Production of Uniform 3D Microtumors in Hydrogel Microwell Arrays for Measurement of Viability, Morphology, and Signaling Pathway Activation

Manjulata Singh,¹ David A. Close,¹ Shilpa Mukundan,¹ Paul A. Johnston,^{1,2} and Shilpa Sant^{1,3,4}

¹Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania.

²University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania.

³Department of Bioengineering, Swanson School of Engineering, University of Pittsburgh, Pittsburgh, Pennsylvania.

⁴McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania.

ABSTRACT

Despite significant investments in cancer research and drug discovery/development, the rate of new cancer drug approval is $\leq 5\%$ and most cases of metastatic cancer remain incurable. Ninety-five percent of new cancer drugs fail in clinical development because of a lack of therapeutic efficacy and/or unacceptable toxicity. One of the major factors responsible for the low success rate of anticancer drug development is the failure of preclinical models to adequately recapitulate the complexity and heterogeneity of human cancer. For throughput and capacity reasons, high-throughput screening growth inhibition assays almost exclusively use two-dimensional (2D) monolayers of tumor cell lines cultured on tissue culture-treated plastic/glass surfaces in serum-containing medium. However, these 2D tumor cell line cultures fail to recapitulate the three-dimensional (3D) context of cells in solid tumors even though the tumor microenvironment has been shown to have a profound effect on anticancer drug responses. Tumor spheroids remain the best characterized and most widely used 3D models; however, spheroid sizes tend to be nonuniform, making them unsuitable for high-throughput drug testing. To circumvent this challenge, we have developed defined size microwell arrays using nonadhesive hydrogels that are applicable to a wide variety of cancer cell lines to fabricate size-controlled 3D microtumors. We demonstrate that the hydrogel microwell array platform can be applied successfully to generate

hundreds of uniform microtumors within 3–6 days from many cervical and breast, as well as head and neck squamous cell carcinoma (HNSCC) cells. Moreover, controlling size of the microwells in the hydrogel array allows precise control over the size of the microtumors. Finally, we demonstrate the application of this platform technology to probe activation as well as inhibition of epidermal growth factor receptor (EGFR) signaling in 3D HNSCC microtumors in response to EGF and cetuximab treatments, respectively. We believe that the ability to generate large numbers of HNSCC microtumors of uniform size and 3D morphology using hydrogel arrays will provide more physiological *in vitro* 3D tumor models to investigate how tumor size influences signaling pathway activation and cancer drug efficacy.

INTRODUCTION

It is widely accepted that tumor growth and progression are controlled by the tumor microenvironment,^{1–6} which consists of cellular and noncellular components. Cellular components include tumor cells, stromal cells (fibroblasts, epithelial cells, and infiltrating immune cells), soluble factors secreted by them, extracellular matrix (ECM), and the biophysical/mechanical forces and cues generated by cell–cell and cell–ECM contacts. Noncellular components include pH, hypoxia/necrosis, and diffusion gradients for oxygen, nutrients, and waste products. All of these components are interconnected and communicate with each other. Development of biomimetic *in vitro* models with controlled tumor microenvironments is critical for the mechanistic understanding of the molecular events in tumorigenesis and metastasis to identify new targets and for testing the efficacy of potential new therapies *in vitro* under more physiologically relevant conditions.

Two-dimensional (2D) cell-based *in vitro* models are popular for preclinical cancer drug efficacy and safety testing due to the relative ease of their implementation and the throughput and capacity they provide for high-throughput screening. Traditional 2D cell culture refers to the flat

monolayer culture of cells plated on plastic dishes or glass substrates that can easily be adapted into multiwell microtiter plates. However, it is evident that 2D cultures fail to mimic the microenvironment context and relevant complexity of solid tumors *in vivo*.

Preclinical animal models for cancer rely heavily on mouse tumor models that are expensive, thereby limiting the numbers of agents that can be tested, and are encumbered by ethical considerations. In addition, the mice often have compromised immune systems and the nonhuman stromal components do not recapitulate human pathophysiology, leading to poor predictions of drug efficacy and patient responses in clinical trials. *In vitro* three-dimensional (3D) constructs of human cancer cell lines serve better to mimic the cell–cell interactions, cell–matrix interactions, and heterogeneous microenvironment of solid tumors observed *in vivo*.

Among various 3D models, tumor aggregates, often referred to as spheroids or microtumors, are multicellular structures that have been widely used to study (1) cell–cell interactions,^{7,8} (2) microenvironmental cues important for tumor growth,⁵ (3) to understand complex phenomena such as angiogenesis,^{9–14} and (4) to test drug penetration, tumor responses, and drug resistance.^{15,16} Microtumors have been shown to recapitulate various aspects of solid tumors *in vivo*, including their responses to cancer drugs (*e.g.*, development of multidrug resistance), and can serve as good models for preclinical drug testing. Microtumors can be generated using a variety of methods, including the use of nonadhesive surfaces, spinner flasks, NASA rotary systems, and hanging drop methods. However, these techniques are both time-consuming and labor intensive and, most importantly, they generate aggregates with a wide range of irregular sizes and shapes, making assay standardization difficult.^{7,17} Tumor size heterogeneity can play a critical role in the establishment of differential gradients within the tumor microenvironment, such as hypoxia, pH, nutrients, growth factors, cytokines, waste products, and metabolic stress, and consequently their responses to drug treatment.¹⁸ Thus, there is an unmet need for methods to generate sufficient tumor aggregates of controlled sizes so that they can be utilized for preclinical cancer drug efficacy screening.

Emerging microfabrication and micromolding techniques¹⁹ offer an opportunity to control the size of 3D tissue constructs more efficiently.^{20,21} In this study, we report the fabrication of hydrogel arrays containing defined size microwells (150–600 μm) for the high-throughput production of microtumors. We demonstrate the use of this platform to generate uniform microtumors from a variety of human cancer cell lines originating from cervical, breast, and head and neck squamous

cell carcinoma (HNSCC). We further demonstrate the use of these 3D microtumors in a variety of assay formats to measure cell viability, microtumor morphology, and both activation and inhibition of the epidermal growth factor receptor (EGFR) signaling pathway in response to EGF and cetuximab, respectively.

MATERIALS AND METHODS

Materials

All the chemicals were purchased from Sigma-Aldrich Chemical Company unless mentioned otherwise. All the cell culture media, Dulbecco's phosphate-buffered saline (DPBS), and serum were purchased from Mediatech, Inc. Breast cancer cell lines (MCF7, T47D, and BT474) were obtained from Dr. Steffi Oesterreich (Magee Women's Research Institute, UPCI, Pittsburgh), whereas head and neck cancer cell lines (Cal 33, Cal 27, and BICR56) were obtained from Dr. Ann-Marie Egloff (Departments of Otolaryngology and Microbiology and Molecular Genetics, University of Pittsburgh). Live–dead (calcein AM/ethidium homodimer) assay kit was purchased from Invitrogen. Propidium iodide (PI) was purchased from Immunochemistry Technologies, LLC. Hydrocortisone was purchased from Sigma-Aldrich.

Fabrication of Polydimethylsiloxane Stamps Containing Micropillar Arrays

Polydimethylsiloxane (PDMS) stamps were fabricated using four different silicon masters, each containing microwell arrays of different diameters (150, 300, 450, and 600 μm), as described previously.^{21,22} Briefly, a 10:1 mixture of prepolymer silicone elastomer base solution and curing agent (Sylgard 184; Dow Corning Corporation) was degassed for 10 min in a vacuum chamber to remove any bubbles, and then poured onto the silicon master patterned with an SU-8 photoresist. It was further cured at 75°C for 45 min and PDMS stamps containing micropillars were peeled from the masters (*Fig. 1A*, schematic). PDMS pillars of four different diameters viz. 150, 300, 450, and 600 μm were fabricated by this method.

