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Thick-tissue bioreactor as a platform for long-term organotypic culture and drug delivery

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Abstract

We have developed a novel, portable, gravity-fed, microfluidics-based platform suitable for optical interrogation of long-term organotypic cell culture. This system is designed to provide convenient control of cell maintenance, nutrients, and experimental reagent delivery to tissue-like cell densities housed in a transparent, low-volume microenvironment. To demonstrate the ability of our Thick-Tissue Bioreactor (TTB) to provide stable, long-term maintenance of high-density cellular arrays, we observed the morphogenic growth of human mammary epithelial cell lines, MCF-10A and their invasive variants, cultured under three-dimensional (3D) conditions inside our system. Over the course of 21 days, these cells typically develop into hollow “mammospheres” if cultured in standard 3D Matrigel. This complex morphogenic process requires alterations in a variety of cellular functions, including degradation of extracellular matrix that is regulated by cell-produced matrix proteinases. For our “drug” delivery testing and validation experiments we have introduced proteinase inhibitors into the fluid supply system, and we observed both reduced proteinase activity and inhibited cellular morphogenesis. The size inhibition results correlated well with the overall proteinase activities of the tested cells.

Keywords

microfluidic; bioreactor; bioMEMS; organotypic cell culture; long-term cell culture; breast cancer; proteinase activity; mammosphere; MCF-10A

Introduction

Traditional cell culture approaches that use monolayer culture within tissue culture dishes or well plates cannot recreate the complex chemical and physical microenvironment that exists in tissue. In addition, cells themselves are in constant communication with each other and the surrounding matrix, modifying it or modifying their own behavior based on the changes in their adjacent microenvironment(1). Realistic assessment of cellular processes regulating tumorigenesis and particularly of *in vitro* anti-cancer treatments must take both cell-cell and cell-matrix components into account. Furthermore, culturing cells within three-dimensional (3D) systems facilitates cellular growth that better recapitulates *in vivo* cellular behavior, molecular functions, and gross morphological features(2;3). Moreover, cellular response to

perturbations in the system, such as application of anti-tumor therapeutics, can differ depending upon the culture system used. For instance, in MCF-7 cancer cell lines it has been shown that the effectiveness of anti-tumor treatments on cells that were cultured in 3D matrix was reduced by orders of magnitude compared to the treatments on the same cells cultured in a monolayer(4;5). There is an obvious need to develop cell culture techniques incorporating three-dimensional matrix, where both chemical and physical microenvironments more closely approximate physiological conditions. It is also important for these 3D culturing systems to competently provide cell maintenance conditions over time spans of several weeks in order to allow completion of cellular morphogenic programs(2;3;6).

In recent years multiple approaches have been developed that employ microfluidic devices to recreate the 3D microenvironment *in vitro* and to grow large populations of cells at tissue-like densities(7). Microfluidic technology and devices applied to cell culture offer important advantages over standard culture techniques. There is significant reduction in size and in reagent use; cells can potentially be cultured at higher densities with more realistic cell-to-fluid volume ratios that do not dilute paracrine and autocrine factors; media flows are laminar and the flow rates can be easily adjusted in real-time for proper delivery of media, proteins, or assay reagents; and various spatial and temporal chemical gradients can be established within the microfluidic cell culture devices(8;9). Our group is focused on developing a microfluidics-based bioreactor for three-dimensional long-term culture of large populations of cells or small portions of excised tissue in a controlled microenvironment.

There have been several approaches used in the development of microfluidics-based cell culture bioreactors that address issues of long-term culture, the majority of which concentrate on interrogation of cells growing in a monolayer for up to 12 days. However, as *in vitro* monolayer cultures do not always faithfully mimic cellular *in vivo* behavior, the current trend is to grow cells in a more “natural,” tissue-like, 3D microenvironment, while still utilizing the advantages afforded by microfluidics. Even though this new technology is still under development, it has been successfully applied to maintaining viability of tumor spheroids for several hours for cancer treatment screening(10), monitoring the weeks-long process of differentiation from myoblasts to myotubes(11), and observing adipogenic and osteogenic differentiation of mesenchymal stem cells (MSCs)(12). Some groups have attempted to construct microfluidic bioreactors suitable for maintaining proper functionality of small tissue samples for periods of time ranging from a couple of hours to 3 days(13). Dhurjati *et al.*(14) introduced a large-format microfluidic bioreactor (25 cm² culture area) within which fetal osteoblasts were cultured uninterrupted for four months (120 days). Bauer *et al.*(15) used collagen-filled PDMS microchannel plates(16) and similarly filled commercial polystyrene microchannel plates to grow 3D co-cultures of human mammary fibroblasts and T47D breast carcinoma cells for 5 days, and demonstrated the effects of inhibition of paracrine signaling on fibroblast-induced carcinoma cell mitogenesis. All of these systems performed well for the intended purpose; however, many needed expensive external active fluid-pumping sources, and some required complex multilayer assembly, *e.g.*, 10 layers(17), or additional mechanisms to provide shear stresses.

