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TITLE:

Modeling Breast Cancer in Human Breast Tissue Using a Microphysiological System

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KEYWORDS:

breast cancer, microphysiological system, human breast tissue, adipose-derived stem cells, primary culture, drug development, metabolic crosstalk

SUMMARY:

This protocol describes the construction of an in vitro microphysiological system for studying breast cancer using primary human breast tissue with off the shelf materials.

ABSTRACT

Breast cancer (BC) remains a leading cause of death for women. Despite more than $700 million invested in BC research annually, 97% of candidate BC drugs fail clinical trials. Therefore, new models are needed to improve our understanding of the disease. The NIH Microphysiological Systems (MPS) program was developed to improve the clinical translation of basic science discoveries and promising new therapeutic strategies. Here we present a method for generating MPS for breast cancers (BC-MPS). This model adapts a previously described approach of culturing primary human white adipose tissue (WAT) by sandwiching WAT between adipose-derived stem cell sheets (ASC)s. Novel aspects of our BC-MPS include seeding BC cells into non-diseased human breast tissue (HBT) containing native extracellular matrix, mature adipocytes, resident fibroblasts, and immune cells; and sandwiching the BC-HBT admixture between HBT-derived ASC sheets. The resulting BC-MPS is stable in culture ex vivo for at least 14 days. This model system contains multiple elements of the microenvironment that influence BC including adipocytes, stromal cells, immune cells, and the extracellular matrix. Thus BC-MPS can be used to study the interactions between BC and its microenvironment.

We demonstrate the advantages of our BC-MPS by studying two BC behaviors known to influence cancer progression and metastasis: 1) BC motility and 2) BC-HBT metabolic crosstalk. While BC motility has previously been demonstrated using intravital imaging, BC-MPS allows for high-resolution time-lapse imaging using fluorescence microscopy over several days. Furthermore, while metabolic crosstalk was previously demonstrated using BC cells and murine pre-adipocytes differentiated into immature adipocytes, our BC-MPS model is the first system to demonstrate this crosstalk between primary human mammary adipocytes and BC cells in vitro.

INTRODUCTION

Each year, more than 40,000 US women die of breast cancer (BC)[1]. Despite more than $700 million invested in BC research annually, 97% of candidate BC drugs fail clinical trials[2,3]. New models are needed to improve the drug development pipeline and our understanding of BC. The NIH Microphysiological (MPS) Program delineated the features required for breakthrough models for improving translating basic science into clinical success[4]. These included the use of primary human cells or tissues, stable in culture for 4 weeks, and inclusion of native tissue architecture and physiological response.

Current in vitro BC models, such as two-dimensional culture of BC cell lines, membrane insert co-culture, and three-dimensional spheroids and organoids, do not meet the NIH's MPS criteria because none of these recapitulate native breast tissue architecture. When extracellular matrix (ECM) is added to these systems, breast ECM is not used; instead, collagen gels and basement membrane matrices are used.

Current in vivo systems, such as patient derived xenografts (PDX), similarly do not meet the NIH's MPS criteria because murine mammary tissues vastly differ from human breasts. Moreover, immune system-BC interactions are increasingly recognized as key in tumor development, but the immunocompromised murine models used for generating PDX tumors lack mature T cells, B cells, and natural killer cells. Furthermore, while PDX allows for primary breast tumors to be maintained and expanded, the resulting PDX tumors are infiltrated with primary murine stromal cells and ECM[5].

To overcome these challenges, we have developed a novel, ex vivo, three-dimensional human breast MPS that meets the NIH MPS criteria. The foundation of our breast MPS is made by sandwiching primary human breast tissue (HBT) between two sheets of adipose-derived stem cells (ASCs), also isolated from HBT (**Figure 1**). Plungers for transferring the cell sheets to sandwich the HBT can be 3D printed or made from simple acrylic plastics (**Figure 1H,I**). This technique adapts our previously described approach for culturing primary human white adipocyte tissue[6,7]. The breast MPS can then be seeded by a BC model of choice, ranging from standard BC cell lines to primary human breast tumors. Here, we show that these BC-MPS are stable in culture for multiple weeks (**Figure 2**); include native elements of HBT such as mammary adipocytes, ECM, endothelium, immune cells (**Figure 3**); and recapitulate the physiological interactions between BC and HBT such as metabolic crosstalk (**Figure 4**). Lastly, we show that BC-MPS allows for the study of amoeboid movement of BC cells throughout HBT **(Figure 5**).

