Engineered microfluidic bioreactor for examining the three-dimensional breast tumor microenvironment

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The interaction of cancer cells with the stromal cells and matrix in the tumor microenvironment plays a key role in progression to metastasis. A better understanding of the mechanisms underlying these interactions would aid in developing new therapeutic approaches to inhibit this progression. Here, we describe the fabrication of a simple microfluidic bioreactor capable of recapitulating the three-dimensional breast tumor microenvironment. Cancer cell spheroids, fibroblasts, and endothelial cells co-cultured in this device create a robust microenvironment suitable for studying in real time the migration of cancer cells along matrix structures laid down by fibroblasts within the 3D tumor microenvironment. This system allows for ready evaluation of response to targeted therapy. *Published by AIP Publishing.*
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I. INTRODUCTION

The tumor microenvironment (TME) plays a crucial role in tumor growth and metastasis, and for this reason, it is important to study cancer cells in reference to the dynamic contents of their environment. The complexity of cancer cannot be accurately modeled as the sum of the behavior of its components but must be modeled in reference to the products of the interactions of the components. Thus, many facets of cancer progression cannot be captured by limiting experiments to two-dimensional representations on plastic or glass substrates with only a single cell type. Hanahan and Weinberg have categorized six major “hallmarks” of cancer: continuous proliferation; loss of response to growth inhibitors; activation of invasion and metastasis;

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induction of a state of replicative immortality; induction of the growth of blood vessels into and around the tumor; and failure to die.¹ At least three of these categories involve direct interactions with other cell types and tissues. Failure to die may require evasion of the immune response. Induction of the growth of blood vessels in the tumor environment requires recruitment of endothelial cells to form blood vessels for nourishment and metastatic potential. Activating invasion and metastasis requires communication with multiple cell types between the time a cancer cell begins migrating from the initial tumor and the time it arrives at a secondary site. Indeed, it has widely been accepted that cancer cells can “recruit” other cell types and cause them to behave in a pro-tumorigenic fashion.² For example, endothelial cells are readily recruited and organized into blood vessels to provide nourishment to the tumor. Stromal cells, specifically fibroblasts, have been shown to provide oncogenic signals for induction of tumorigenesis, have been implicated in the provision of drug-resisting capabilities to the tumor, and are suspects in the promotion of angiogenic and pro-metastatic factors.^{2–4} Furthermore, leukocyte subsets can enhance or inhibit tumor growth and progression, such as tumor-associated macrophages or tumor-entrained neutrophils, respectively. Thus, the “soil” of the pre-metastatic niche either permits or blocks tumor cell seeding, as described years ago as the “seed and soil” principle.^{5,6} Yet, the processes involved in the inter-cellular communications pathways remain evasive.

Secreted factors from both the tumor and the surrounding stroma play major roles in cancer progression leading to metastasis. For example, epidermal growth factor (EGF) has been shown to be an important element in the progression of breast cancers.⁷ Other secreted factors, such as matrix metalloproteases (MMPs), tumor necrosis factor alpha (TNF- α), and transforming growth factor beta (TGF- β) all have been shown to play roles in cancer progression.⁸ Further, chemokines, a class of small, chemotactic cytokines, have been highly implicated in pathways involving cancer metastasis to secondary organs.⁹ For example, in breast cancer, the CXCL12 (SDF-1)/CXCR4 pathway has been shown to be crucial in metastasis to bone. Moreover, the bone microenvironment, which has a relatively high concentration of CXCL12, can serve as a secure location for migrating breast cancer cells that express the CXCR4 receptor. This pathway also includes activation of downstream factors such as PI3K, MAPK, and Ras, all of which are also significantly involved in breast cancer metastasis.¹⁰ However, cues regulating the emanation of these signals from individual cell types in the TME have not been fully elucidated. By studying these interactions in an environment that closely mimics the *in vivo* situation, we can more accurately identify key factors in cancer progression and metastasis.