Our Thick-Tissue Bioreactor (TTB) is a compact, stand-alone, gravity-fed microfluidic system that 1) can be easily sterilized, assembled under sterile conditions, and transported between culture incubator and the imaging station; 2) maintains cell viability for long-term cell culture, with the future goal of long-term maintenance of tissue (*i.e.*, tumor) biopsy samples; and 3) allows convenient visualization of the response of cells and tissues to drug agent challenges. To validate the suitability of our custom-built TTB to maintain long-term organotypic culture and regulate drug delivery, we utilized the long-term culture and morphogenic program of breast epithelial cell lines, MCF-10A and their invasive and

tumorigenic variants (MCF-AT1 and MCF-CA1d), grown in 3D gelled tissue matrix (*i.e.*, Matrigel)(6;18). The MCF-10A human mammary epithelial cell lines undergo a distinct morphogenesis pattern that results in the formation of hollow “mammospheres” in standard 3D Matrigel culture systems over the course of 21 days(6). This complex process requires alterations in a variety of cellular functions, including proliferation, migration, matrix degradation, and cell survival, making this a useful system for testing a long-term cell culture bioreactor and the long-term effects of inhibitory agents, such as matrix proteinase inhibitors, targeted towards regulating these cellular processes. Validation of drug delivery into the system was accomplished by applying matrix proteinase inhibition to the system to impair the morphogenic process and compare it against mammosphere formation in the absence of proteinase inhibition.

Materials and Methods

Design and fabrication of Thick-Tissue Bioreactor (TTB)

The Thick-Tissue Bioreactors (TTB) were fabricated in polydimethylsiloxane (PDMS) using standard soft lithography and replica molding techniques. The device consists of two parts: 1) a 1 mm thick PDMS membrane that contains four Cell/Biopsy growth chambers that are 3 mm in diameter and separated by 5.85 mm, and 2) a 4 mm thick PDMS top piece that contains four microfluidic supply networks, one for each chamber (Figure 1A and B). The cell culture membrane is bonded to a 2 × 3, 1 mm thick glass slide to provide structural support and to allow cell imaging through an inverted microscope. Growth chambers are in direct contact with supply networks or, alternatively, are separated from microfluidic supply channels by a polycarbonate filter (Osmonics, Inc.) with 0.4 μm pores, which maintains diffusional exchange of nutrients and waste products and prevents leakage of extracellular matrix (ECM) into the microfluidic channels. These pores are too small to allow cell migration. The supply network for each of the cell growth chambers consists of eight parallel microfluidic channels 100 μm wide × 100 μm tall, separated by 100 μm walls. These are connected together to larger 800 μm wide input / output regions of the network (shown in blue in Figure 1A and B). Splitting a larger supply channel for each of the cell culture chambers into several parallel paths allows maintenance of structural integrity of the supply network, prevents possible channel collapse during the reactor assembly and clamping, and provides alternative pathways for the perfusing media in case some of the Matrigel escapes and blocks one of the smaller channels. The entire reactor is clamped together under sterile conditions in a custom-built polycarbonate and stainless steel clamp that allows for easy alignment of the reactor components, provides structural stability to the assembly, and evenly distributes holding pressure across the PDMS surfaces (Figure 1C and D).

All four individual reactors in a single TTB cartridge are gravity-fed from a modified 14 mL tube with a loose-fitting cap (*i.e.*, a “snap cap tube”) mounted in a custom-built holder. We described previously our modifications to the tube (19). The output of the feeding tube is directly connected to a manifold assembly containing 100 μm wide and 50 μm tall microfluidic channels that split the input flow into four even streams for each of the reactors within the cartridge. A second output manifold (on the waste side of the reactor) is used as in-series fluid flow resistance to passively regulate the flow rate, to recombine the outflow from four reactors, and to direct effluent into a waste collector (Labtainer™ from HyClone) or, alternatively, to sample extraction systems (Figure 1D and E) that collect fluid while preventing retrograde contamination of the reactor(19). The fluid level in the feeding tube was typically checked daily and replenished every other day to be maintained at 15 cm above the bioreactor surface. All tubing was 0.020 ID, 0.060 OD Saint-Gobain Tygon microbore tubing (Part # AAQ04103) from Fisher Scientific.

