporting Information. Some treatments lacked a sinusoidal curve and therefore IC₅₀ values could not be calculated. For simple comparison between culture models, the minimum effective concentration (MEC) of each drug was calculated. The MEC is the lowest concentration of drug that resulted in a statistically significant reduction in survival index (ANOVA with Dunnett multiple comparisons test p-value < 0.05 vs. DMSO). This is reported for all compounds and culture models for C4-2B (Table 2) and Ishikawa cells (Table 3). NucSI data can be found in Tables S6 and S7, Supporting Information.

In general, 3D HA models tended to increase the resistance of cells to compounds when compared to 2D culture. When treated with 10 μ M paclitaxel, cells tended to first die at the spheroid periphery (Figure 4d) indicating increased resistance from diffusion limitation. Co-culture with stromal cells also tended to increase cancer cell drug resistance compared with 3D mono-culture. In the endometrial cancer model, co-culture with stromal cells elicited insensitivity to all drugs except docetaxel. In the case of clinically used ECa compounds, comparison of *in vitro* data to clinical data showed that 3D HA mono-culture performed comparably to 2D cell culture for the majority of compounds, indicating that they predict efficacy of compounds with proven clinical relevance. Interestingly, sunitinib, a promising compound currently in clinical trials, was ineffective in the HA mono-culture model. For PCa, 3D HA culture performed similarly to 2D culture for all clinically used drugs except mitoxantrone, reported to be of modest efficacy as a single agent.^[47] Notably, the 3D-alginate model showed very high susceptibility of ECa cancer cells to all compounds, but performed similarly to 3D HA cultures of PCa. Rapid gelation kinetics of the 3D-alginate culture and significant heterogeneity of the hydrogel layer lead to inconsistent cytotoxicity data (see Figures S8–S11 and S12, Supporting Information). These technical difficulties could be, in part, responsible for the poor performance of this culture model, but nonetheless highlights the difficulty in using alginate for high throughput screening.

The poor performance of mitoxantrone on PCa indicates that the 3D HA culture system is a more stringent test for drug efficacy than the 2D model. Indeed, mitoxantrone, a drug that failed clinical trials in ECa, was ineffective in 3D HA culture. However, lapatinib, which also failed clinical trials was effective at high concentration in the 3D HA mono-culture. In addition, the HA culture models showed no cytotoxicity for failed PCa therapeutics dasatinib and sunitinib. Each of these drugs was cytotoxic in 2D culture. These examples underscore the increased accuracy and predictive capability of the 3D HA culture platform over 2D culture. By identifying drugs unlikely to translate from pre-clinical to clinical trials, the costs associated with advanced testing of these drugs could be reduced.

While the 3D HA culture system was more accurate for failed drugs, several drugs showed no cytotoxicity in any culture condition. This included carboplatin, ifosfamide, temsirolimus and combination of ifosfamide + cisplatin for both C4-2B and Ishikawa; cisplatin, and estramustine for Ishikawa; and lapatinib for C4-2B (data not shown). This is likely due to genetic and proteomic variation within the cell lines eliciting resistance to certain drug mechanisms. Indeed, it has been reported that the Ishikawa cell line is resistant to cisplatin treatment.^[48] These variations can also have the opposite effect: in the case of lapatinib, the Ishikawa cells may represent the 3.3% of patients to which lapatinib was effective. Comparison of *in vitro* cytotoxicity to overall clinical response assumes derived cell lines are representative of patient populations. Flexibility of our 3D culture system allows incorporation of additional cell lines to better represent characteristics of the patient population. This will be necessary for development of an assay system which has broader applicability to multiple subtypes of cancer. In addition, similar hydrogel systems have been used to culture patient derived xenograft (PDX) PCa lines,^[32] which have not adapted to growth in 2D culture. Indeed, PDX-maintained lines more closely resemble patient tumors than 2D culture derived cell lines.^[49]

In summary, we developed a highly reproducible, multilayer 3D culture model that is compatible with existing automated high-throughput dispensing, imaging and analysis methodologies. These culture models recapitulate cytotoxicity of compounds used clinically for both PCa and ECa. In addition, these models performed exceptionally well in identifying drugs that failed clinical trials. The robustness of this system supports incorporation of additional tumor cell lines, PDX lines, other cell types, growth factors and ECM components necessary to mimic the *in vivo* tumor microenvironment. This novel, high-throughput-compatible HA hydrogel system is an effective model for high content analysis of multiple cancer types in mono- or co-culture.