Microfluidics is an emerging technology used to observe and control experiments on an extremely small scale. There are several benefits to the use of microfluidic devices over standard *in vitro* or *in vivo* assays. For example, with controlled microfabrication of functional, three-dimensional polymeric materials, smaller quantities of reagents are required and results can be obtained swiftly and with high throughput. Additionally, microfluidic bioreactors offer the unique ability to precisely control specific variables in these experiments (such as flow rates and gradients) without errors inherent in other models (e.g., a mouse dying prematurely of a cause other than the one being investigated). These properties of microfluidic bioreactors make it easier to isolate and observe changes in one experimental variable and to obtain results faster than would otherwise be possible.

In fact, a more biologically relevant microenvironment is crucial for understanding disease progression and therapeutic mechanisms. Mice are highly utilized in cancer research, even though it is known that mice metabolize drugs differently than humans due to genetic differences inherent between the two species.¹¹ These species differences result in a major complication in preclinical trials to evaluate drug efficacy, which is typically why orthogonal models are used and several experiments conducted to determine the pharmacodynamics and pharmacokinetics of the therapy. In contrast, microfluidic bioreactors can be used to simulate an *in vivo* TME by integrating the appropriate tissue environment and cell types in order to study factors that are at play in cancer progression. This simulation of the *in vivo* environment allows for more physiologically relevant study of tumor progression and drug response, at a fraction of the time and cost inherent in live animal models.

The flexibility of microfluidic applications is also helpful in understanding individual facets of the tumor microenvironment. For example, Bersini *et al.* and Jeon *et al.* created microvasculature systems using microfluidic bioreactors to study breast cancer extravasation into differing microenvironments.^{11,12} They found that stromal factors within the target microenvironment, as well as flow profiles within the vasculature, have significant roles in the successful extravasation of cancer cells.^{12,13} Other devices have been fabricated to study the interactions between stromal cells and the tumor, leading to advances in understanding cell-cell interactions within the microenvironment.^{14,15} Microfluidics have even been applied to the capture and purification of circulating tumor cells (CTCs), a rare event that may lead to further understanding cancer progression into the secondary site.¹⁶ As a result, microfluidic bioreactors can serve as valid models for examining the three-dimensional tumor microenvironment and the factors that play a role in cancer progression.¹⁷

The work we describe in this manuscript details the creation of a microfluidic device capable of simulating many properties of the tumor microenvironment. We combine an extracellular matrix (ECM), spheroids (aggregates of cancer cells thought to behave like an *in vivo* tumor), fibroblasts, and endothelial cells into a microfluidic device that allows for visualization of the migration of cancer cells out of a tumor spheroid and into the stromal matrix. We anticipate that this device may be used for analysis of metabolomics, qPCR, and proteomics, enabling a more in-depth look at the gene expression, protein secretion, and metabolic activity of the cells present in this microenvironment.

II. EXPERIMENTAL

A. Cell culture

Human breast adenocarcinoma cells, MDA-MB-231 (ATCC), were grown in T-75 flasks in complete Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with pen-strep/L-glutamine (PSLG) antibiotics. Standard culturing methods were used, passaging cells every 3–4 days, prior to confluency. MDA-MB-231 cells were transduced with a green fluorescent protein (GFP) lentiviral vector and selected via antibiotic resistance. Murine NIH-3T3 cells (ATCC) were cultured in the same manner as the adenocarcinoma cells. The NIH-3T3 cells were transfected with mCherry via lentivirus and selected using fluorescence-activated cell sorting (FACS) analysis. Normal-tissue associated fibroblasts (NAFs) were isolated from patient breast tissue and cultured under similar conditions as the aforementioned cell types. Human primary microvascular endothelial cells (HMVEC) (Life Technologies) were cultured in complete endothelial basal media (Lonza).