Fabrication of Hydrogel Microwell Arrays

The generated PDMS stamps were subsequently used for fabrication of nonadhesive polyethylene glycol dimethacrylate (PEGDMA) microwell arrays on a glass slide. For covalent attachment of PEG hydrogel microwells to the glass slide, sodium hydroxide-treated glass slides were coated with 3-trimethoxysilyl polymethacrylate (TMSPMA).²³ A micropillar PDMS stamp was placed on an evenly distributed PEGDMA 1000 (Sigma-Aldrich) solution containing 1% (w/w) photoinitiator

Fig. 1. Fabrication of polydimethylsiloxane (PDMS) mold and polyethylene glycol dimethacrylate (PEGDMA) hydrogel arrays. **(A)** Schematic diagram to illustrate fabrication of PDMS molds using Sylgard 184 silicone elastomer on to SU-8 patterns of microwell sizes 150, 300, 450, and 600 μm . **(B)** Top and cross-sectional view of PDMS molds imaged by scanning electron microscopy (SEM) showed uniform sizes and smooth surfaces of posts in each size of mold. **(C)** Schematic illustrating preparation of PEGDMA hydrogel microwell arrays onto TMS-PMA-coated glass slides using PDMS molds by photocross-linking. **(D)** Photomicrographs of PEGDMA hydrogel arrays showing uniform-sized microwells in each device. **(E)** Dimension measurements illustrating uniform width and height of PDMS posts and uniform diameter of hydrogel microwells.

Irgacure-1959 (Ciba AG CH-4002) on the glass slide, and then photocross-linked by exposure to UV light (350–500 nm wavelength, 5 W/cm²) for 45 s using the OmniCure Series 2000 curing station (EXFO). After photocross-linking of PEGDMA, the PDMS stamp was peeled from the substrate. This

led to fabrication of the 2 $\times$ 2 cm² device of hydrogel microwell arrays. Depending on the size of the PDMS micropillars, each 2 $\times$ 2 cm² device contained $\sim$ 2,100, 625, 484, and 289 of the 150, 300, 450, and 600- μm diameter microwells.

Cell Culture

All cells were cultured under sterile conditions and maintained in 5% CO₂ at 37°C in a humidified incubator. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% Hyclone fetal bovine serum (FBS). The cell lines of MCF7, T47D, and BT474 (breast cancer); HeLa (colon cancer); and Cal 33, Cal 27, and FaDu (head and neck cancer) were cultured using DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin. SCC 9 (head and neck cancer) was cultured using DMEM/F12 containing 10% heat-inactivated FBS and 1% penicillin-streptomycin and 0.4 $\mu\text{g}/\text{mL}$ hydrocortisone. PE/CA-49J (head and neck cancer) was cultured using Iscove's DMEM with 2 mM glutamine, 10% heat-inactivated FBS, and 1% penicillin-streptomycin.

Generation of Uniform Size Microtumors from Different Cell Lines

A variety of epithelial cancer cell lines representing cervical, breast cancer, and HNSCC were used to test the efficiency of the hydrogel microwells to generate uniform size microtumors. For cell seeding, microwell arrays (2 $\times$ 2 cm²) with well sizes of 150, 300, 450, or 600 μm were sterilized in six-well microplates using 70% isopropanol under UV light for 30 min and washed with DPBS thrice. Single-cell suspension (100 μL , 10 $\times$ 10⁶ cells/mL) was seeded onto each array and cells were allowed to settle in the microwells by gravity for 30 min. This step was

repeated one more time to ensure complete filling of the microwells. The microwell array was then washed gently thrice with DPBS to remove undocked cells. The cell-seeded devices were cultured with respective media in a humidified incubator at 37°C. The culture media were changed every

day during the culture time. To generate microtumors of defined sizes, Cal 33 cells were seeded as described above using four different microarrays with defined microwell sizes of 150, 300, 450, and 600 μm .

Characterization of Microtumors

Microtumor formation inside the wells was monitored using an inverted microscope (Primovert; Zeiss).

Live and Dead Assay

Viability of Cal 33 microtumors of sizes 150–600 μm inside the arrays was investigated using the Live–Dead assay reagents as per the manufacturer's recommended protocol (Invitrogen). Briefly, the entire device containing microtumors was washed with DPBS two to three times and incubated for 20–30 min with diluted calcein AM (2 μM , stains live cells green) and ethidium homodimer (4 μM , stains dead cells red). The images of the array were captured on an upright microscope using a $2.5\times$ objective (Olympus FLUOVIEW 1000).

Growth Kinetics of Microtumors

Growth kinetics of Cal 33 microtumors in PEGDMA hydrogel microarrays was studied by measuring their diameters and number of cells per microtumor over a period of 6 days in culture. After day 1 or 6 of culture, microtumors were harvested by gently flushing them outside the hydrogel microwells. Images of harvested microtumors were captured by using an inverted microscope (Primovert; Zeiss). Diameters of the microtumors were estimated by image analysis (ImageJ, NIH, $n \geq 50$ microtumors). The microtumor volumes were then calculated from the measured diameters, assuming a spherical morphology. To calculate number of cells per microtumor, 50–150 microtumors of each size were harvested on days 1 and 6 ($n \geq 50$ microtumors). Next, the microtumors were trypsinized for 15 min to obtain single-cell suspensions, and the cell number was counted using a hemocytometer to calculate the number of cells/microtumor and the packing density (number of cells/ μm^3).

Spatial Distribution of Cells Within Microtumors (Ki-67/PI)

Cal 33 microtumors grown in two different microwell sizes (150 and 600 μm) were harvested on day 6. Live microtumors were incubated with PI (1:1,000) diluted in growth media inside the CO_2 incubator for 2 h. Subsequently, they were washed and fixed with 4% paraformaldehyde for 20 min, followed by washing with DPBS thrice. Microtumors were further fixed with 95% methanol on ice for 15 min. They were then stained with primary antibody Ki-67 (H-300) (Santa Cruz; 1:100) diluted in blocking buffer by incubation at 4°C overnight. After washing with DPBS thrice, the microtumors

were stained with Alexa Fluor-conjugated anti-rabbit for 1 h at room temperature.

Diffusion of Oxygen Across the Microtumors

The availability of oxygen to the cells in the microtumor core was determined using ruthenium-tris (4,7-diphenyl-1,10-phenanthroline)dichloride (Ru-dpp; Sigma-Aldrich). Due to dynamic quenching, fluorescence of the dye (abs. λ_{max} 455 nm, luminescence λ_{max} 613 nm) is inversely dependent on oxygen concentration and the signal intensity is greatly reduced in the presence of oxygen.²⁴ Cal 33 microtumors of sizes 150 and 600 μm were incubated in 1×10^{-4} μM Ru-dpp-containing culture medium for 3 h. Background fluorescence was omitted by imaging culture media containing Ru-dpp without microtumors and microtumors without Ru-dpp. Fluorescent images of Ru-dpp-stained microtumors were acquired on a confocal microscope (Olympus Fluoview; Olympus) using a 543 nm He-Ne laser to excite the dye and 604 LP emission filters.