Experimental Section

Antibodies

Primary antibodies used for immunofluorescent staining were MUC1 (clone 214D4, Cat. No. 05-652, EMD Millipore), EIG121 (Cat. No. ab156275, Abcam), EGFR (clone H11, Cat. No. MS-316-P1, NeoMarkers), PSA (Cat. No. NB200-135, Novus Biologicals), CD105 (clone 209701, Cat. No. MAB1320, R&D Systems), vimentin (clone EPR3776, Cat. No. ab92547, Abcam), CD10 (clone F-4, Cat. No. sc-46656, Santa Cruz Biotechnology), and HDAC2 (Cat. No. sc-7899, Santa Cruz Biotechnology). Antibodies without an indicated clone ID are rabbit polyclonal. Secondary antibodies included Alexa Fluor[®] goat anti-mouse IgG 488 (Cat. No. A11029), Alexa Fluor[®] goat anti-rat IgG 488 (Cat. No. A11006), and Alexa Fluor[®] goat anti-rabbit IgG 568 (Cat. No. A11011), all obtained from Life Technologies. Antibodies were used at the following combinations and concentrations: EGFR (1:200) and goat-anti-mouse-AF488 (1:200), PSA (1:100) and goat-anti-rabbit-AF568 (1:100), CD105 (1:50) and goat-anti-rat-AF488 (1:200), vimentin (1:200) and goat-anti-rabbit-AF568 (1:100), MUC1 (1:100) and goat-anti-mouse-AF488 (1:200), EIG121 (1:50) and goat-anti-rabbit-AF568 (1:100), CD10 (1:100) and goat-anti-mouse-AF488 (1:200), HDAC2 (1:50) and goat-anti-rabbit-AF568 (1:100).

Cell culture

Established cell lines with known genomic characterization were used. For ECa, Ishikawa cells, isolated from a well differentiated stage 2 endometrial adenocarcinoma^[50] were matched with the endometrial stromal sarcoma cells line ESS-1, derived from a high-grade endometrial stromal sarcoma.^[51] For PCa, C4-2B cells, a castration-resistant bone metastatic cell line derived from LNCaP cells^[52] were coupled with the bone marrow stromal cell line HS27a, immortalized by expression of human papilloma virus E6/E7 genes.^[53] The C4-2B and ESS-1 cell lines were kindly provided by MD Anderson Cancer Center and Russell Broaddus (MD Anderson Cancer Center, Houston, TX), respectively. HS-27a and Ishikawa cell lines were purchased from American Type Culture Collection and Sigma-Aldrich, respectively. C4-2B cells were maintained in RPMI 1640 (Life Technologies) supplemented with heat-inactivated FBS (10%, v/v) and L-Glutamine (2 mM) (Life Technologies). Ishikawa cells were maintained in DMEM/F12 (Life Technologies) supplemented with heat-inactivated FBS (10%, v/v). ESS-1 cells were grown in RPMI 1640 (Life Technologies) supplemented with heat-inactivated FBS (20%, v/v). HS-27a cells were maintained in low glucose DMEM (Life Technologies) supplemented with heat-inactivated FBS (10%, v/v). All cultures were maintained at 37°C in a humidified atmosphere of air / CO₂ (95:5, v/v).

Hydrogel production and characterization

Thiolated hyaluronan (Glycosil[®], carboxymethylated, thiolated hyaluronic acid), thiolated porcine gelatin (Gelin-S[®]), and polyethylene glycol diacrylate (PEGDA) MW 3400 (Extralink[®]) were manufactured at BioTime Inc. (Alameda, CA) as previously described^[28]. Five independent lots of thiolated hyaluronan and porcine gelatin were synthesized, and characterized for dissolution time, pH, appearance, sterility, endotoxin levels, and thiolation levels. All five lots were within quality limits defined by BioTime for these specifications. The shear elastic modulus of HyStem[®] (Glycosil[®] + Extralink[®]) is 340 Pa and HyStem-C[®] (Glycosil[®] + + Gelin-S[®] + Extralink[®]) is 83 Pa.^[54] These hydrogels have a water content of approximately 98% and undergo limited swelling: approximately 20% over 50 days.^[55]