B. Spheroid preparation

MDA-MB-231-GFP⁺ cells were detached from the T-75 flask using trypsin and transferred to a 15 ml conical tube, which was then centrifuged at 500 rpm for 2 min. The supernatant was aspirated, and the cells were re-suspended in complete DMEM and counted in a hemocytometer. The cells were then diluted to a density of 100 cells/ μ l. A hanging drop technique was utilized to quickly create robust spheroids. Briefly, the cells in suspension were pipetted in 1 μ l droplets onto the inverted lid of a 100 mm well dish. The lid was then carefully re-inverted back onto its base, which had been filled with 5 ml of 1 $\times$ phosphate-buffered saline (PBS) solution to prevent dehydration. The well dish was placed in an incubator overnight, where the cells would aggregate into a spheroid at the bottom of each droplet. While typically used in an experiment the day after culturing, the spheroids would reliably stay viable for up to 3 days after initiation of culture.

C. Staining of primary cells

For experiments utilizing primary human fibroblasts or primary human microvascular endothelial cells, cells were incubated in a fluorescent dye to stain these cells reliably for a period of a week. Normal human fibroblast (NAF) cells were stained with 20 μ M CellTracker Red (CTR, ex/em: 577/602 nm) (ThermoFisher), while human microvascular endothelial cells

(HMVECs) were stained with 20 μM CellTracker Blue (CTB, ex/em: 371/464 nm) (ThermoFisher). Briefly, cells in a T-75 flask were first washed with $1\times$ PBS. An amount of appropriate dye was added to 10 ml of the respective culture media to make a concentration of 20 μM in both cases, and the dye-containing media was introduced to the flask. The cells were incubated for 45 min before removing the media, isolating the cells, and placing them in the microfluidic device.

D. Creation of microfluidic device

The microfluidic device was fabricated using standard photolithography methods and cast in (poly)dimethylsiloxane (PDMS) (Fig. 1). More detailed descriptions of microfluidic fabrications have been previously published.^{18–20} Inlet and outlet holes were punched using standard biopsy punches. The completed device was bonded to a blank piece of PDMS using corona discharge plasma bonding (Harrick Plasma).²¹ This blank piece of PDMS was in turn bonded to a glass slide. Bonding the device first to a blank piece of PDMS rather than directly onto the glass slide was performed both to prevent cells from adhering to the glass slide as well as to create a more *in vivo*-like microenvironment.

For sterilization, all channels of the completed device were flushed with 70% ethanol and heated for one hour to allow the ethanol to evaporate. The device was then flushed with 20% FBS-containing, phenol-red free DMEM (PSLG added) (Gibco), and submerged in a Petri dish filled with the same media in an incubator overnight. This submersion was performed to allow the media to saturate the PDMS and the system to equilibrate.

E. Extracellular matrix preparation and cell loading

After 24 h of equilibration, media was removed from the channels of the bioreactor to prepare for the three-dimensional tumor microenvironment. In experiments that incorporated endothelial cells, 6 μl of a 10 000 cells/ μl CTB-labelled HMVEC cell suspension was introduced to one of the side channels. These cells were incubated for 3 h before continuing the loading procedure.

FIG. 1. Creation of two-layer microfluidic device. (a) Representation of AutoCAD layout of the device. The dimensions of the center channel (purple) are 5.3 mm length, 2 mm width, and 180 μm height. Side channel (blue) heights were 100 μm . Star-shaped microposts (yellow) were spaced 5 μm apart to separate the channels and prevent crossing of extracellular matrix from one channel to another. Not to scale. (b) Schematic close-up depiction of the functioning device. The center channel is filled with extracellular matrix (purple), containing the spheroid (green) and fibroblasts (red). The punch-hole through which the spheroid enters the device is outlined as a faint, dashed circle. In a side channel, endothelial cells (light blue) have formed a barrier on the microposts to act as a barrier membrane for any cancer cells trying to extravasate from the microenvironment. Not to scale. (c) Cross section view of the device as depicted in (b). Not to scale. (d) Image of a microfluidic device prior to loading with ECM. The blue fluid was used to emphasize outline of channels. The red arrow denotes the hole by which a spheroid is introduced into the ECM-loaded device.