PROTOCOL

All human tissues were collected in accordance to protocol #9189 as approved by the Institutional Review Board Office of LSUHSC.

1. Seeding of Adipose-derived Stem Cells (ASCs) for cell sheets

1.1. Purchase ASCs from commercial sources or isolate from primary human breast tissue by following established protocols[8,9]. Seed human breast ASCs at 70% density (~80,000 cells/cm^2 surface area) onto 6-well standard tissue culture plates in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, and 1% Penicillin/Streptomycin (BC-MPS Media). Ensure that the ASCs to be used for forming the cell sheets should be less than passage 10.

1.1.1. For each well of breast cancer microphysiological system (BC-MPS) that will be generated, seed cells in 1 standard tissue culture well and 1 well of similar size on poly(N-isopropylacrylamide) (pNIPAAm)-coated tissue culture plastic plate. One 6-well plate of BC-MPS will require one standard tissue culture 6 well plate (bottom) and one pNIPAAm-coated plate (top). The pNIPAAm plates can be purchased from commercial sources or can be coated in-lab[10–12].

1.2. Culture ASCs in a tissue culture incubator at 37 ° C and 5% CO_2. Change the media every 2-3 days in a biosafety cabinet.

1.3. Culture the ASCs until they become 100% confluent and form a striated pattern. Depending on the confluence at which they were seeded, this will take 7-10 days. Confluent cell sheets are required to anchor the buoyant adipose breast tissue.

2. Preparation of supplies needed for BC-MPS

2.1. To prepare gelatin for 6-well plates make a 12% gelatin solution in distilled H_2O. Mix 19 g of gelatin type B (gel strength of ~225 Bloom) and 125 mL of H_2O in a 250 mL glass media bottle. Add 1.6 mL of 1 M NaOH to the solution to neutralize the pH.

2.2. Autoclave the solution under a liquid cycle for 25 min to sterilize the solution.

2.3. Allow the solution to cool to 37 °C. In a sterile biosafety cabinet add 16 mL of sterile 10x Hanks balanced salt solution (HBSS) to the solution to obtain a final volume of 160 mL.

NOTE: The gelatin solution can be used right away or stored at room temperature for later use. The prepared gelatin is stable at room temperature for 1 month. If only a few plungers will be needed then a 10 mL solution can be made and sterile filtered on the same day as making BC-MPS as previously described[7].

2.3.1 To prepare gelatin solution by filter sterilization, dilute 1 mL of 10x HBSS with 9 mL of H_2O to make a 1x HBSS solution in a 15 mL conical tube. Add 100 µL of 1 M NaOH to each 10 mL tube and then add 0.7 g of gelatin to the solution. Mix the solution vigorously to dissolve the gelatin.

2.3.2 Incubate the tube in a 75 °C water bath for 20 min shaking every 5 min. Use a 10 mL syringe and a 0.22 µm syringe filter to filter the gelatin solution. Filter the gelatin solution directly onto the plungers in a biosafety cabinet.

2.4. 3D print or use simple acrylic to make the plungers used for transferring the cell sheets. Ensure that the plungers fit loosely in the desired size of well. A standard 3D printed plunger is shown in **Figure 1**. The plungers can be designed using standard computer aided design software such as TinkerCad and printed using filament 3D printers that are compatible with polylactic acid (PLA)[13, 14].

2.5. To prepare a rack for holding the plungers place a 15 mL tube rack in a biosafety cabinet. Place plungers upside down in the rack, so that the bottom of the plungers is facing up. Place a plastic box with a lid for transporting BC-MPS in the biosafety cabinet. It is recommended that the box be at least 35.5 cm length x 28 cm width x 8.25 cm height to fit 4 plates of BC-MPS. Remove the lid from the box and spray down the rack, plungers, and box with 70% EtOH.

2.6. Prepare sterile razor blades and forceps by autoclaving or by placing them in a biosafety cabinet and washing with 70% EtOH.

2.7. Spray the plungers and box with 70% EtOH and turn on the UV light in the biosafety cabinet for at least 30 min to sterilize the supplies.

3. Prepare gelatin plungers and apply to the upper cell sheets

3.1. If the gelatin solution was previously prepared, heat it in a 37 ° C water bath to melt it.

3.2. Pipette the gelatin solution onto the plungers in the biosafety cabinet using a 5- or 10-mL serological pipette. A plunger for 1 well of a 6-well plate will require ~2.5 mL of gelatin.