Seeding and Counting Cal 33 Microtumors into Assay Plates

Cal 33 microtumors were harvested from 300- μm PEGDMA hydrogel microarray devices after 4 days in culture by washing with PBS and were allowed to settle by gravity, and then the PBS was aspirated and the microtumors were resuspended in serum-free media (SFM) and transferred into microfuge tubes. The microtumors were then serially diluted 1:2 and 50 μL transferred into the wells of white, Greiner 384-well μClear bottom cell culture microplates for quantitation. The number of microtumors per well was determined by three methods: visual scoring, automated image analysis, and by using the CellTiter-Glo[®] Luminescent (Promega) assay to measure the number of viable cells in each well. To score visually, the number of microtumors per well was simply counted by a technician using a light microscope. For automated image analysis, transmitted light images were acquired using an ImageXpress Micro (IXM) automated imaging platform (Molecular Devices, LLC) using a $4\times$ objective. Four images per well were captured to ensure coverage of the entire well and spheroid counts were determined using the multiwavelength cell scoring (MWCS) image analysis module. For the CellTiter-Glo luminescent cell viability assay, after the wells were scored visually and images acquired with the IXM, 25 μL of CellTiter-Glo reagent was added to each well. The plate was then centrifuged at 50 g for 1 min and incubated on a shaking platform for 15 min at room temperature. Relative luminescence units were captured using a SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices, LLC).

Culturing Cal 33 Microtumors in Assay Plates Coated with Agar

To make agar-coated assay plates, a 2% agarose solution was prepared in DMEM. The solution was then allowed to mix on a heated stirrer plate set to 65°C to ensure that the agarose had dissolved. The agarose solution was then autoclaved at 121°C for 45 min. After allowing the solution to cool to ~65°C, 25 µL was transferred to each well of a black, Greiner 384-well µClear bottom cell culture microplate and allowed to solidify for 1 h. Harvested microtumors were then washed and diluted in complete DMEM, and 50 µL of spheroid suspension was plated into each well of the agar-coated plate. Every 24 h, transmitted light images were acquired using an IXM automated imaging platform (Molecular Devices, LLC) using a 4× objective. Microtumor plates were incubated at 37°C, 5% CO₂, and 95% humidity for the duration of the experiment.

EGF Pathway Activation in Cal 33 Microtumors, Fixation, and Hoechst Staining

Cal 33 microtumors were harvested from 300-µm PEGDMA hydrogel microarray devices after 4 days in culture by washing with PBS and were allowed to settle by gravity, and then the PBS was aspirated and the microtumors were resuspended in SFM and transferred into microfuge tubes. The Cal 33 microtumors were again allowed to settle by gravity and, after the SFM was aspirated, the microtumors were resuspended in SFM ± 50 ng/mL (0.7 nM) EGF. Control tubes did not receive EGF treatment. Microtumors were stimulated with the EGF for 15 min at 37°C before being fixed and stained with an equal volume of PBS containing 7.4% formaldehyde and 4 µg/mL Hoechst 33342. Fixing and staining were carried out overnight at room temperature and in the dark. The microfuge tubes were briefly centrifuged to pellet the microtumors, and the fixation buffer was then aspirated and the microtumors were washed twice with 500 µL of PBS by repeated brief centrifugation in the microfuge and resuspension in PBS.

Indirect Immunostaining of Total and Phospho-ERK1/2 in Cal 33 Microtumors

After fixation, Hoechst staining, and subsequent washes in PBS, Cal 33 microtumors were briefly centrifuged to pellet the microtumors, resuspended in 95% ice-cold methanol, and permeabilized on ice for 1 h. After a brief centrifugation in the microfuge, the methanol was removed and the microtumors were washed twice with 500 µL Tween 20 blocking buffer (0.1% Tween 20 in PBS), and then incubated in the blocking buffer for 30 min at room temperature. After removal of the blocking buffer, the microtumors were resuspended in 100 µL of PBS containing either 1:100 p44/42 MAPK (Erk1/2) rabbit

polyclonal antibody #9102 (Cell Signaling Technology) or 1:400 Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® rabbit mAb #4370 (Cell Signaling Technology) and incubated for 1 h at room temperature on a rocker platform. After a brief centrifugation in the microfuge, the primary antibodies were then removed by aspiration and the microtumors were washed twice with 500 µL Tween 20 blocking buffer, and then incubated in blocking buffer for 15 min at room temperature. After removal of the blocking buffer, the microtumors were resuspended in 100 µL PBS containing 1:1,000 Alexa Fluor® 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Life Technologies). The microtumors were incubated with secondary antibody for 1 h at room temperature in the dark, and then washed twice with 500 µL Tween 20 blocking buffer. An appropriate volume of spheroid suspension was then transferred into the wells of a black, Greiner 384-well µClear bottom cell culture microplate. Images of the Cal 33 microtumors were then acquired on an IXM automated imaging platform (Molecular Devices, LLC) using either a 4× or 10× objective and a DAPI/FITC filter set.

Inhibition of EGF Pathway Activation in Cal 33 Microtumors by Cetuximab

Cal 33 microtumors were harvested from 300-µm PEGDMA hydrogel microarray devices after 4 days in culture by washing with PBS and were allowed to settle by gravity, and then the PBS was aspirated and the microtumors were resuspended in SFM and transferred into microfuge tubes. The microfuge tubes were centrifuged briefly to pellet the microtumors, and then they were resuspended in serum-free media containing 0.7 µM cetuximab and they were incubated together for 1 h at 37°C. Control tubes did not receive cetuximab antibody. The microtumors were then carefully washed twice with 1 mL serum-free media before stimulation with EGF as described above.

Image Acquisition on the IXM Automated High Content Imaging Platform

The IXM is an automated field-based high-content imaging platform (Molecular Devices, LLC) integrated with the MetaXpress Imaging and Analysis software. The IXM optical drive includes a 300 W Xenon lamp broad-spectrum white light source and 2/3" chip cooled CCD camera and optical train for standard field of view imaging and an IXM-transmitted light option with phase contrast. The IXM is equipped with a 4× Plan Apo 0.20 NA objective, a 10× Plan Fluor 0.3 NA objective, a 20× Ph1 Plan Fluor ELWD DM objective, a 20× S Plan Fluor ELWD 0.45 NA objective, a 40× S Plan Fluor ELWD 0.60 NA objective, and a single slide holder

adaptor. The IXM has a transmitted light module and is equipped with the following ZPS filter sets for fluorescent imaging: DAPI, FITC/ALEXA 488, CY3, CY5, and Texas Red. Images of Cal 33 microtumors were acquired using either a 4× or 10× objective in both the transmitted light and fluorescent acquisition modes. The infrared laser autofocus was used to detect the bottom of the plate and well, and then a series of 10–20 Z-stack images were acquired, each separated by a step size of 20 μm in a range equally distributed above and below a set Z-position. A journal was then used to either select the best focus image from the Z-stack or to collapse all of the images in the Z-stack into a single maximum projection image. No background subtraction was applied to the images and no flat-field correction was applied to either the best focus or maximum projection image output by the journal.