For all devices, whether or not HMVECs were added, an extracellular matrix (ECM), consisting of 56% advanced HEPES buffer solution, 24% Type 1 rat tail collagen (Corning), and 20% growth-factor reduced Matrigel (BD Biosciences), was created by mixing appropriate volumes of the reagents in an Eppendorf tube. These ratios were adapted from work by Hockemeyer *et al.* as ideal for creating a microenvironment in a bioreactor of this type.¹⁴ Mixing was done on ice to prevent preliminary polymerization of the matrix. Fibroblasts, either the mCherry-labeled NIH-3T3s or the CTR-labeled NAFs (depending on the experiment), were trypsinized, counted, and added to the ECM in a quantity that would result in a fibroblast (3T3 or NAF) to cancer cell (MDA-MB-231) ratio of $\sim 4:1$ in the device. $9\ \mu\text{l}$ of this ECM-fibroblast solution was then quickly added to the center channel of the device. A single MDA-MB-231 tumor spheroid was gently transferred into the device by lowering a hanging droplet onto the hole above the device's center channel [marked by the red arrow in Fig. 1(d)]. This allowed the droplet to fall into the hole, taking with it the intact spheroid. After the spheroid transfer was confirmed by microscopy, the device was incubated in a Petri dish for 45 min to allow the ECM to polymerize and the MDA-MB-231 spheroid to integrate into its new environment. Afterwards, the device was submerged in 20% FBS-containing, phenol-red free DMEM (PSLG added) and was imaged daily using an inverted fluorescence microscope with a $10\times$ objective (Zeiss). One should note that in several figures, the fluorescent images appear unfocused; this is because each image represents only a two-dimensional, focal-plane slice of the three-dimensional microenvironment. Images with unfocused areas are indicative of multiple cells which have migrated outside the focal plane (into and out of the page) but are still within the frame. As a result, the two-dimensional image blurs the fluorescence from cells outside of this plane (see Fig. 4).

F. Metabolomics assays

For analysis of metabolites, conditioned media from devices was isolated and filtered through a 3000 Dalton cut-off Amicon[®] Ultra-15 membrane device (Millipore). This allowed for effective separation of larger secreted factors, such as chemokines and most extracellular proteins, from smaller metabolites. The filtrate from the separation step was spun down at 500 rpm for 5 min. Metabolites from $250\ \mu\text{l}$ filtrate were extracted using an 80:20 MeOH/H₂O protein precipitation followed by overnight incubation at -80°C . These samples were then spun down at 15 000 rpm for 15 min and the supernatant transferred to a new Eppendorf tube. The supernatants containing the metabolites were dried *in vacuo* and were re-suspended in $150\ \mu\text{l}$ of 0.1% formic acid in water with 2% acetonitrile, followed by centrifugation for 5 min at 15 000 rpm to remove insoluble material.

Global untargeted metabolomics analyses were performed using full MS and data-dependent acquisition (DDA) analyses on a Q-Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Vanquish UHPLC binary system and auto-sampler (Thermo Fisher Scientific, Germany). Extracts ($5\ \mu\text{l}$ injection volume) were separated by reversed-phase liquid chromatography on a Hypersil GOLD $1.9\text{-}\mu\text{m}$, $2.1\text{ mm} \times 100\text{ mm}$ column (Thermo Fisher Scientific) held at 40°C . Reverse-Phase separation was performed at $250\ \mu\text{l}/\text{min}$ using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) with the following gradient: 1% B for 1 min, 1%–35% B over 9 min, 35%–70% B over 5 min, 70%–99% B over 4 min, 99% B held 3 min, 99%–1% B over 3 min, and 1% B held 5 min (gradient length 30 min).

Full MS analyses were acquired over a mass range of m/z 70–1050 under both electrospray ionization (ESI) positive and negative profile modes. Full mass scan was used at a resolution of 120 000 with a scan rate at $\sim 3.5\text{ Hz}$. The automatic gain control (AGC) target was set at 1×10^6 ions, and maximum ion injection time (IT) was at 100 ms. Source ionization parameters were optimized with the spray voltage at 3.0 kV, and other parameters were as follows: transfer temperature at 280°C ; S-Lens level at 40; heater temperature at 325°C ; sheath gas at 40, Aux gas at 10, and sweep gas flow at 1.