High throughput dispensing

Glycosil[®], Gelin-S[®] and Extralink[®] were reconstituted (1%, w/v) in degassed water for use in HA hydrogels. Two hydrogel compositions were used: for the first and second layers (see below), a 4:1 volumetric ratio of Glycosil[®]:Extralink[®] (HA-PEGDA) was used; for the third layer (see below), a 2:2:1 volumetric ratio of Glycosil[®]:Gelin-S[®]:Extralink[®] (HA-collagen) was prepared. Since the addition of Extralink[®] drives gelation, it is added just prior to robotic dispensing. Dispensing was performed with a Multidrop[™] Combi Reagent Dispenser (Cat # 5840300, ThermoFisher Scientific, Waltham, MA, USA) with a small tube dispensing cassette on the fast dispensing setting into Aurora 384 well plates (Cat # 1052, Brooks Automation, Inc., Chelmsford, MA, USA). After dispensing each layer, tubing was immediately washed with PBS (10 mL). For the multi-layer HA-based culture system, three distinct layers were dispensed. The first (acellular cushion layer) consisted of 12 µL HA-PEGDA per well. Plates were spun at 160 ×g for 2 minutes in a plate centrifuge (Allegra 6 with GH-3.8 rotor, Beckman-Coulter, Brea, CA, USA). Five min after gelation, the second

(cancer layer), consisting of 14 μL HA-PEGDA combined with 5,000 cancer cells (C4-2B or Ishikawa) per well, was dispensed. Five min after gelation of the second layer, the third (stromal layer), consisting of 5 μL HA-collagen without cells, was dispensed. Total volume of hydrogel and number of cells per plate are as follows: the cushion layer was 4 mL HA + 1 mL PEGDA per 384 well plate, the cancer layer was 2.14 million cells in 5 mL HA + 1.25 mL PEGDA per plate, and the stromal layer was 0.94 mL HA, 0.94 mL thiolated gelatin and 0.47 mL PEGDA per plate. After gelation of the final layer, growth media (50 μL) was added to each well. After 2 days of incubation, media was removed and replaced with fresh growth media (50 μL). For co-cultures, media replacement contained 2,500 stromal cells (HS27a or ESS-1). 2D mono-culture was performed by mixing cancer cells into cancer cell media (300 cells per 50 μL media). Of this mixture, 50 μL was dispensed per well. Seeding of cells for 3D-alginate culture was performed by pre-dispensing CaCl_2 (1 μL of 5%, w/v) in PBS. Then, 5,000 C4-2B or Ishikawa cells per 26 μL 3% (w/v) alginate in PBS (typically, 2.11 million cells in 11 mL per plate) were pre-mixed then dispensed into wells. After 30 min, growth media (50 μL) was added to each well.

Layer thickness assessment

Layer thickness was determined by the addition of fluorescent bead boundaries between hydrogel layers. A 1:100 dilution of 6 μm Fluoresbrite Carboxy YO beads (Cat# 19395, Polysciences, Inc., Warrington, PA, USA) was made into reconstituted Glycosil®. A 1:1000 dilution of a 20 mM stock of 9-anthracenylmethyl acrylate in DMSO (Cat # 577111, Sigma-Aldrich, St. Louis, MO, USA) was added to all layers. Bead boundary layers were added to the bottom of the well as well as after dispensing of each of the cushion, cancer and stromal layers as described above. After dispensing each bead boundary layer, plates were spun at $160 \times g$ for 2 min in a plate centrifuge. Subsequent dispensing was performed 5 min after gelation of the previous layer. Imaging of the hydrogel layers was performed with a Nikon A1-Rsi confocal microscope (Nikon Corporation, Tokyo, Japan) with a $10\times$ objective and $10\times$ ocular lens for $100\times$ final magnification. Fields were imaged with 405 nm and 568 nm lasers and the appropriate emission filters to detect acrylated anthracene and fluorescent beads, respectively. Each well was imaged with a Z-stack from 2200 μm to 3700 μm with 25 μm slices, encompassing all hydrogel layers. All images were extracted and processed using CellProfiler.^[56] Average anthracene fluorescence was determined using the MeasureImageIntensity module and the total number of beads per image was calculated using the IdentifyPrimaryObjects module. Per-image bead count data was ordered by Z-slice and grouped by well. Identification of the local maxima of fluorescent beads within each well group was used to determine the Z-slice corresponding with each new hydrogel layer. The number of images between local maxima multiplied by the Z-slice thickness determined the layer thickness. Anthracene fluorescence by Z-slice was used to verify total thicknesses calculated. A total of three 384-well plates each for 5 lots of hydrogel was dispensed and imaged.