Image Analysis Using the MWCS Image Analysis Module

We used the MWCS image analysis module to quantify the number of Cal 33 3D microtumors per well and to measure the total ERK1/2 and pphspho-ERK1/2 expression levels in the digital images acquired on the IXM as described above. For transmitted light images, we set up the instrument acquisition to invert the contrast such that the 3D Cal 33 microtumors were bright on a dark background. To identify and count 300 μm microtumors, we defined the approximate minimum width to be 150 μm and the approximate maximum width to be 300 μm and the threshold intensity above local background to be 100. The MWCS image analysis module provided outputs for the number of microtumors detected and their average diameters (μm) and areas (μm²). To measure total ERK1/2 and pERK1/2 expression levels, we used Hoechst 33342 to stain and identify the Cal 33 nuclei of the microtumors and used this fluorescent signal in Ch1 to define a microtumor mask. Objects in Ch1 that exhibited the appropriate fluorescent intensities above background and size (width, length, and area) characteristics were identified and classified by the image segmentation as microtumors and used to create a mask for each spheroid. For Hoechst-stained microtumors, we defined the approximate minimum width to be 75 μm and the approximate maximum width to be 250 μm and the minimum threshold intensity above local background to be 25. The spheroid mask was used to quantify the amount of target Ch2 total ERK1/2 or pphspho-ERK1/2-associated FITC fluorescence within the spheroid mask. For the total ERK1/2 or pphspho-ERK1/2 signals in the FITC channel, we defined the approximate minimum width to be 125 μm and the approximate maximum width to be 300 μm and the minimum threshold intensity above local background to be 200. The MWCS image analysis module outputs quantitative data, including the

average and integrated fluorescent intensities of the Hoechst-stained objects (microtumors) in Ch1; the number of microtumors detected in Ch1; the average spheroid diameters (μm) and area (μm²); and the integrated and average fluorescent intensities of the Ch2 signals (total ERK1/2 or pphspho-ERK1/2) within the detected spheroid mask.

RESULTS AND DISCUSSION

Fabrication and Characterization of PEGDMA Hydrogel Microarray

Figure 1A shows the schematic for patterning PDMS micropillar molds using SU-8 photoresist consisting of defined microwell patterns. PDMS micropillars of strictly controlled sizes were successfully fabricated using 150, 300, 450, and 600-μm microwells patterned on the respective silicon masters. Before hydrogel microwell array formation, PDMS stamps bearing micropillars were characterized for their size and surface properties using scanning electron microscopy (SEM) (Fig. 1B). All the pillars of respective size devices were uniform and showed smooth surface morphology in SEM images. The cross section of PDMS stamp also showed uniform depth of each post (Fig. 1B). Hydrogel microwell arrays of each size were then generated on TMSPMA-coated glass slides (Fig. 1C) by UV-initiated free radical polymerization of methacrylate end groups present on the PEG chains. Methacrylate groups present in both, the TMSPMA coating of the glass slides and PEGDMA, take part in free radical polymerization to effectively anchor the hydrogel microarrays to the glass slide as described earlier.²⁵ Photomicrographs of PEGDMA hydrogel arrays indicated that the formed microwells were uniformly sized in each size device (Fig. 1D). Measurement of PDMS post and hydrogel microwell dimensions revealed that they are of uniform height and diameter, respectively (Fig. 1E).

Microtumor Formation in Various Cancer Cell Lines

The PEGDMA hydrogel arrays were used to fabricate microtumors from cancer cell lines of various origins within 3–6 days of culture (Fig. 2). To illustrate that the hydrogel microarrays generate uniform microtumors from a variety of cancer cell lines, microwell arrays of 300 μm size were used. The UV-sterilized hydrogel microwell arrays were seeded with cervical (HeLa), breast (MCF7, T47D, and BT474), and head and neck cancer cell lines (Cal 33, Cal 27, PE/CA-PJ-49, SCC9, and BICR56) suspended in growth media and cultured for 3 days (Fig. 2A). Initial cell seeding densities were optimized to provide a single microtumor per microwell. Lower seeding densities of 1×10^6 cells/mL/2 × 2 cm² device resulted in multiple aggregates per microwell, especially in large size

Fig. 2. Application of PEGDMA hydrogel microwell array for generation of microtumors using various cancer cell lines. **(A)** Schematic showing formation of microtumors by seeding cell suspension on to hydrogel microwell array. Various cervical, breast cancer **(B)**, and head and neck cancer cells **(C)** seeded on hydrogel arrays of 300- μ m microwell size formed uniform microtumors inside the devices (top panel) and were successfully harvested after 3 days (bottom panel). Bright-field image analysis showed uniform-sized microtumor formation in hydrogel microwell and compact morphology of harvested microtumors. Scale bars in top and bottom panels of **(B)** and **(C)** are 100 and 500 μ m, respectively.

(600 μ m) wells (data not shown). However, a seeding density of 2×10^6 cells/device resulted in uniform-sized single microtumors per microwell. The top panels of *Figure 2B* and *C* show images of microtumors formed from HeLa, MCF7, T47D, BT474, Cal 33, Cal 27, PE/CA-PJ-49, SCC9, and BICR56 with a single microtumor/well after 3 days in culture. The bottom panels of *Figure 2B* and *C* show images of the highly uniform 3D microtumors harvested from these devices on day 3. It is evident that the nonadhesive nature of hydrogel microwells reduced cell-polymer interaction such that cell-cell interactions were promoted, leading to the formation of single microtumors in each microwell of the array that were highly uniform in size and shape (*Fig. 2B, C*). The size of generated microtumors depends on the cell seeding density as well as cell lines. It should be noted that microtumors generated from the

same cell line under same culture conditions (cell density, microwell size, time of culture) show highly uniform size distribution as shown in the bottom panel of harvested microtumors of each cell line (*Fig. 2B, C*). However, microtumor size was variable across different cancer cell lines (cultured under same conditions of cell density, time, etc., as in *Fig. 2*). Micrographs in *Figure 2* show that in general, head and neck cancer cells compact much more than breast cancer cell lines and some HNC cells compact more than other (e.g., Cal 33 vs SCC9 and BICR56). The observed variability in the size of photomicrographs is due to the difference in compactness of the microtumors across the various cell lines. This may be due to focal adhesion characteristics of individual cell lines. Some cell lines form tight adherence junction and compact microtumors, while the other ones form loose aggregates.²⁶ However, for any cell line, optimization of cell seeding density and culture time using reported hydrogel microwells will easily lead to formation of microtumors with tightly controlled sizes. In the past, nonadhesive surfaces have been used in stem cell research to generate uniform embryoid bodies.^{21,27} Our results indicate that we can successfully use nonadhe-

sive PEG hydrogel microarrays to generate hundreds of compact microtumors from various cancer cell lines with uniform size and shape, which can be used as solid tumor models for testing cancer drugs or to investigate signaling pathway activation.

Fabrication of Size-Controlled Microtumors of Head and Neck Cancer

Tumor size is crucial in the formation of nutrient/oxygen gradients, hypoxia, and spatial cellular organization. As tumors grow beyond a certain size, they exhibit decreased O_2 tension (hypoxia), experience metabolic stress, may generate reactive oxygen species, and adapt their signaling pathways to survive under hypoxia.²⁸ Signaling through hypoxia-inducible factors leads to upregulation of proangiogenic

signaling molecules such as vascular endothelial growth factor and cytokines (IL-8) that can initiate the angiogenic switch and aggressive behavior.²⁹ Uncontrolled tumor growth is also known to increase interstitial pressure and tissue stiffness.³⁰ Indeed, consistent association of large tumor size, rapid growth rates, and metastatic risk in the majority of clinical cancer cases suggests that the molecular basis of these phenomena may be linked.³¹ Most importantly, tumor size may also affect the penetration/interaction of therapeutic agents within these tumors due to diffusion limitations. Thus, 3D models with precise control over tumor size and shape are critical to recapitulate these processes *in vitro*. Using PEGDMA hydrogel microarrays of defined size microwells, we were successfully able to fabricate Cal 33 HNSCC microtumors of sizes 142.28 ± 4.44 , 241.37 ± 12.97 , 316.85 ± 19.51 , and 452.82 ± 53.15 μm when grown for 6 days in hydrogel microwells of 150, 300, 450, and 600 μm size, respectively. It was observed that the microtumor size was controlled by the microwell diameter (Fig. 3A, B). On the other hand, multicellular aggregates generated by the NASA rotary system, spinner flask, and liquid overlay methods generate spheroids with a broad size range (50–500 μm), depending upon cell seeding density, rotation speed, and time of culturing.^{7,17} Depending on the size of the microwells, each $2 \times 2 \text{ cm}^2$ device contained $\sim 2,100$, 625, 484, and 289 of the 150, 300, 450, and 600 μm microtumors. To evaluate the viability of the cells in the Cal 33 microtumors, whole microarrays

containing microtumors were stained with the calcein AM/ethidium homodimer live/dead reagents. Fluorescent images of the Cal 33 microtumors were intensely green with no or insignificant red fluorescence, indicating that the vast majority of cells inside the microwells were alive rather than dead (Fig. 3B). Smaller microtumors (150–300 μm) were compact compared with the larger 600 μm ones. Likewise, transmitted light images of the Cal 33 microtumors harvested on day 3 also demonstrated the ability of microwells to generate uniform microtumors of defined sizes and shapes (Fig. 3C).