Tandem spectra were acquired using a data-dependent scanning mode in which one full MS scan (m/z 70–1050) was followed by 2 MS/MS scans. MS/MS scans are acquired in profile

mode using an isolation width of 1.3 m/z, stepped collision energy (NCE 20, 40, and 60), and a dynamic exclusion of 6 s. MS/MS spectra were collected at 15 000 resolution, with an AGC target set at 2×10^5 ions, and maximum IT of 100 ms.

G. Metabolite data processing and analysis

To investigate changes in the metabolic profile induced by external stimuli, two different conditions, before and after 48 h treatment with 0.7 μ M of the pan-PI3K inhibitor BKM120 (Buparlisib), were used in devices containing an MDA-MB-231 cancer cell spheroid and NIH-3T3 fibroblasts. For internal validation, the two conditions were both run in three process replicates, with each sample being injected twice, for a total of 6 points per condition. UPLC-MS/MS raw data were imported, processed, normalized, and reviewed using Progenesis QI v.2.1 (Nonlinear Dynamics, Newcastle, UK). All sample runs were aligned against a QC pool reference run, and peak picking was performed on individual aligned runs to create an aggregate data set. Features (retention time and m/z pairs) were combined using both adduct and isotope deconvolution. Data were normalized to all compounds as an abundance ratio between the run being normalized and a reference run. Statistically significant changes were identified using multivariate statistical analysis, including principal component analysis (PCA), and *p* values were generated using analysis of variance (ANOVA) or pairwise comparison. Three process replicates and two technical replicates from each sample type were used to calculate the fold change and *p* value, and the features were considered differentially expressed only if they met both criteria of fold change $\geq |1.5|$ and significance ($p \leq 0.05$). Feature lists generated from different individual comparisons were visually compared using Venn diagrams generated by the Venny software package.²²

Metabolomics pathway analysis was performed by Mummichog software version 2.0 using default parameters. Compound ions measurement files exported from Progenesis QI analysis software were used to generate the mummichog input files. Mummichog tested the enrichment of input metabolites against random data resampled from the list of compounds by permutations and produced an empirical *p* value for known biological pathways. Input metabolites in the significant pathways (*p* value ≤ 0.05) were linked against known metabolic pathways.²³

III. RESULTS AND DISCUSSION

A. Efficacy of microbio reactor

The bioreactor was validated as an environmentally relevant model, allowing for in-depth analysis of the tumor microenvironment and visualization of cancer progression and migration. Figure 2 shows still images from one such experiment, where an MDA-MB-231 breast tumor spheroid and NIH-3T3 fibroblasts were maintained for a period of fifteen days in the microbio reactor. The spheroid was observed to be growing and exploring its microenvironment over the time-period. This shows the device as a valid model for studying cell-cell interactions within the tumor microenvironment and may provide a useful model to query mechanisms behind cell recruitment and cancer migration from the primary tumor site.

IV. INVESTIGATING EFFECTS OF CELL-CELL INTERACTIONS

As mentioned above, the microbio reactor presented here offers insights into the process of cancer cell migration. Not surprisingly, we observed that heterotypic cell-cell contacts can begin quickly after initiation of the co-culture of different cell types in the bioreactor, in some cases, as quickly as four hours [Figs. 3(a) and 3(b)].

These contacts and interactions may serve as a foundation for cancer cell recruitment of the stromal cells via inter-cellular signaling. For example, we also observed distinct differences in 3T3 fibroblast phenotype when cultured in the presence or absence of a cancer cell spheroid. When in the presence of a spheroid, the fibroblasts created complex, fibril-like structures within the extracellular matrix, their length reaching distances of up to 2.5 mm in some cases [Figs. 3(c)–3(f)]. When NIH-3T3 fibroblasts were cultured within an ECM in the bioreactor in the

FIG. 2. Still images of cells in microfluidic reactor. Images show the growth of a cancer cell spheroid (green) over the course of two weeks. The MDA-MB-231 tumor spheroid grows during the time period, but cells do not migrate outside of the original field of view until NIH-3T3 fibroblasts (red) reach the spheroid. Scale bar $\sim 100\ \mu\text{m}$.

absence of a cancer spheroid, however, the cells created a flat monolayer similar to normal culture in a T-75 flask [Figs. 3(g)–3(h)].