Live cell/dead cell/nuclei confocal microscopy

After cell growth, culture media was replaced with calcein AM (4 μM , Cat# C1430, Life Technologies, Carlsbad, CA, USA) to stain live cells, ethidium homodimer-1 (4 μM , EthD, Cat# E1169, Life Technologies, Carlsbad, CA, USA) to stain dead cells and bisbenzimidazole

trihydrochloride (4 μM , Hoechst 33342, Cat# B2261, Sigma-Aldrich, St. Louis, MO, USA) to stain all nuclei, in PBS (Cat# 17-512F, Lonza Group, Basel, Switzerland). After 1 hr of incubation, cells were imaged by confocal microscopy. For low-throughput characterization of cell survival and cancer spheroid formation a Nikon A1-Rsi confocal with a 10 $\times$ objective was used. For high-throughput imaging of cancer spheroids, an IN Cell 6000 Analyzer (Cat# 29-0433-23, GE Healthcare, Chalfont, Buckinghamshire, UK) with a 10 $\times$ objective was used. Stromal cells were imaged at the base of the meniscus of the stromal cell hydrogel layer in triplicate wells. 3D cancer spheroids were imaged at 3 Z-locations 75 μm (50 μm for high-throughput) apart in the center of triplicate wells. Cancer cell mono-cultures in 2D were imaged at the cell monolayer in the center of triplicate wells.

Image analysis and survival assessment

Single-channel live/dead/nuclei confocal images were analyzed with CellProfiler using the IdentifyPrimaryObjects and MeasureObjectSizeShape modules. Cancer spheroid size was calculated by multiplying the maximum diameter in pixels of each CellProfiler-detected object in the calcein AM channel by the microns per pixel of the confocal microscope and objective to get diameter in microns. Diameters were plotted as Tukey box plots using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Assessment of cell survival involved calculation of total area in each channel by multiplying the number of identified objects by the average area of objects in pixels. Two survival indexes were then calculated. The first is the metabolic survival index (MetaSI) which compares the live cell staining to dead cell staining, defined as:

$$\text{Metabolic survival index} = \frac{\text{Total calcein area}}{\text{Total calcein area} + \text{total EthD area}} \times 100$$

The second survival index, the nuclear survival index (NucSI), compares total nuclei with dead cell staining, defined as:

$$\text{Nuclear survival index} = \frac{\text{Total Hoechst33342 area}}{\text{Total Hoechst33342 area} + \text{total EthD area}} \times 100$$

Immunofluorescence

Cells in 2D culture were immunostained with antibodies against cellular markers using conventional methods. Briefly, cells were fixed for 10 min with paraformaldehyde (4%, w/v, PFA) solution in phosphate buffer, and then rinsed with PBS. Cells were permeabilized with Triton X-100 (0.2%, v/v) in PBS for 5 min at 25 $^{\circ}\text{C}$, then blocked with a solution of Triton X-100 (0.2%, v/v) and goat serum (3%, w/v) in PBS for 15 min, aspirated, incubated with primary antibody in blocking solution for 30 min, rinsed 3 $\times$ 5 min with PBS, incubated with secondary antibody in blocking solution for 30 min, rinsed 3 $\times$ 5 min with PBS, and counterstained with DAPI (3 $\mu\text{g}/\text{mL}$) and Alexa Fluor[®] 647 phalloidin (5 units mL^{-1}) (Life Technologies) according to manufacturer's directions. Samples from 2D cultures were sealed with ProLong Gold (Life Technologies) according to manufacturer's directions, and imaged on a Nikon A1-Rsi confocal microscope using appropriate laser lines. Cells in 3D

culture were immunostained by the same general methods, but with these slight modifications: fixation by either PFA or 1:1 methanol:acetone solution, Triton X-100 concentration increased to 0.3% (v/v), blocking for 1 hr, primary and secondary antibody incubation for 2 hrs at 37 °C, and gentle rotation on an x-y rotating table during staining steps.