Bioreactor assembly and clamping

The PDMS membrane with cell chambers resting on a glass slide were sterilized by autoclaving, and the remaining partially assembled bioreactor components (clamps, lids, supply networks, tubing, etc.) were sterilized using gamma irradiation. Other sterilization methods were tested and abandoned because ethanol and ethylene oxide readily partition into plastic components(20), and autoclaving is not suitable for the assemblies containing materials with drastically different coefficients of thermal expansion. The 2 partially assembled reactor portions (illustrated in Supplemental Figure S1) allowed for ease in loading the 3D matrix and cell components and the subsequent easy alignment and combination of the two portions into a complete system. The sterilized individual TTB components were fully assembled in a standard cell culture biosafety laminar flow hood using standard aseptic techniques with care to maintain the sterile conditions of the internal working components of the TTB that are in contact with cells and media. After the cell chambers were loaded with Matrigel/cell mixture, they were initially sealed using the top PDMS piece, which contains the microfluidic supply network with dry channels, to avoid possible cross-contamination between the four adjacent cell growth chambers, and also to avoid the possibility of trapping air bubbles in the supply channels. Next, the glass slide containing the loosely sealed bioreactor components was clamped in position via a pressure plate and a lid, which was securely attached to the pair of stainless steel holders with 4–40 stainless steel screws. Both the lid and the pressure plate were machined from ¼" thick abrasion-resistant polycarbonate plate purchased from McMaster-Carr (Part # 8707K131).

Cell lines and culture

MCF-10A and MCF-10A pBabe (vector control) cell lines were obtained from Joan Brugge (Harvard Medical School, Boston, MA)(6). Ras-transformed MCF-10AT1 and the tumorigenic MCF-10CA1a and MCF-10CA1d variants were obtained from Dr. Fred Miller (Barbara Ann Karmanos Cancer Center, Wayne State University, Detroit, MI)(18). These MCF-10A and cell line variants form varying degrees of invasive structures in 3D Matrigel, depending upon the degree of transformation and tumorigenicity of the cell line. Cells were maintained in "Growth Media" (DMEM/F12, 5% horse serum, 0.1 µg/ml insulin, 0.5 µg/ml hydrocortisone, 0.1 µg/ml cholera toxin, and 20 ng/ml EGF) unless specified otherwise.

3D Matrigel culture/mammosphere formation

All cell culture experiments were done in parallel using both our TTB bioreactors and standard 8-well chamber slide cell culturing techniques. Cell chambers of both the TTB bioreactor systems and the 8-well chamber slide were coated with 2 µl and 30 µl of low growth factor Matrigel, respectively. Then the cells were re-suspended in Matrigel (3×10^5 cells/ml) and plated into bioreactor cell chambers (~5 µl/well) or 8-well cell chambers (~70 µl/well) and allowed to gel for 1 hour at 37°C. Based on chamber dimensions, these volumes of Matrigel resulted in an equal thickness of 1 mm for both culture systems. Cells were maintained with Growth Media by either media changes every 3 days (8-well chamber) or continuous perfusion in the bioreactor. Mammospheres were cultured in the absence or presence (when indicated) of the following 1x proteinase inhibitor (PI) cocktail: 12.5 µM GM-6001 (metallo-); 125 µM E-64 (cysteine); 50 µM Pepstatin A (aspartate); 1 µM Leupeptin (cathepsin D); 1.1 µM Aprotinin (serine).

Drug toxicity measurements

For MCF-10 CA1d cell monolayer drug toxicity testing using docetaxel, 1.0×10^4 cells were plated into each TTB cell chamber and 3.0×10^4 cells/well into an 8-well chamber slide. Cells were cultured with increasing concentrations of docetaxel (0, 1, 10, 100 and 1000 ng/ml) as indicated for 24 hours. For establishing the threshold of non-toxicity of the

proteinase inhibitor cocktail, we plated MCF-10A cells and cell line variants into 8-well chamber slides (5000 cells/chamber), and treated them in the absence or presence of increasing concentrations of the proteinase inhibitor cocktail (0.5 x, 1x and 2x) for 48 hours. We assessed cell viability using Live/Dead Assay (Molecular Probes) according to manufacturer's directions, using 1 μ M of Calcein AM (*i.e.*, live stain indicator) and 2 μ M of Ethidium homodimer-1 (*i.e.*, dead stain indicator).