The fibrils that developed in the presence of the cancer spheroid were observed to provide a scaffolding for the cancer cells' migration. In experiments performed with fibroblasts, the MDA-MB-231 cancer cells preferentially migrated only in areas occupied by fibroblast fibrils and, given the opportunity, moved first along the fibroblasts in a single-file manner (Fig. 4). These results suggest that even though cancer cells may possess an intrinsic capability to metastasize, the cells may wait until external stimuli are provided or other environmental conditions are met before initiating migration leading to metastasis.

The mechanism(s) behind the change in fibroblast phenotype and their subsequent facilitation of the migration of cancer cells is still unknown, and the identification of crucial pathways in this transition would be further used for understanding of the role of the cancer microenvironment in metastasis.

V. IMPLEMENTATION OF PRIMARY CELLS

A change in labeling technique was required to evaluate the interactions of primary cultures of human fibroblasts and human endothelial cells, since these are short-term cultures and selection of cells stably expressing a transduced tag is not practical. We used *intravital* labeling for these experiments, which limits the duration of the experiments to 7 days because the dyes begin to diffuse out of the cells by 7 days of culture. The microfluidic bioreactor included primary human fibroblasts (labeled with CellTracker Red) and primary human microvascular endothelial cells (labeled with CellTracker Blue), along with the GFP-tagged MDA-MB-231 tumor spheroid, in the bioreactor. Figure 5 shows some images of these cell types within the device, where the primary HMVECs are located in a side channel of the device, and the primary NAFs and MDA-MB-231 GFP⁺ spheroid have become integrated into the ECM in the center channel. These experiments show a potential for a microfluidic bioreactor of this type to support multiple primary human cell lines simultaneously, in order to create a more coherent, biologically relevant microenvironment.

VI. METABOLOMICS ASSAY

We have recently demonstrated that microfluidic reactors can provide sufficient volumes of conditioned media to support mass-spectrometric metabolomic analysis of the cellular response

FIG. 3. Cell-cell interactions within the TME. MDA-MB-231 cancer cells (green) are seen interacting with NIH-3T3 fibroblasts (red). (a) Interactions between the two cell types are seen as early as 4 h into an experiment. Inset shows an enlarged field near the spheroid detailing a specific contact between a cancer cell and a fibroblast. (b) In a separate experiment, by day two of co-culture of MDA-MB-231 cells and NIH-3T3 fibroblasts, multiple cell-cell contacts are seen as the cancer cells grow within their surrounding microenvironment. Scale bar $\sim 100 \mu\text{m}$. Differences in the NIH-3T3 fibroblast (red) phenotype in response to the presence or absence of a spheroid were also observed. (c) Fibroblast fibril reaching approximately 2.5 mm across the bioreactor (spheroid present but out of frame to the top of the image). Image created using the MosaicJ plug-in in ImageJ.²⁴ (d)–(f) Images of different fibril-like morphologies adopted by fibroblasts when in the presence of a spheroid (out of frame). Structures are complex, well-defined shapes within the ECM. (g) and (h) Flattened morphologies adopted by fibroblasts when in the absence of a tumor cell spheroid. Scale bar $\sim 100 \mu\text{m}$.

to a challenge.²⁵ To determine whether a similar analysis might be capable of addressing questions regarding the mediators of this interaction between the MDA-MB-231 cancer cells and stromal fibroblasts, we performed a metabolomics analysis on the cells, matrix, and media from the micro-bioreactors from two separate experiments over a time course of 48 h in triplicate. For metabolomics analysis, media effluent from devices containing a cancer cell spheroid and NIH-3T3 fibroblasts were collected after exposure to control (C48) or $0.7 \mu\text{M}$ of the pan PI3K inhibitor, BKM120 (T48), for 48 h. The media were then analyzed for metabolites using reverse phase liquid chromatography mass spectrometry (IM-MS-MS), in positive and negative mode in triplicate.