Drug treatments

Both 2D and 3D cancer cell mono-cultures were tested with a panel of 232 unique chemotherapeutic compounds. These drugs included 114 compounds from the NCI Approved Oncology Drugs set (National Cancer Institute, Bethesda, MD, USA) as well as an additional 118 unique compounds culled from the University of Texas Health Science Center custom clinical oncology drug set, both generously provided by Dr. Clifford Stephan (TAMHSC, Houston, TX, USA). The full list of compounds can be found in tables S2 and S3, Supporting Information. After 1 day of growth for 2D or 4 days of growth for 3D mono-culture, each drug (50 nL of 10 mM stock solutions in DMSO) were added to the media (50 μ L) in each well using a Tecan Freedom EVO liquid handling robot (Tecan Group Ltd., Männedorf, Switzerland) for a final concentration of 10 μ M in each case. One well was treated per drug in quadruplicate plates. An additional 16 wells were treated with staurosporin (10 μ M) or doxorubicin (10 μ M) as positive killing controls and 106 wells with DMSO (0.1%, v/v) alone as negative killing controls. After 3 days of drug treatment cells were subjected to live/dead/nuclei staining, high throughput image analysis and survival assessment as described above.

The different culture models were tested in dose response studies with a panel of single or combination treatments with clinical relevance for PCa or ECa (Table S4 and S5, Supporting Information). Clinical trials were culled from the Embase biomedical database. Trials were included if the drugs listed were the only chemotherapeutics, and the authors reported patient tumor response and/or prostate specific antigen response (PSA, PCa only). As the C42B cell line represents a metastatic stage disease, trials of localized PCa were not included in the analysis. The total number of reported patients with a tumor response (complete + partial) or a PSA reduction of >50% in all trials of a specific drug were pooled and divided by the total number of assessable patients in each case, resulting in an estimate of clinical efficacy. No compensation was made for variations in patient population (e.g. previous chemotherapy, tumor stage, *etc.*) or in administered drug concentration or schedule.

All dose response drugs were obtained from the NCI open chemical repository. The 2D and 3D culture models described above were treated with a range of 1 μ M to 10 μ M drug concentration in duplicate wells in duplicate plates. An additional 8 wells per plate were treated with an equal volume of DMSO as a negative cytotoxicity control. After 3 days of drug treatment cells were subjected to live/dead/nuclei staining, high throughput image analysis and survival assessment as described above. The minimal effective concentrations (MEC) were defined as the lowest concentration of drug resulting in significant cytotoxicity compared to DMSO control.

Statistical analyses

All statistical calculations were performed with GraphPad InStat3 (GraphPad Software, Inc., La Jolla, CA, USA). Spheroid diameters were compared using Mann-Whitney two-tailed analyses. Statistical analysis of cytotoxicity and minimum effective concentration was performed by comparing metabolic or nuclear survival indices with a one-way analysis of variance with Dunnett multiple comparisons test against the DMSO control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Multi-layer co-culture system and layer characterization

a: Illustration of bead boundary method for hydrogel layer thickness assessment (left) and confocal 3D reconstruction (right). Fluorescent beads (red) were encapsulated in boundary layers between HA hydrogel layers (blue) in order to measure layer thicknesses. **b:** Illustration of multi-layer 3D co-culture system (left) and confocal 3D reconstruction (right). A representative, manually pipetted three layer co-culture system of C4-2B (7 days growth) and HS27a (5 days growth) cells was stained with calcein AM (green), EthD (red) and Hoechst (blue). Scale bar 50 μm .

Figure 2. Cancer and stromal cells survive and grow in monoculture

Confocal images represented as calcein AM (green), ethidium homodimer-2 (red) and Hoechst 33342 (blue) stains with magnified inset, MetaSI represented as mean $\pm$ standard deviation and live cell cluster diameter represented as Tukey box plots. **a:** Ishikawa cells in 3D HA mono-culture. **b:** C4-2B cells in 3D HA mono-culture. **c:** ESS-1 cells in 2.5D mono-culture on HA-collagen. **d:** HS27a cells in 2.5D mono-culture on HA-collagen. **e:** Manually dispensed cells in co-culture, Ishikawa with ESS-1 and C4-2B with HS27a. **f:** Quantitation of cancer cell cluster diameter of cells from **e**. **g:** MetaSI of cells from **e**. **h:** Quantitation of cancer cell cluster diameters from high throughput dispensing in mono- or co-culture. **i:** MetaSI of cancer cell clusters from high throughput dispensing in mono- or co-culture. Full image scale bars represent 50 μ m, magnified inset scale bars represent 25 μ m. Two-tailed Mann-Whitney p value < 0.001 (***) compared to day 1 (**a**, **b**) and mono- vs co-culture (**h**).