Protease activity measurements

Mammospheres were first established for 6 days using a modified overlay method(6). Briefly, after laying down a plug of 1:1 Matrigel/BD Puramatrix Peptide Hydrogel (BD Biosciences) mixture in both the bioreactor (2 μ l) and 8-well (10 μ l) cell chamber systems, cells (3×10^5 cells/ml) were plated into the bioreactor cell chamber (500 cells/5 μ l Matrigel:hydrogel mixture/well) or the 8-well cell chamber (5000 cells/20 μ l Matrigel:hydrogel mixture/well) and allowed to gel for 1 hour at 37°C. Cells were cultured for 6 days to form mammospheres in phenol red free Growth Media containing 2% of Matrigel. To assess proteinase activity, on day 6 the media was aspirated and mammospheres were overlaid with 25 μ g/mL of DQ gelatin (Ex/Em 495/515 nm; Molecular Probes) in peptide hydrogel. Following 48 hours of incubation, we assessed protease activity by the increase of fluorescence detected using a Zeiss LSM 510 scanning confocal microscope.

Phalloidin and real-time SYTO-63 staining

To visualize mammosphere formation of live cell cultures, *in situ*, cell nuclei were stained using 1:5000 dilution of Syto 63 (Molecular Probes). Alternatively, mammospheres were fixed at set time points with 10% buffered formalin and stained using 1:400 dilution of phalloidin in 0.5% Triton-X and 1:3500 of Hoescht to visualize nuclei. A 1 mL syringe of diluted fluorophore was pumped through the parallel channels and incubated for 1 hour, after which the reactor system was washed with 1 ml of 1X PBS. The PBS-filled tubing was closed with Luer-lock stoppers. Confocal images within bioreactors were taken with a Zeiss LSM 510 scanning confocal microscope.

Determination of extracellular matrix proteinase activity

Media from MCF-10A and cell line variants cultured to confluence in 100 mM plates was replaced with serum-free experimental buffer (DMEM/F12 containing 5 ng/ml EGF/ 10 μ g/ml Insulin), and conditioned media was collected following 48 hours of incubation. 1/10 total volume was concentrated and analyzed for the ability to cleave 3 mMol dye-quenched (DQ)-gelatin (Molecular Probes) in $\text{Zn}^{2+}/\text{Ca}^{2+}$ Tricine buffer (50 mM Tricine, pH 7.4, 0.2 M NaCl, 10 mM CaCl_2 , 50 μ M ZnSO_4 and 0.005% Brij 35) in the absence and presence of the protease inhibitor cocktail (2.2 μ M Aprotinin, 25 μ M GM-6001, 250 μ M E-64, 100 μ M Pepstatin A and 2 μ M Leupeptin; Calbiochem)(21). Fluorescence (excitation: 485 nm; emission: 538 nm) was measured at 37°C, as a function of time using an MFX Microtiter Plate Fluorometer (Dynex Technologies, Inc., Chantilly, VA). Fluorometric measurements were used to calculate an initial rate of enzyme activity (fluorescence units/minute). As a positive control, DQ-Gelatin was cleaved to completion with 10 ng active-MMP-9.

Determination of microfluidic flow rates

Flow measurements through the TTB were made using the NanoFlow sensor from IDEX Health & Science (formerly, Upchurch Scientific) with a sampling rate of 2 Hz. The NanoFlow sensor was delivered pre-calibrated from the supplier, and the flow rate was confirmed using precision syringe pumps. Tests were performed on a fully assembled system (without cells) inside a custom-built chamber at a constant temperature of 37°C and

100% humidity, mimicking conditions inside the tissue culture incubator. The basic technology for regulating flow through the device involved 1) a fixed height of the gravity supply reservoir and 2) experimental determination of the required fluid resistance offered by the 4-way input splitter manifold in conjunction with the fluid resistance offered by the 4-way output combiner manifold. The 50 μm height of the input manifold microchannels was chosen to provide sufficient fluid resistance to ensure passive volume sharing between the four supply channels of the bioreactor array. To provide a reproducible method of varying bioreactor fluid flow between different experiments, we fabricated a number of different output manifolds, each with the same topology and the same 100 μm channel width as the input manifold but with different channel heights (range 5–60 μm). By selecting an appropriate output manifold we could provide calibrated fluid flow to the bioreactor. The head pressure was kept constant at 15 cm (see Figure 2).