The metabolomics data [Fig. 6(a)] show distinct global metabolomics profiles in the presence or absence of the inhibitor. Pair-wise comparisons of the metabolomics signatures for

FIG. 4. MDA 231 cancer cells preferentially migrate on NIH-3T3 fibroblast fibrils within the bioreactor. (a) Brightfield image of the microfluidic device co-cultured with MDA-MB-231 spheroid and NIH-3T3 fibroblasts (Day 13). The barrier created between the spheroid and fibroblasts at the bottom of the device (green arrow) is apparent. The fibroblasts have also created a fibril reaching to the top of the device (red arrow). (b) 3D image made using the Z-Project application in ImageJ, same experiment as in (a). Cancer cells (green) can be seen crawling up the fibroblast fibril (red) for a vertical distance of over 2 mm. (c) Separate experiment. Cancer cells (green) migrating along fibroblast fibrils (red). Cancer cells were capable of crawling distances of over 2 mm on these fibrils during the course of 6 days. (d) However, cancer cells preferentially do not migrate to areas where fibroblasts are not present. Scale bar $\sim 100 \mu\text{m}$.

cancer cells without (C-control) or with (T-treated) PI3K inhibitor treatment for 48 h illustrate a statistically significant number of metabolite compounds observed in response to treatment and were prioritized using the significance criteria ($p \leq 0.5$ and a fold change of $\geq |1.5|$, testing performed in triplicate). Figure 6(b) shows Venn diagrams which allow for the second-order visualization of the theoretical meta-analysis applied to identify metabolite compounds unique and specific for these experiments (i.e., compounds that are detected only in the inhibitor-treated but not in the control).^{26,27} Using metabolomics data network analysis based on metabolite accurate mass measurements (unique feature) matched against known canonical pathways using Mummichog algorithm version 2.0, we predicted significantly altered metabolic pathways upon inhibitor treatment [Fig. 6(c)].²³ A list of the pathway activities significantly affected by the treatment is listed in Table I in the [supplementary material](#). More metabolomic information is also found in Figs. 1–3 of the [supplementary material](#). Of note, we observed that PI3K activity alters tyrosine metabolism and beta-alanine metabolism as was shown earlier by Caiola *et al.*²⁷

FIG. 5. Images incorporating all human cells in the device. (a) Day 2: CellTracker Red labelled NAFs in the middle of the device (spheroid out of frame). The cells appear to be organizing in a morphology that later may develop into fibrils. (b) Day 1: GFP⁺ spheroid with CellTracker Red labelled hNAFs fibroblasts in device. (c) Day 5: CellTracker Blue labelled HMVECs along the side channel of the device. Arrows point to some of the endothelial cells in the frame. Scale bar $\sim 100 \mu\text{m}$.

FIG. 6. UPLC-MS/MS global metabolomic profile analysis upon treatment with the PI3K inhibitor BKM120, for 48 h. (a) Global principal component analysis (PCA) of inhibitor-treated and untreated cells inside the microfluidic bioreactor, illustrating that distinct metabolic signatures or profiles were observed due to treatment (T) as compared to control (C) from samples taken before (t0) and after 48 h of exposure (t48). Ellipses included to show valid grouping of replicated samples. (b) Second-order visualization of the theoretical meta-analysis applied to identify metabolite compounds whose changes between t0 and t48 were unique and specific for the inhibitor treatment. Pair-wise comparisons of the metabolomics signatures for cancer cells with (T-treated) or without (C-control) inhibitor treatment for 48 h (Tt48 vs Tt0 and Ct48 vs Ct0) illustrate significantly different, changing metabolic compounds, some unique and characteristic to the treatment (significant criteria: $p \leq 0.05$, fold change $\geq |1.5|$) (RPLC-positive). (c) Top significant pathways predicted by network analysis based on metabolite accurate mass measurements (unique feature) matched against known canonical pathways, for treated samples using Mummichog algorithm described in Li *et al.*²³