Characterization of Cal 33 Microtumors

Microfabricated 150 and 600 μm Cal 33 HNSCC tumors were characterized for their growth and proliferation within the microwells for up to 6 days in culture (Fig. 4). The diameters and the number of cells/microtumor in different size microwells from 1- and 6-day-old microtumors were compared (Fig. 4B, C). Images of the 150 and 600 μm Cal 33 microtumors (Fig. 4A) and the quantitative data for cell diameters and counts (Fig. 4B–D) indicated that all of the microtumors proliferated and increased in size throughout the duration of culture. The mean diameters of Cal 33 microtumors harvested on day 1 increased from 130 ± 8.35 , 202.00 ± 17.91 , 272.27 ± 14.53 , and 438.39 ± 32.0 μm to 142.28 ± 4.44 , 241.37 ± 12.97 , 316.85 ± 19.51 , and 452.82 ± 53.15 μm , respectively, for microtumors from 150, 300, 450, and 600- μm hydrogel microwell arrays harvested on day 6. Morphological analysis indicated that although microtumors grown in both 150 and 600- μm wells were compact on day 1, 600 μm microtumors appeared to be less compact than the 150 μm ones on day 6. Packing density of microtumors calculated as number of cells/ μm^3 of all the sizes also supported the microscopic observation. In general, on day 6, small microtumors (150–300 μm) exhibited more than twofold higher packing density compared with large ones (Fig. 4D). This may be due to oxygen and nutrient diffusional limitations imposed by larger sizes. To further support this, 6-day-old small (150 μm) and large (600 μm) microtumors were incubated in oxygen-sensitive ruthenium probe and were imaged by the confocal laser scanning microscope (Fig. 4E). Small 150 μm microtumors did not show any ruthenium fluorescence, while large 600 μm microtumors revealed increased fluorescence

Fig. 3. Generation of size-controlled microtumors of HNSC, Cal 33. (A) Cells were seeded in hydrogel arrays with 150, 300, 450, and 600- μm microwell sizes at a seeding density of 10×10^6 cells/mL/ $2 \times 2 \text{ cm}^2$ to fabricate microtumors. (B) Live–dead staining performed on day 3 indicated good cell viability in microtumors across all the devices. (C) Photomicrographs of harvested Cal 33 microtumors on day 3. Scale bars: 500 μm .

Fig. 4. Characterization and growth kinetics of size-controlled microtumors. **(A)** Photomicrographs of 150 and 600 μm tumors showing growth from day 1 to 6. Growth kinetics of microtumors of all sizes was assessed by measuring **(B)** diameter, **(C)** number of cells/microtumor on days 1 and 6, and **(D)** number of cells/ μm^3 on day 6 (cell density/microtumor, packing density). Data are represented as mean $\pm$ SD ($n=50$; $*P<0.05$ compared with day 1). **(E)** Growth induces diffusional limitations in solid tumors. Oxygen availability inside the microtumors was assessed by staining with Ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride (Ru-dpp) for 3 h and imaging under the confocal microscope. Six hundred micron size tumors showed more number of Ru-dpp-positive cells in the core compared with 150 μm tumor, suggesting limited oxygen diffusion with increase in microtumor size. **(F)** Limited oxygen supply to the cells inside the tumor mass leads to necrosis. Effect of diffusional barriers on spatial distribution of cells in microtumors of 150 μm and 600 μm was investigated by immunostaining with proliferation marker, Ki-67, and apoptosis marker, propidium iodide (PI). Six hundred micrometer tumors showed proliferating cells on the outer surface and more necrotic cells in the core, whereas 150 μm microtumors showed proliferating cells. Scale bars in left and right panels are 100 and 500 μm , respectively. **(G)** Confocal microscopy image showing three-dimensional (3D) projection of a microtumor in xz and yz directions (from outer surface to the center). The center panel shows two-dimensional projection of maximum intensity of Z-stack. Microtumors were stained with live-dead stain (calcein AM shown as green and ethidium homodimer shown as red).

in the central core, suggesting a lack of oxygen availability due to diffusion limitations in the larger microtumors (Fig. 4E). It is also known that as a tumor grows in size, it produces differential proliferation zones with proliferating cells at the outer boundary and necrotic/apoptotic cells in the core of the tumor.³² When 6-day-old small and large microtumors were stained with Ki-67 (marker for proliferating cells) and PI (apoptotic marker), fluorescent images of small microtumors exhibited predominantly Ki-67-positive proliferating cells (Fig. 4F). In contrast, fluorescent images of large microtumors displayed the presence of PI-stained apoptotic cells in the central core (Fig. 4F, G). This suggests that the microtumors generated using hydrogel microwell arrays mimic the microenvironment observed in solid tumor, including hypoxia and a spatial cellular arrangement into differential zones of proliferation.

Cal 33 HNSCC microtumors were harvested from 300- μm PEGDMA hydrogel microarray devices after 4 days in culture and were serially diluted before being seeded into 384-well assay plates (Fig. 5A). We used three different methods to count the number of Cal 33 microtumors per well; we manually counted the number of microtumors per well on an inverted phase-contrast light microscope, we acquired $4 \times 4 \times$ transmitted light images per well on the IXM platform and used the MWCS image analysis module to count the number of microtumors per well, and we added the CellTiter-Glo (CTG) detection reagent to quantify cellular ATP levels (Fig. 5A). Both the manual and the automated light microscopy methods exhibited a very similar linear increase in the microtumor counts per well with decreasing serial dilution. The image analysis method produced slightly higher microtumor counts, likely because some microtumors that straddled the separation zones between

Fig. 5. Three hundred eighty-four-well assays using 3D head and neck squamous cell carcinoma (HNSCC) microtumors harvested from hydrogel microarrays. **(A)** Counting 3D HNSCC microtumors. Cal 33 microtumors were harvested from 300- μm PEGDMA hydrogel microarray devices after 4 days in culture and were serially diluted before being seeded into 384-well assay plates. Three different methods were used to count the number of Cal 33 microtumors per well; we manually counted the number of microtumors per well on an inverted phase-contrast light microscope, we acquired 4×4 transmitted light images per well on the IXM platform and used the multiwavelength cell scoring (MWCS) image analysis module to count the number of microtumors per well, and we added the CellTiter-Glo (CTG) detection reagent to quantify the cellular ATP levels. The mean $\pm$ SD ($n=4$) spheroid counts/well or the CellTiter-Glo relative light units (relative luminescence units [RLUs]) produced by the 3D Cal 33 microtumors from quadruplicate wells are presented. **(B)** Imaging 3D HNSCC microtumors seeded in wells coated with agar. Three hundred eighty-four-well agar-coated microtiter assay plates were prepared as described in the Materials and Methods section. HNSCC microtumors harvested from hydrogel arrays were washed and diluted in complete DMEM+10% fetal bovine serum (FBS), and 50 μL of spheroid suspension was plated into each well of the agar-coated plate. Spheroid plates were incubated at 37°C, 5% CO_2 , and 95% humidity for the duration of the experiment, and every 24 h, $4 \times$ transmitted light images were acquired on the ImageXpress Micro (IXM) automated imaging platform as described in the Materials and Methods section. **(C)** Measuring 3D HNSCC microtumor diameter and area by image analysis; $4 \times$ transmitted light images acquired on the IXM automated imaging platform that had been set up to automatically invert the contrast of the images, light on dark background. We used the MWCS image analysis module to count the number of microtumors and to output width and area parameters as described in the Materials and Methods section. The mean $\pm$ SD ($n=4$) diameters (μm) and areas (μm^2) of 3D Cal 33 microtumors from quadruplicate wells are presented. The microtumor area of 3D HNSCC is plotted against the Y-axis on the left, and the diameter is plotted against the Y-axis on the right.