Addressing possible partitioning of molecules into the PDMS bioreactor walls

We used Rhodamine B, a water soluble dye well known to partition into PDMS(22), to evaluate various methods of treating PDMS microfluidics surfaces in order to minimize the possible absorption of bioactive molecules. Microfluidic channels 50 μm tall and 50 μm wide were fabricated using soft lithography and replica molding as described above. A set of 8 channels was molded in a circular Petri dish from the same wafer with the same batch of PDMS mixed at the same time. After curing in the oven at 65°C overnight, rectangular blocks of PDMS containing 2 channels each were cut out, access holes were punched, and the devices were allowed to rest in air at room temperature for 1 week. Then devices were divided into four groups (2 channels in each) based on treatment: 1) native untreated PDMS, 2) oxidized PDMS stored dry, 3) oxidized PDMS stored wet, and 4) freshly oxidized PDMS. Oxidation was accomplished in a low pressure air-filled plasma oven (PDC-32G, Harrick Co.) for 30 seconds on “High” power setting (18W delivered to RF coils), and PDMS channels were bonded to a flat piece of PDMS to seal them. Groups 1 and 2 were stored dry, while group 3 channels were loaded with DI water and the whole device subsequently stored under water. After 1 week of storage under specified conditions for groups 1–3, channels from the 4th group were freshly oxidized and bonded. Then the channels from all four groups were loaded with 100 μM solution of Rhodamine B (MW=479.02 g/mole) in DI water. To minimize evaporative effects, access ports were sealed with Scotch tape and devices were stored in 100% humidity for 24 hours. After the incubation period, channels were then emptied and washed with 3 mL of DI water each. After drying with nitrogen gas, devices were sectioned and examined on an Axiovert 25 fluorescent microscope equipped with a Q-Color 5 CCD camera and TxRed fluorescent filter set. Line profiles were collected and analyzed with ImageJ.

COMSOL modeling

Simplified 2D and 3D models of the TTB were created in COMSOL Multiphysics environment (Supplemental Figure S3) to estimate diffusive fluxes of delivered agents (*i.e.*, nutrients, growth factor, and pharmaceutical treatments) and their evolution over time inside the cell culture chamber. The 2D model consisted of a 1 mm $\times$ 3 mm rectangle representing a cross-sectional view of the cell chamber in its widest part, with eight 100 μm $\times$ 100 μm rectangles centered on top of the cell chamber simulating a cross-sectional view of the supply network. Since the fluid in the supply channels during our experiments was always moving, the concentration of the “drug” inside the modeled supply channels was assumed to be constant. For model simplicity, the supply concentration was kept at an arbitrary unit of one, while concentration inside the cell chamber was initially zero. Delivered treatment inside the supply channels was allowed to freely diffuse through the filter into the collagen-filled cell chamber with the diffusion coefficient of $D = 2.75 \times 10^{-12} \text{ m}^2/\text{s}$. This diffusion coefficient is for the diffusion of a ~2 MDa FITC-labeled dextran molecule inside

collagen(23), representing the worst case scenario for delivery of a large molecule agent as compared to the more rapid diffusion of smaller-sized molecules, such as growth factors, antibodies, or chemical inhibitors that might be applied inside our thick-tissue bioreactor.

In the 3D model, the cell chamber was represented by a short cylinder 1 mm tall and 3 mm in diameter. Since the concentration inside the supply network channels was assumed to be constant, and in order to simplify computations and the consequent visualization, in the 3D model these channels were substituted with eight simple 100 μm wide planes acting as constant concentration boundaries positioned on top of the culture chamber. As in the case of the 2D model, the diffusion coefficient inside the chamber was assumed to be $2.75 \times 10^{-12} \text{ m}^2/\text{s}$. This allowed us to visualize possible spatial perturbations of the delivered concentration gradient due to the cylindrical nature of the chamber coupled with eight rectangular channels.