a

Ishikawa

b**C4-2B**

Figure 3. Cells maintain phenotypic markers in HA-based mono- and co-culture
a: Ishikawa cells express MUC1 (green) and EIG121 (red) in all culture conditions. **b:** C4-2B cells express EGFR (green) and PSA (red) in all culture conditions. **c:** ESS-1 cells express CD10 (green), and HDAC2 (red) in all culture conditions. HDAC2 expression is nuclear in mono-culture, but nuclear and cytoplasmic in co-culture. **d:** HS27a cells express CD105 (bottom) and vimentin (top) in all culture conditions. Nuclei shown in blue. Scale bar 50 μ m.

Figure 4. Dose response of clinically relevant compounds for testing in culture models

a: Representation of each culture model tested with clinically relevant drugs. Cells were grown on tissue culture plastic (2D), encapsulated in a single alginate layer (3D-alginate; blue), in mono-culture (HA mono-culture) or in co-culture with corresponding stromal cells (HA co-culture) in HA multi-layer system (cushion layer tan, cancer layer green, stromal layer red). **b,c:** Typical dose response curves for cancer cell cytotoxicity. **d:** Calcein AM (green), EthD (red) and Hoechst (nuclei) staining of cells treated with 10 μ M paclitaxel showing outside-in cellular death. Scale bar 50 μ m.

Table 1
Drugs with differential cytotoxicity in 2D vs 3D, organized by mechanism of action

Cells in 2D or 3D monoculture were treated with 10 μ M drug for 3 days. Survival indexes statistically different from DMSO according to ANOVA with Dunnett post-test considered cytotoxic. Comparison of 2D and 3D cytotoxicity showed several categories of drugs with differential kill.

Ishikawa cells		
2D more resistant	Mechanism	Compound
	DNA damage agent	Valrubicin
	PI3K pathway inhibitor	GDC 0941
3D more resistant	Mechanism	Compound
	Alkylating agent	Mechlorethamine HCl
	Aurora kinase inhibitor	ZM447439
	Cox-2 inhibitor	Celecoxib
	DNA replication inhibitor	Hydroxyurea
		Mitoxantrone
	EGFR family inhibitor	Lapatinib
	GSK-3 inhibitor	SB 216763
	HSP inhibitor	Geldanamycin
		NVP AU922
	IR inhibitor	BMS-536924
	mTOR inhibitor	Rapamycin
		Temsirolimus
	PI3K pathway inhibitor	MK-2206
	PKC pathway inhibitor	PKC412
		U 73122
	Potassium channel opener	BMS 204352
	ROCK inhibitor	GSK 269962A
	ROS generator	Elesclomol
	SRC inhibitor	Dasatinib
		SU 6656
	VEGFR and PDGFR family inhibitor	CHIR 258
		Sunitinib
		Vandetanib
	Xanthine oxidase inhibitor	Allopurinol
C4-2B cells		
2D more resistant	Mechanism	Compound
	ALK inhibitor	NVP TAE684
	Alkylating agent	Pipobroman
	Autophagy inducer	STF-62247
	Bcl2 Inhibitor	ABT-263

	Cell cycle inhibitor	Roscovitine
	Cox-2 inhibitor	Celecoxib
	DNA damage agent	Bleomycin sulfate
		Teniposide
		Valrubicin
	DNA repair inhibitor	Compound 401
	DNA replication inhibitor	Clofarabine
		Floxuridine
		Fluorouracil
		Gemcitabine HCl
		Hydroxyurea
	EGFR family inhibitor	Canertinib
		Gefitinib
	ER inhibitor	Raloxifene
	IR inhibitor	BMS-536924
	MAPK pathway inhibitor	PD 169316
	MEK inhibitor	CI 1040
		RDEA119
	PI3K pathway inhibitor	GDC 0941
		MK-2206
		PI-103
		TGX 221
		ZSTK474
	VEGFR and PDGFR family inhibitor	Sunitinib
		Vandetanib
3D more resistant	Mechanism	Compound
	Bcl2 Inhibitor	HA14-1
	PKC pathway inhibitor	U 73122
	Potassium channel opener	BMS 204352
	ROS generator	Elesclomol
	SRC inhibitor	SU 6656