the quadrants were captured in two of the four images and were scored twice. The CTG luminescence signal also provided a robust response that increased linearly with the more microtumors seeded per well (Fig. 5A). The noninvasive transmitted light microscopy images enable investigators to examine the effects of culture conditions and/or cancer drugs on 3D microtumor size and morphology, while the use of cell viability reagents such as CTG could be used to assess either the growth or killing of cells in the microtumors. Cal 33 microtumors that are seeded into tissue culture-treated assay plates begin to attach to the well substrate and will spread out to form a 2D cell monolayer in <24 h. If, however, the bottoms of the wells are coated with agar, the Cal 33 microtumors maintain their 3D morphology for up to 3 days in tissue culture (Fig. 5B). During the 72 h in culture on agar, there was an apparent time-dependent radial spreading and migration of cells at the periphery of the microtumor (Fig. 5B) and a corresponding increase in both the microtumor diameter and area (Fig. 5C).

Activation and Inhibition of EGF Pathway Activation in Cal 33 Microtumors by Cetuximab

The monoclonal antibody, cetuximab, which blocks EGFR signaling by antagonizing ligand binding to EGFRs, was

developed and approved to target the high levels of elevated EGFR expression frequently ($>90\%$) detected in HNSCC.^{33–36} Cal 33 microtumors that were harvested from 300- μm PEGDMA hydrogel microarray devices after 4 days in culture were treated $\pm$ EGF (50 ng/mL) for 15 min and fixed in paraformaldehyde and stained with Hoechst. The fixed microtumors were then permeabilized in 95% ice-cold methanol, and then the total ERK1/2 or phospho-ERK1/2 expression levels were determined by indirect immunofluorescence antibody staining, image acquisition on the IXM, and image analysis using the MWCS module (Fig. 6). Images of Hoechst-stained nuclei (Ch1) or FITC-stained total or phospho-ERK1/2 expression in Cal 33 microtumors were segmented by the MWCS image analysis module to generate whole-spheroid masks to quantify the number of microtumors and the integrated or average fluorescent intensity signals (Fig. 6D). Total ERK1/2 staining of the Cal 33 microtumors was not apparently affected by EGF treatment (Fig. 6A, C, F). In contrast, EGF treatment of Cal 33 microtumors significantly increased the levels of phospho-ERK1/2 staining (Fig. 6B, C, F; $P<0.001$). Pretreatment of Cal 33 microtumors with cetuximab completely abolished the EGF-induced increase in phospho-ERK1/2 staining (Fig. 6C, F;

Fig. 6. Activation and inhibition of the epidermal growth factor receptor (EGFR) signaling pathway in 3D microtumors harvested from hydrogel microarrays. **(A)** Gray scale and color composite images of total ERK1/2 expression in 3D HNSCC microtumors. Cal 33 microtumors were harvested from 300- μ m PEGDMA hydrogel microarray devices after 4 days in culture and were then treated $\pm$ 50 ng/mL EGF for 15 min before fixation in paraformaldehyde containing Hoechst DNA stain. Fixed microtumors were then permeabilized with 95% methanol and stained for total ERK1/2 expression levels by indirect immunofluorescence staining using a total ERK1/2-specific antibody; 10 $\times$ fluorescent images of the Hoechst-stained nuclei (Ch1) and total ERK1/2-FITC (Ch2) staining in the Cal 33 HNSCC microtumors were acquired on the IXM automated imaging platform as described in the Materials and Methods section. Gray scale images of the transmitted light Hoechst-stained nuclei (Ch1) and total ERK1/2-FITC (Ch2) staining of Cal 33 microtumors $\pm$ EGF treatment are presented together with the corresponding color composite images. **(B)** Gray scale and color composite images of phospho-ERK1/2 expression in 3D HNSCC microtumors. Cal 33 microtumors were harvested from 300- μ m PEGDMA hydrogel microarray devices after 4 days in culture and were then treated $\pm$ 50 ng/mL EGF for 15 min before fixation in paraformaldehyde containing Hoechst DNA stain. Fixed microtumors were then permeabilized with 95% methanol and stained for phospho-ERK1/2 expression levels by indirect immunofluorescence staining using a phospho-ERK1/2-specific antibody; 10 $\times$ fluorescent images of the Hoechst-stained nuclei (Ch1) and phospho-ERK1/2-FITC (Ch2) staining in the Cal 33 HNSCC microtumors were acquired on the IXM automated imaging platform as described in the Materials and Methods section. Gray scale images of the transmitted light Hoechst-stained nuclei (Ch1) and phospho-ERK1/2-FITC (Ch2) staining of Cal 33 microtumors $\pm$ EGF treatment are presented together with the corresponding color composite images. **(C)** Color composite images of total and phospho-ERK1/2 expression in 3D HNSCC microtumors $\pm$ pretreatment with cetuximab. Cal 33 microtumors were harvested from 300- μ m PEGDMA hydrogel microarray devices after 4 days in culture, were resuspended in serum-free media, and transferred into microfuge tubes. The microfuge tubes were centrifuged briefly to pellet the microtumors, and then they were resuspended in serum-free media containing 0.7 μ M cetuximab and they were incubated together for 1 h at 37°C. Control tubes did not receive cetuximab antibody. The microtumors were then carefully washed twice with 1 mL serum-free media before stimulation $\pm$ EGF 50 ng/mL for 15 min as described above. The Cal 33 microtumors were then fixed and stained with Hoechst, permeabilized with 95% methanol, and then stained for total and phospho-ERK1/2 expression levels by indirect immunofluorescence staining as described above; 10 $\times$ fluorescent images of the Hoechst-stained nuclei (Ch1) and total or phospho-ERK1/2-FITC (Ch2) staining in the Cal 33 HNSCC microtumors were acquired on the IXM automated imaging platform as described in the Materials and Methods section. Color composite images of Hoechst-stained nuclei and total or phospho-ERK1/2-FITC staining of Cal 33 microtumors $\pm$ cetuximab and $\pm$ EGF treatment are presented. **(D)** Gray scale and pseudocolor images of 3D HNSCC microtumors and the corresponding masks derived by the image analysis module. To measure total ERK1/2 and pERK1/2 expression levels, we used Hoechst 33342 to stain and identify the Cal 33 nuclei of the microtumors and used this fluorescent signal in Ch1 to define a microtumor mask (gray mask). Objects in Ch1 that exhibited the appropriate fluorescent intensities above background and size (width, length, and area) characteristics were identified and classified by the image segmentation as microtumors and used to create a mask for each spheroid. For Hoechst-stained microtumors, we defined the approximate minimum width to be 75 μ m and the approximate maximum width to be 250 μ m and the minimum threshold intensity above local background to be 25. The spheroid mask was used to quantify the amount of target Ch2 total ERK1/2 or phospho-ERK1/2-associated FITC fluorescence within the spheroid mask. For the total ERK1/2 or phospho-ERK1/2 signals in the FITC channel, we defined the approximate minimum width to be 125 μ m and the approximate maximum width to be 300 μ m and the minimum threshold intensity above local background to be 200. The MWCS image analysis module outputs quantitative data, including the average and integrated fluorescent intensities of the Hoechst-stained objects (microtumors) in Ch1; the number of microtumors detected in Ch1; the average spheroid diameters (μ m) and area (μ m²); and the integrated and average fluorescent intensities of the Ch2 signals (total ERK1/2 or phospho-ERK1/2) within the detected spheroid mask. **(E)** Number of 3D HNSCC microtumors in the $\pm$ pretreatment with cetuximab and $\pm$ EGF treatment groups. Images of Cal 33 microtumors $\pm$ cetuximab and $\pm$ EGF treatment as described in **C** were analyzed using the MWCS image analysis module as described in the Materials and Methods section. Hoechst-stained objects in Ch1 that exhibited the appropriate fluorescent intensities above background and size (width, length, and area) characteristics were identified and classified by the image segmentation as microtumors and enumerated. The mean $\pm$ SD ($n=3$) number of 3D Cal 33 microtumors per image from triplicate wells are presented. A two-way ANOVA among the three treatment groups for the total ERK and phospho-ERK1/2 antibodies indicated that the differences in the number of Cal 33 microtumors analyzed were not significant ($P>0.05$). A two-way ANOVA among the three treatment groups for the total ERK and phospho-ERK1/2 antibodies indicated that the differences in the number of Cal 33 microtumors analyzed were not significant ($P>0.05$). **(F)** Average total or phospho-ERK intensity of 3D HNSCC microtumors in the $\pm$ pretreatment with cetuximab and $\pm$ EGF treatment groups. Images of Cal 33 microtumors $\pm$ cetuximab and $\pm$ EGF treatment as described in **C** were analyzed using the MWCS image analysis module as described in the Materials and Methods section. Hoechst-stained objects in Ch1 that exhibited the appropriate fluorescent intensities above background and size (width, length, and area) characteristics were identified and classified by the image segmentation as microtumors and used to create a mask for each spheroid. The MWCS image analysis module used the spheroid mask to quantify the amount of target Ch2 total ERK1/2 or phospho-ERK1/2-associated FITC fluorescence within the microtumor. The mean $\pm$ SD ($n=3$) average fluorescent intensities of the Ch2 signals, either total ERK1/2 or phospho-ERK1/2, within the detected spheroid masks of 3D Cal 33 microtumors from triplicate wells are presented. A two-way ANOVA of the total ERK antibody data indicated that the differences in the average fluorescent intensities of the total ERK Ch2 signals were not significant ($P>0.05$) for any of the comparisons among the three treatment groups. A two-way ANOVA of the phospho-ERK antibody data indicated that the differences in the average fluorescent intensities of the phospho-ERK Ch2 signals were also not significant ($P>0.05$) for the unstimulated to EGF+cetuximab treatment groups. In contrast, however, a two-way ANOVA of the phospho-ERK antibody data indicated that the differences in the average fluorescent intensities of the total ERK Ch2 signals were significant ($P<0.001$) for comparisons of the unstimulated to EGF and for the EGF to EGF+cetuximab treatment groups. A two-way ANOVA of the total ERK antibody data indicated that the differences in the average fluorescent intensities of the total ERK Ch2 signals were not significant ($P>0.05$) for any of the comparisons among the three treatment groups. A two-way ANOVA of the phospho-ERK antibody data indicated that the differences in the average fluorescent intensities of the phospho-ERK Ch2 signals were also not significant ($P>0.05$) for the unstimulated to EGF+cetuximab treatment groups. In contrast, however, a two-way ANOVA of the phospho-ERK antibody data indicated that the differences in the average fluorescent intensities of the total ERK Ch2 signals were significant ($P<0.001$) for comparisons of the unstimulated to EGF and for the EGF to EGF+cetuximab treatment groups.