Results

Effect of flow rates within the TTB

We tested several configurations of the output manifold for their ability to regulate microfluidic flow rates within the TTB, including flow channel height variation and incorporation of valves (see Figure 2 and Supplemental Figure S2). Commercial manifolds yielded a media flow rate much above the target rate of 1 ml/day (Supplemental Figure S2A). We examined whether the simple screwdriver-adjusted TURN valves could regulate media flow and drug delivery under dynamic conditions when cell population is constantly changing ((9); Supplemental Figure S2B). These valves worked extremely well for starting and stopping the flows; however, regulation of the flow through each of the individual manifold outputs using TURN valves proved to be challenging. When we used gravity-induced flow going through the input manifold to the bioreactor cartridge, any adjustments with a single TURN valve in each individual leg of the manifold splitter resulted in the flow redistribution through other reactors in the cartridge, making it difficult to accurately adjust flow through each of the individual reactors within the cartridge. As an alternative to individual channel TURN valve-adjusted flow regulators, we investigated use of pre-fabricated microfluidic flow resistance manifolds (Figure 2). Flow rates as a function of the waste manifold channel height (*i.e.*, flow resistance) were measured with the NanoFlow flow meter (IDEX Health & Science, formerly Upchurch Scientific; Figure 2B). We found that by selecting a manifold with an appropriate channel height, we could produce a sufficient resistance to flow that, in combination with the fixed height of the supply reservoir, could be used to passively limit the flow through the bioreactor (Figure 2C). This technique has proved to be quite adequate and more economical in terms of fabrication time and complexity than the TURN valve based method. We found that 20 μm channel heights of the output manifold, while maintaining the same 100 μm width, provided the total target media flow rate of 1 mL/day.

Surface treatments of PDMS addressing possible loss of reagents/nutrients

To address concerns that some molecules could be absorbed into the PDMS surface of the cell culture chamber and thus potentially alter the actual concentration of chemicals delivered into the cell culture chamber or released by the cells during 3-week-long periods, we performed a series of experiments that used the water soluble Rhodamine B dye, which readily partitions into PDMS, as a tool to investigate methods of making the walls of PDMS microfluidic structures less likely to absorb bioreactor molecules. These experiments focused on various common surface treatments which can be easily applied to PDMS microchannels. As shown in Supplemental Figure S4, in the absence of any oxygen plasma treatments, Rhodamine easily partitions into PDMS and diffuses into the bulk of the

material(22), while plasma treatment creates a silica barrier that significantly impedes such a behavior. We observed a 4-fold reduction in the amount of Rhodamine able to partition into bulk PDMS after fresh plasma oxidation of microfluidic channels. This plasma oxidation-produced barrier remains effective even after week-long storage under wet conditions (simulating a TTB reactor loaded with liquid at all times), as evidenced by the fact that these wet-stored devices maintain the same 4-fold reduction in Rhodamine partitioned into the bulk. Thus, plasma treatment of PDMS surfaces facilitates not only wettability and loading of the TTB but also produces a barrier impeding chemical partitioning and loss during long-term culture conditions.

TTB supports long-term organotypic growth

The ability of the TTB to sustain growth of organotypic cultures over extended periods of time was demonstrated using the MCF-10A cell line and its invasive and tumorigenic variants. Long-term culture of the MCF-10A cell line within 3D Matrigel follows a morphogenic program that results in a mammosphere structure with a hollow lumen over the course of 3 weeks. Mammosphere morphogenesis can be visualized in our closed bioreactor system using a live cell stain to visualize nuclei combined with confocal microscopy. The TTB slide and clamp system were purposefully designed to allow placement into standard microscopy systems and, considering the short working distances of confocal microscopy, to allow real-time *in situ* imaging of the cellular processes at different times during mammosphere growth. The TTB cell chamber depth was standardized to the ECM gel depth of the organotypic culture system that we utilized. Furthermore, the MCF-10A variants have varying irregular features that are readily visualized in the closed TTB system. A series of confocal images for MCF-10A, MCF-10AT1, and MCF-10CA1d variants is shown in Figure 3. These mammospheres have successfully matured from individual cells within the TTB over a period of 21 days. The complete stack of confocal images is shown in Supplemental Figure S5.

Drug delivery within the TTB system

As a first step towards validation of drug delivery in our TTB, we applied increasing concentrations of a commonly used chemotherapeutic, docetaxel, for 24 hours to MCF CA1d cells within the TTB and a traditional 8-well culture chamber(24;25). Docetaxel treatment resulted in a dose-dependent increase in cell death of MCF-10 CA1d cells in both systems at concentrations that are commonly toxic to a wide range of cells ((26;27) Supplemental Figure S6). For the next step, as we were interested in monitoring cellular changes within a 3D organotypic microenvironment of a long-term cell culture system, we evaluated the ability of a known inhibitor to blunt cellular growth in our reactor system and compared results with our side-by-side tests using traditional cell culture techniques. Mammosphere formation is dependent upon a number of cellular processes and is regulated by extracellular proteinase activity. A matrix proteinase inhibitor cocktail known to target a broad spectrum of extracellular matrix proteinase activity was used (Aprotinin, for serine; GM6001, for metallo-; E-64, for cysteine; and Pepstain A, for aspartic; Calbiochem)(28). As we wanted to demonstrate long-term delivery of drug into our system, we first determined the amount of drug that could be continuously added to traditional cell culture without affecting long-term cell viability. For these experiments, we applied the matrix proteinase inhibitor cocktail to monolayer cell cultures in incrementally higher concentrations and evaluated cell viability using the Live/Dead Assay (Molecular Probes). High concentration ranges of the proteinase inhibitor cocktail that have proven effective to inhibit cellular processes such as cellular migration are toxic to cells with 48 hours of continuous application (28); however, the concentration we chose for long-term drug delivery evaluation within the TTB system did not have an impact on cell viability (see Supplemental Figure S7) yet retained its inhibitory properties.