3.3. Once the gelatin has solidified on the plungers (~30-45 min) move the pNIPAAm-coated ASC plates to the biosafety cabinet. Gently place the plungers into the wells of the pNIPAAm-coated ASC plates so that the gelatin contacts the ASCs. Place a metal washer (~7.5 g) on the plunger to weigh down the plunger so that gelatin is in direct contact with the ASC cell sheet. Leave the plungers on the ASC cell sheets at room temperature for 30 min.

3.4. Gently move the plate with the plungers into the sterile box in the biosafety cabinet and place the lid on it. Move the box to a 4 °C fridge for 30 min. If a 4 °C fridge is not available place the plate on ice in an ice bucket in the biosafety cabinet for 30 min.

4. Preparation of cancer cell lines

4.1. Seed the cancer cell lines in a standard T75 tissue culture dish and keep them in culture so that they are ~80% confluent on the day BC-MPS will be processed. (~200,000 cancer cells will be needed per BC-MPS). Grow the cancer cells in normal media.

NOTE: To identify and isolate the cancer cells it is recommended that the cells are transfected with a fluorescent protein to distinguish them from the ASCs and breast tissue. The number of cancer cells needed per BC-MPS has been optimized for MCF7 and MDA-MB-231 cells. Other cell lines may require further optimization.

4.2. On the day that the BC-MPS is being prepared move the flask containing the cancer cells to the biosafety cabinet. Aspirate the media from the flask. Wash the flask with 5 mL of PBS.

4.3. Add 1 mL of cell detachment solution to the flask containing the cancer cells and then incubate the flask in a 37 °C incubator until the cells have detached (~2-5 min).

4.4. In the biosafety cabinet add 9 mL of PBS to the flask, transfer the cells in PBS into a 15 mL conical tube and count the cell number using a hemocytometer.

4.5. Centrifuge the cancer cells at 500 x *g* for 5 min at room temperature.

4.6. Resuspend the cells in BC-MPS media so that there are 2 x 10^6 cells/mL.

4.7. Place the cancer cells in a 37 °C water bath until they are ready to be added to the human tissue.

5. Processing of human breast tissue

5.1. In a biosafety cabinet wash the human breast tissue (HBT) 3x with 10 mL of sterile PBS.

5.2. Use sterile forceps and a razor blade to coarsely mince the BC-MPS and try to remove as much fascia and connective tissue as possible. Failure to remove the connective tissue may result in the ASC upper layer to not properly anchor the HBT.

NOTE: The fascia and connective tissue can be identified by its white or clear appearance compared to the yellow color of the adipose tissue.

5.3. Once the connective tissue has been removed use a sterile razor blade to finely mince the tissue until it has a homogenous liquid consistency. The tissue is finely minced when it can easily be pipetted using a 25 mL serological tip.

5.4. Use a sterile razor blade to cut the tip off a p1000 pipette tip to assist in pipetting the minced HBT.

5.5. In a 1.5 mL tube mix the minced HBT, cancer cell lines, and BC-MPS media (for 1 well of a 6 well plate this requires 200 µL of minced HBT, 100 µL of of BC-MPS media, and 100 µL of cancer cells).

5.6. Move the ASC plate that will be used for the bottom cell sheet from the incubator to the biosafety cabinet. Aspirate the media from the bottom ASC plate. Pipette the mixture onto the center of the well of the bottom ASC plate using a p1000 pipette tip that had the distal end cut off from step 5.4

5.7. Move the box containing the upper ASC plate with the plungers on it to the biosafety cabinet. Gently remove the gelatin plungers from the pNIPAAm-coated plate and place on top of the HBT mixture. Add BC-MPS media to the well (for 1 well of a 6-well plate add 2 mL of media). Carefully move the bottom ASC plates with the BC-MPS mixture and plungers to the sterile plastic box and place the lid of the box on for transport.

NOTE: The 6-well plate lid will not fit back on the ASC plate while the plungers are on. Care must be taken to avoid contaminating the culture.

5.8. Incubate the bottom plate in the box in an incubator at 37 ° C until the gelatin has melted, and the top ASC layer has begun to adhere to the bottom layer (~30 min).

5.9. Move the box with the plates to the biosafety cabinet. Gently remove the plungers from the bottom plates. The tissue with the cancer cells will be anchored to the bottom of the well.

5.10. Place the lid of the 6-well plate back onto the bottom plate and incubate at 37 °C to completely melt the gelatin and allow the top layer to completely anchor to the bottom layer (~30-60 min).