$P < 0.001$), without affecting the number of microtumors in each treatment group (Fig. 6E).

There are 600,000 new cases and ~ 300,000 HNSCC-related deaths per year, making it the eighth leading cause of cancer worldwide.^{37–39} The major factors that influence HNSCC carcinogenesis and pathology are smoking, alcohol use, genetics, and human papillomavirus infection.^{37–39} Despite reduced cigarette smoking rates, the incidence of HNSCC continues to

rise in developed countries.^{37–40} The pathological diversity and heterogeneous genetic background of HNSCC have made the development of effective drug therapies challenging. Only six drugs are currently approved by the FDA for HNSCC treatment; methotrexate, 5-fluorouracil, bleomycin, cisplatin, docetaxel, and cetuximab.^{37–39} Only 10%–25% of HNSCC patients respond to single-drug treatments, and drug combinations generally do not prolong survival over single-agent

therapy.^{37,38} The current surgery, radiation, and chemotherapy regimens have resulted in only a limited improvement in HNSCC prognosis and 5-year survival rates are at 50%.^{37–39,41–43} Since patients with recurrent or metastatic HNSCC have median survival rates of 6–12 months,^{37,38,41} the need for new and effective therapies is critical. We believe that the ability to generate large numbers of HNSCC microtumors of uniform size and 3D morphology using hydrogel arrays described herein will provide more physiological, *in vitro* 3D tumor models to test new cancer drug efficacy. Furthermore, the availability of 3D HNSCC microtumors of defined sizes will facilitate the investigation of how tumor size influences signaling pathway activation and the efficacy of cancer drugs. We further believe that use of such *in vitro* 3D microtumor models will improve the success rate of cancer drug discovery.

ACKNOWLEDGMENTS

The authors thank Mr. Akhil Patel (School of Pharmacy) and Center for Biologic Imaging (CBI), University of Pittsburgh, for SEM images of the PDMS molds. The authors also thank Prof. Steffi Oesterreich (Women's Cancer Research Center, University of Pittsburgh Cancer Institute) and Dr. Ann Marie Egloff (Departments of Otolaryngology and Microbiology and Molecular Genetics at University of Pittsburgh) for providing breast cancer and HNC cell lines. S.S. acknowledges funding support from NIH (EB018575) and start-up funds from the Department of Pharmaceutical Sciences at University of Pittsburgh. P.A.J. acknowledges funding from a Development Research Project grant from the Head and Neck Cancer SPORE of the University of Pittsburgh Cancer Institute.

DISCLOSURE STATEMENT

The authors declare no conflict of interests.