Next, we assessed whether this non-toxic concentration of proteinase inhibitor cocktail was still effective as an extracellular matrix inhibitor within organotypic cell cultures. First, mammosphere formation of MCF-10A cells achieved with the traditional cell culture methods was blunted with addition of this non-toxic proteinase inhibitor cocktail (Figure 4A). Second, we measured proteinase inhibition of proteinase activity in developing organotypic cultures within the TTB. For these experiments, we used the overlay method for establishing 6-day-old mammospheres. Additionally, because it is known that reduced growth factor Matrigel contains trace matrix proteinase content, and because we wanted to improve the baseline sensitivity of the proteinase activity assay, we cultured the MCF-10A cells and variants in a Matrigel:hydrogel mixture. The defined artificial matrix (*i.e.*, hydrogel) contains low endogenous proteolytic activity that allowed for visualization of cell-associated proteolytic activity. The 1:1 mixture did not affect long-term mammosphere formation within the time frame evaluated. Following 6 days of culture, mammospheres were overlaid with hydrogel containing fluorogenic probe (DQ-gelatin). The mammospheres were cultured in the presence and absence of matrix proteinase inhibitor cocktail for 48 hours. As shown in Figure 4B, MCF-10AT1 organotypic cultures demonstrated robust proteinase activity, as indicated by the increase of fluorescein fluorescence of the matrix-embedded DQ-gelatin. However, there was a notable reduction in proteinase activity surrounding mammospheres cultured in the presence of proteinase inhibitors. Our results demonstrate effective protease inhibition using these concentrations of the proteinase inhibitor cocktail.

We next evaluated long-term drug delivery within the TTB. Using organotypic cultures of the MCF-10A human mammary epithelial cell line and their invasive counterparts grown within the TTB, we assessed mammosphere formation in the absence and presence of continuous application of the protease inhibitor cocktail that targets a broad spectrum of extracellular matrix proteinase activity into the system. As shown in Figure 5, this protease inhibitor cocktail down-regulated morphogenesis of the MCF-10A variants within the TTB. MCF-10A cell lines showed on average a ~32% reduction in mammosphere diameter, whereas the MCF-10 -AT, -CA1a, and -CA1d demonstrated a more robust average reduction in mammosphere diameter of ~ 73%, 61%, and 67%, respectively. Interestingly, the sensitivity of the proteinase inhibitory effects correlates well with endogenous matrix proteinase activity of these cell lines. Conditioned media collected from MCF-10A and MCF-10 -AT, -CA1a, and -CA1d grown under 2D monolayer culture conditions was compared for its ability to cleave denatured collagen (*i.e.*, DQ-gelatin). MCF-10A had the lowest measureable endogenous matrix proteinase degrading ability at a rate of 112.2 ± 22.4 (AU/min $\pm$ SE). MCF-10 -AT, -CA1a, and -CA1d cell line variants had higher rates of measured degradation ability at 168.2 ± 18.8 , 123.2 ± 27.8 , and 169.7 ± 31.3 (AU/min $\pm$ SE). Since the endogenous proteinase activity for MCF-10A is less when compared to its tumorigenic variants, it would be logical to expect that the decrease in mammosphere size would also be the least out of the whole cell panel. Overall, these results suggest that *in vitro* acinar development and the subsequent down-regulation of mammosphere formation were correlated with pharmacological down-regulation of matrix proteinase activity in these cell systems. Additionally, our results are supported by a short, 5-day 3D static culture of T47D breast carcinoma cells in collagen inside a microfluidic channel (15), where a 45% decrease in the size of cell clusters formed was observed after the incubation with a single MMP inhibitor GM6001. In general, our studies demonstrate the utility of the TTB for long-term organotypic culture and continuous drug delivery within a closed system with capabilities for optical analysis of cellular functions.