VII. CONCLUSIONS

The microfluidic bioreactor presented here can serve as an important tool for answering complex questions pertaining to the mechanisms behind cell-cell interactions in the microenvironment, including cancer progression and migration. The device is fabricated using an inexpensive, readily available, biocompatible polymer that allows for an isolated, yet biologically relevant, microenvironment. This type of device fabrication also enables the creation of several devices in a short time period, which could result in the ability to analyze multiple experimental variables at once and thereby serve as a high-throughput screen. Furthermore, such microfluidic bioreactors are disposable, so that re-using devices made under different techniques (such as laser ablation in silicon or reactive ion etching) is unnecessary, as is dealing with the concomitant sterilization procedures. This allows for reduced time on the part of the researcher and prevents contamination issues that could invalidate experimental results.

We observed several interesting properties of the co-cultured cells within the TME in this microbioreactor system. NIH-3T3 fibroblasts, when exposed to a tumor cell spheroid, create fibril-like structures reaching throughout the ECM. In contrast, fibroblasts cultured without a spheroid do not form fibrils in the device. Furthermore, we observed cancer cells migrating on these fibroblast structures in a manner that mimics migration associated with metastasis.²⁸ It is known that stromal cells such as fibroblasts play a major role in cancer progression, and study of these cell-cell interactions within this type of bioreactor could yield significant advances in our understanding of cell-cell interactions in the microenvironment.²

It is also possible to easily change the structure of experiments within a device of this type. While we focused primarily on the interactions between cancer cells and surrounding fibroblasts, we also were able to culture endothelial cells within a side channel of the bioreactor. Further studies incorporating endothelial cells could provide data regarding breast cancer extravasation from the primary tumor site through the endothelium. It may also be possible to include leukocytes or other immune cells within the bioreactor, in order to have a more cohesive view of interactions between cancer cells, cancer associated fibroblasts, endothelial cells, and immune cells during cancer progression.

While this device has limitations in reference to quantitation of movement over the context of several devices, in that the position of the spheroid in reference to the fibroblasts will vary a bit from one device to another device, one observes this same type of variation in human tumors. Moreover, the behavior of the tumor cells in the spheroid varies in reference to the nearness of the fibroblasts. This has been shown by other groups^{29,30} and our data support these earlier findings, now using a microfluidic bioreactor. Another novel strength of this device is the wide range of end-point assays that can be performed on the cells and conditioned media. Metabolomics, qPCR, and proteomics assays can all be performed on the system to yield highly annotated molecular analyses to understand biologically driven processes. For example, inhibition of PI3K has been shown to have significant effects on glucose metabolism. Here, in a limited sample size experiment, we observed that BKM120 enhanced fructose and mannose metabolism while reducing purine metabolic pathways (see the [supplementary material](#)). Altogether, the ability to run multiple assays on human cells co-cultured under *in-vivo*-like conditions could provide insight into differences in gene and protein expression and changes in response to drugs, to further explore the role that metabolites and secreted factors play in cancer progression and cell recruitment.

In conclusion, the microfluidic device presented here is a valid tool to study the processes involved in cancer progression and metastasis in relation to the tumor microenvironment. Pathways crucial to fibroblast recruitment, initiation of angiogenesis, mechanisms of drug resistance imparted to the tumor by the stroma, and analysis of the effects of the tumor on the stromal cells can all be studied using this microbioreactor. The small size and unique capabilities and reliability of this device also enable parallel testing of several different conditions, providing accurate results at a fraction of the costs and time inherent in other models.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for global metabolic profile analysis. It also shows the significant pathway activity by treatment with the PI3K-inhibitor BKM120.

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There are no conflicts to declare.

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