REFERENCES

- Zumsteg A, Christofori G: Corrupt policemen: inflammatory cells promote tumor angiogenesis. *Curr Opin Oncol* 2009;21:60–70.
- Weigelt B, Bissell MJ: Unraveling the microenvironmental influences on the normal mammary gland and breast cancer. *Semin Cancer Biol* 2008;18:311–321.
- Pietras K, Ostman A: Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res* 2010;316:1324–1331.
- Finger EC, Giaccia AJ: Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. *Cancer Metastasis Rev* 2010;29:285–293.
- Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW: The organizing principle: microenvironmental influences in the normal and malignant breast. *Differentiation* 2002;70:537–546.
- Bin Kim J, Stein R, O'Hare MJ: Three-dimensional *in vitro* tissue culture models of breast cancer—a review. *Breast Cancer Res Treatment* 2004;85:281–291.
- Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA: Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol* 2010;148:3–15.
- Mueller-Klieser W: Tumor biology and experimental therapeutics. *Crit Rev Oncol Hematol*. Nov–Dec 2000;36:123–139.
- Ehsan SM, Welch-Reardon KM, Waterman ML, Hughes CCW, George SC: A three-dimensional *in vitro* model of tumor cell intravasation. *Integr Biol* 2014;6:603–610.
- Szot CS, Buchanan CF, Freeman JW, Rylander MN: In vitro angiogenesis induced by tumor-endothelial cell co-culture in bilayered, collagen I hydrogel bioengineered tumors. *Tissue Eng Part C Methods* 2013;19:864–874.
- Lu P, Weaver VM, Werb Z: The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* 2012;196:395–406.
- Hielscher AC, Gerecht S: Engineering approaches for investigating tumor angiogenesis: exploiting the role of the extracellular matrix. *Cancer Res* 2012;72:6089–6096.
- Infanger D, Pathi S, Fischbach C: Microenvironmental regulation of tumor angiogenesis: biological and engineering considerations. In: *Biophysical Regulation of Vascular Differentiation and Assembly*. Gerecht S (ed.), pp. 167–202. Springer, New York, 2011.
- Stroock AD, Fischbach C: Microfluidic culture models of tumor angiogenesis. *Tissue Eng Part A* 2010;16:2143–2146.
- Weigelt B, Lo A, Park C, Gray J, Bissell M: HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment. *Breast Cancer Res Treatment* 2010;122:35–43.
- Ong SM, Zhao ZQ, Arooz T, et al.: Engineering a scaffold-free 3D tumor model for *in vitro* drug penetration studies. *Biomaterials* 2010;31:1180–1190.
- Lin R-Z, Chang H-Y: Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol J* 2008;3:1172–1184.
- West CML, Moore JV: Mechanisms behind the resistance of spheroids to photodynamic treatment: a flow cytometry study. *Photochem Photobiol* 1992;55:425–430.
- Bae H, Chu H, Edalat F, et al.: Development of functional biomaterials with micro- and nanoscale technologies for tissue engineering and drug delivery applications. *J Tissue Eng Regen Med* 2014;8:1–14.
- Fukuda J, Khademhosseini A, Yeh J, et al.: Micropatterned cell co-cultures using layer-by-layer deposition of extracellular matrix components. *Biomaterials* 2006;27:1479–1486.
- Karp JM, Yeh J, Eng G, et al.: Controlling size, shape and homogeneity of embryoid bodies using poly(ethylene glycol) microwells. *Lab Chip* 2007;7:786–794.
- Kwon CH, Wheeldon I, Kachouie NN, et al.: Drug-eluting microarrays for cell-based screening of chemical-induced apoptosis. *Anal Chem* 2011;83:4118–4125.
- Khademhosseini A, Yeh J, Jon S, et al.: Molded polyethylene glycol microstructures for capturing cells within microfluidic channels. *Lab Chip* 2004;4:425–430.
- Victor VM, Nuñez C, D'Ocón P, Taylor CT, Esplugues JV, Moncada S: Regulation of oxygen distribution in tissues by endothelial nitric oxide. *Circ Res* 2009;104:1178–1183.
- Revin A, Russell RJ, Yadavalli VK, et al.: Fabrication of poly(ethylene glycol) hydrogel microstructures using photolithography. *Langmuir* 2001;17:5440–5447.
- Kenny PA, Lee GY, Myers CA, et al.: The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol* 2007;1:84–96.
- Hwang YS, Chung BG, Ortmann D, Hattori N, Moeller HC, Khademhosseini A: Microwell-mediated control of embryoid body size regulates embryonic stem cell fate via differential expression of WNT5a and WNT11. *Proc Natl Acad Sci U S A* 2009;106:16978–16983.
- Zhang C, Cao S, Toole BP, Xu Y: Cancer may be a pathway to cell survival under persistent hypoxia and elevated ROS: a model for solid-cancer initiation and early development. *Int J Cancer* 2015;136:2001–2011.
- Liao D, Johnson R: Hypoxia: A key regulator of angiogenesis in cancer. *Cancer Metastasis Rev* 2007;26:281–290.
- Yu H, Mouw JK, Weaver VM: Forcing form and function: biomechanical regulation of tumor evolution. *Trends Cell Biol* 2011;21:47–56.

31. Tse JM, Cheng G, Tyrrell JA, et al.: Mechanical compression drives cancer cells toward invasive phenotype. *Proc Natl Acad Sci U S A* 2012;109:911–916.
32. Vorsmann H, Groeber F, Walles H, et al.: Development of a human three-dimensional organotypic skin-melanoma spheroid model for *in vitro* drug testing. *Cell Death Dis* 2013;4:e719.
33. Chen LF, Cohen EE, Grandis JR: New strategies in head and neck cancer: understanding resistance to epidermal growth factor receptor inhibitors. *Clin Cancer Res* 2010;16:2489–2495.
34. Egloff AM, Grandis JR: Improving response rates to EGFR-targeted therapies for head and neck squamous cell carcinoma: candidate predictive biomarkers and combination treatment with Src inhibitors. *J Oncol* 2009;2009:896407.
35. Leeman RJ, Lui VW, Grandis JR: STAT3 as a therapeutic target in head and neck cancer. *Expert Opin Biol Ther* 2006;6:231–241.
36. Quesnelle KM, Boehm AL, Grandis JR: STAT-mediated EGFR signaling in cancer. *J Cell Biochem* 2007;102:311–319.
37. Brockstein B: Management of recurrent head and neck cancer: recent progress and future directions. *Drugs* 2011;71:1551–1559.
38. Goerner M, Seiwert TY, Sudhoff H: Molecular targeted therapies in head and neck cancer—an update of recent developments. *Head Neck Oncol* 2010;2: 8–12.
39. Stransky N, Egloff, AM, Tward, AD, et al.: The mutational landscape of head and neck squamous cell carcinoma. *Science* 2012;333:1157–1160.
40. Ramqvist T, Dalianis T: Oropharyngeal cancer epidemic and human papillomavirus. *Emerg Infect Dis* 2010;16:1671–1677.
41. Denaro N, Russi EG, Adamo V, Colantonio I, Merlano MC: Postoperative therapy in head and neck cancer: state of the art, risk subset, prognosis and unsolved questions. *Oncology* 2011;81:21–29.
42. Li H, Wawrose, JS, Gooding WE, et al.: Genomic analysis of head and neck squamous cell carcinoma cell lines and human tumors: a rational approach to preclinical model selection. *Mol Cancer Res* 2014;12:571–582.
43. Perez-Ordóñez B, Beauchemin M, Jordan RCK: Molecular biology of squamous cell carcinoma of the head and neck. *J Clin Pathol* 2006;59:445–453.

Address correspondence to:

Shilpa Sant, PhD

Department of Pharmaceutical Sciences

School of Pharmacy

University of Pittsburgh

3501 Terrace Street

527 Salk Hall

Pittsburgh, PA 15261

E-mail: shs149@pitt.edu

Abbreviation Used

2D	= two-dimensional
3D	= three-dimensional
DMEM	= Dulbecco's modified Eagle's medium
DPBS	= Dulbecco's phosphate-buffered saline
ECM	= extracellular matrix
EGFR	= epidermal growth factor receptor
FBS	= fetal bovine serum
HNSCC	= head and neck squamous cell carcinoma
IXM	= ImageXpress Micro
MWCS	= multiwavelength cell scoring
PDMS	= polydimethylsiloxane
PEGDMA	= polyethylene glycol dimethacrylate
PI	= propidium iodide
RLU	= relative luminescence unit
SEM	= scanning electron microscopy
SFM	= serum-free media
TMSPMA	= 3-trimethoxysilyl polymethacrylate