Conclusions

We have developed a portable thick-tissue bioreactor (TTB) capable of maintaining sterile microenvironment and sustaining cell growth, maturation, and organ formation during 3 week-long cell culture. The performance of the TTB was validated with the MCF-10A cell line and its invasive and tumorigenic variants that were capable of developing into hollow lumen spheroids when cultured in 3D matrix over the course of 21 days. Real-time staining and visualization of fully developed mammospheres were possible within the closed bioreactor system. The fully assembled TTB was compatible with regular and fluorescent microscopy and allowed optical assessment of the mammosphere development and proteinase activity using confocal microscopy. The potential drug delivery into our bioreactor system to monitor changes in cellular functions was demonstrated by reduction of mammosphere formation upon delivery of matrix proteinase inhibitors into the closed bioreactor system, as well as in overall reduction of proteinase activity. We created both 2D and 3D COMSOL models to estimate drug delivery and gradient evolution into the cell culture chamber of the bioreactor. The TTB would be readily amenable to other organotypic cell systems. Due to the small cell chamber volumes, it would be particularly useful in systems that utilize cells that are difficult to obtain, such as primary cells, stem cells, or patient tumor cell lines, or in experiments where the cellular secretions need to be analyzed with minimal dilution(29;30). It would be beneficial as a high-throughput microfluidic device compatible with 3D organotypic cell culture that would allow testing of various treatment options to select the one of optimal benefit to the patient. Finally, we demonstrated that microfluidic channels separated by a porous membrane from a small chamber filled with collagen can provide long-term perfusion of cells actively growing in a three-dimensional matrix.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

3D	three-dimensional
PDMS	polydimethylsiloxane
TTB	Thick-Tissue Bioreactor
TURN	Tape Underlayment Rotary Node
ECM	extracellular matrix
MSC	mesenchymal stem cell
MMP	matrix metallo-proteinase
PI	proteinase inhibitor
RF	radio frequency

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Figure 1. Design of the Thick Tissue Bioreactor (TTB)

A) A photograph of the TTB filled with blue and red dyes indicating microfluidic supply network (blue) and the cell culture chambers (red) beneath the perfusion channels; B) A close view of the binary flow divider with ceiling support pillars and a cell culture chamber; C) Block diagram of the typical layout for the clamped TTB indicating major components; D) A photograph of the clamped TTB cartridge with a schematic diagram illustrating the set-up for long-term culture; E) A photograph of the fully assembled TTB prototype with the gravity-driven feeding system, passively regulated flow through the input/output manifold, and a LabtainerTM from HyClone as a waste containment system.

Figure 2. Effect of the channel heights within the output manifold on the total flow rate through the TTB

A) A block diagram of the experimental set-up. B) Typical flow rates through the TTB with the output manifolds of 100 μm channel widths and varying channel heights. C) Flow rate as a function of output manifold channel height. Each point is a 20-minute average with error bars (covered by the dots) representing standard deviations with the corresponding LOD = 0.35 nl/min. D) A photograph of the TTB connected to the input and output manifolds.

Figure 3. Selected confocal slices of MCF cell variants cultured within the TTB for 21 days Mammospheres formed by MCF-10A, MCF-10AT1, and MCF-10CA1d cells labeled with Alexa 488 phalloidin (Green, which is an actin stain) and with SYTO-63 (Red, a nuclear stain for the live cells). (10X). Note the hollow lumens formed by the MCF-10A in contrast to the filled, grape-like cell clusters typical of the tumorigenic MCF-10 CA1d cell line.

Figure 4. Proteinase inhibitor delivery into the TTB

(A) Matrix proteinase inhibitor (PI) treatments of organotypic culture of MCF-10A cell variants (+) shows a reduction in mammosphere size as compared to control (-). (B) Inhibition of proteinase activity was confirmed by confocal microscopy of MCF-10A cell variants that were grown for 5 days within TTB and then overlaid with matrix containing fluorogenic protease activity probe (25 µg/ml DQ-gelatin in hydrogel). Following 48 hours, protease activity was detected by an increase in fluorescence due to proteolysis of DQ-gelatin (Ex/Em 495/515 nm; Molecular Probes). Images representative of one plane of a z-stack (40x).

Figure 5. Results for the proteinase inhibitor treatment of various MCF-10A and cell line variants

Cells cultured within the TTB for 21 days and exposed to PI treatment demonstrate reduction in the size of the formed mammospheres (+) as compared to control (-). The protease inhibitor cocktail is defined in “Methods.” Images were taken *in situ* with the TTB being fully assembled and continuously perfused, as shown in Figure 1D.