Table 2
Minimum effective concentrations (MEC) of clinically relevant compounds in prostate cancer culture models

Compounds used were in clinical use (green) or failed human clinical trials for efficacy (red). Patient tumor and PSA response rate calculated from pooled clinical trial data. MEC calculated as the lowest concentration of drug that resulted in a statistically significant reduction in survival index compared to DMSO control. Minimum effective concentration is expressed in molar concentration and color coded from most sensitive (1E-12 M, red) to least sensitive (1E-5 M, cream). Compounds which did not result in significant decrease in survival index denoted as NS. The ratio of culture models predicting efficacy or failure of drug treatments are listed at the bottom.

Prostate cancer	Overall tumor response	Overall PSA response	Metabolic survival index			
			2D	3D-alginate	3D HA mono-culture	3D HA co-culture
Cabazitaxel	13.30%	39.20%	1.00E-12	1.00E-08	1.00E-08	1.00E-08
Carboplatin + paclitaxel	13%	20%	1.00E-12	1.00E-07	1.00E-07	1.00E-07
Docetaxel	26.90%	44.80%	1.00E-12	1.00E-08	1.00E-07	1.00E-07
Doxorubicin	11.90%	15.50%	1.00E-06	1.00E-05	1.00E-06	1.00E-08
Mitoxantrone	7.80%	20.90%	1.00E-05	1.00E-05	NS	NS
Paclitaxel	50%	29.30%	1.00E-08	1.00E-07	1.00E-08	1.00E-07
Vinblastine	9.10%	3.20%	1.00E-08	1.00E-08	1.00E-07	1.00E-07
Vinorelbine	6%	24.70%	1.00E-07	1.00E-07	1.00E-06	1.00E-06
Ratio predicting efficacy			8/8	8/8	7/8	7/8
Dasatinib	1.70%	4.30%	1.00E-06	NS	NS	NS
Sunitinib	2.90%	5.90%	1.00E-05	NS	NS	NS
Ratio predicting failure			0/2	2/2	2/2	2/2

Table 3
Minimum effective concentrations of clinically relevant compounds in endometrial cancer culture models

Compounds used were in clinical use (green) or failed human clinical trials for efficacy (red). Sunitinib (black) is reported as promising and is in ongoing clinical trials and therefore is not included in prediction ratios. Patient tumor and PSA response rate calculated from pooled clinical trial data. MEC calculated as the lowest concentration of drug that resulted in a statistically significant reduction in survival index compared to DMSO control. Minimum effective concentration is expressed in molar concentration and color coded from most sensitive (1E-12 M, red) to least sensitive (1E-5 M, cream). Compounds which did not result in significant decrease in survival index denoted as NS. The ratio of culture models predicting efficacy or failure of drug treatments are listed at the bottom.

Endometrial cancer	Overall tumor response	Metabolic survival index			
		2D	3D-alginate	3D HA mono-culture	3D HA co-culture
Carboplatin + paclitaxel	68.80%	1.00E-08	1.00E-12	1.00E-07	NS
Docetaxel	20.90%	1.00E-12	1.00E-12	1.00E-07	1.00E-08
Doxorubicin	22.20%	1.00E-06	1.00E-12	1.00E-11	NS
Etoposide	9.10%	1.00E-05	1.00E-12	1.00E-05	NS
Ifosfamide + paclitaxel	45%	1.00E-08	1.00E-12	1.00E-12	NS
Paclitaxel	36.20%	1.00E-08	1.00E-12	1.00E-07	NS
Vinblastine	11.80%	1.00E-08	1.00E-12	1.00E-10	NS
Sunitinib (promising, not in clinic)	21.40%	1.00E-05	1.00E-12	NS	NS
Ratio predicting efficacy		8/8	8/8	7/8	1/8
Lapatinib	3.30%	1.00E-05	1.00E-12	1.00E-05	NS
Mitoxantrone	2.80%	1.00E-06	1.00E-12	NS	NS
Ratio predicting failure		0/2	0/2	1/2	2/2