5.11. Gently move the plates to a biosafety cabinet and aspirate the media with a 10 mL serological pipette. Add 2 mL of fresh media to each well. Aspirate from the edge of the well and pipette onto the edge of the well to avoid dislodging the tissue.

5.12. Maintain the BC-MPS at 37 °C and 5% CO_2 for the desired length of time and change media every 2-3 days.

6. Digestion of BC-MPS for analysis

6.1. When the BC-MPS is ready to be analyzed, move the plate to the biosafety cabinet. Remove media with a serological pipette to avoid accidental dislodging of any tissue.

6.2. Add 1 volume of PBS to each well.

6.3. Remove the PBS with a serological pipette.

6.4. Add 1 mL of cell disassociation solution to each well. Move the plate back to the incubator and incubate at 37 °C for 5 min to allow the cells to detach. In a biosafety cabinet, use a cell scraper to completely detach the cells and the tissue from the culture plate.

6.5. Transfer the solution with the tissue to a 15 mL conical tube using a serological pipette. Add 2 mL of PBS to each well to collect any remaining cells and transfer this solution to the conical tube. If the cells are fluorescent, wrap the tube in aluminum foil to protect it from light.

6.6. Incubate the tube at 37 °C under constant agitation in an orbital shaker at 1 x *g* for 10-20 min to completely dissociate cells from the tissue.

6.7. In a biosafety cabinet use a serological pipette to disrupt any remaining clumps of cells in the tube and filter the sample through a 250 µm tissue strainer into a new 15 mL tube. Pipette the solution slowly through the strainer so that it does not overflow.

6.8. Rinse the strainer with 1 mL PBS to collect any cells still in the strainer.

6.9. Centrifuge the samples at 500 x *g* at room temperature for 5 min. After centrifugation, the adipocytes will be floating on the top layer of the solution while the cancer cells and ASCs will be mixed together in a pellet at the bottom of the tube.

6.10. To isolate the adipocytes gently pour the top layer into a new tube or alternatively cut the tip off a p1000 pipette tip and transfer the top layer to a new tube. Centrifuge the sample at 500 x *g* for 5 min again and use a syringe and needle to remove the solution from below the adipocytes so that only the adipocytes are remaining. The adipocytes are now ready for analysis

6.11. After removing the adipocytes from the solution, separate the ASCs and cancer cells from each other for analysis if the cancer cells were previously transfected with a fluorescent protein. Aspirate the remaining solution from the tube containing the ASCs and cancer cells without disrupting the cell pellet. Resuspend the pellet in PBS or BC-MPS media and use flow cytometry to sort the cells based on the fluorescence.

REPRESENTATIVE RESULTS

Stability in culture

BC-MPS is a stable microphysiological system that can be cultured in vitro for up to at least 14 days. A brightfield image of the ASC cell sheets was taken at 100x magnification to display the striated pattern of the confluent sheet (**Figure 2A**). The ASC cell sheets are stable in culture for at least 4 weeks. BC-MPS at 14 days in culture in one well of a 6 well plate was imaged with a color camera demonstrating that the buoyant HBT is still stably anchored by the ASC cell sheets to the bottom of the well after 14 days (**Figure 2B**). This demonstrates that the cell sheet used to sandwich the adipose tissue is still intact. The triple negative BC cell line MDA-MB-231 expressing RFP was cultured in HBT for 14 days and then imaged with a fluorescent microscope demonstrating the stability of the BC-MPS with cell lines out to at least 14 days (**Figure 2C**). The presence of the BC cells in the tissue demonstrates the ability of cancer cells to invade the HBT.

A triple negative PDX explant was excised from a mouse, stained with Cell Tracker CMTPX and cultured with HBT. Fluorescent microscopy images were taken at day 6 to show the stability of BC-MPS with tumor explants for at least 6 days **(Figure 2D)**. This demonstrates that BC-MPS can be used to culture tumor explants with HBT as well as cell lines.

Native HBT elements

BC-MPS contains the native elements of HBT in culture. BC-MPS containing 100 mg of HBT were cultured in vitro for 14 days. The tissue was imaged using brightfield microscopy after 14 days in culture **(Figure 3A)**. This image demonstrates the preservation of clusters of mature adipocytes at 14 days in culture. The tissue was then fixed with 10% formalin, paraffin embedded, sectioned at 5 µm using a microtome and then stained with Movats pentachrome stain using a standard protocol. The fixed sections were imaged at 100x **(Figure 3B)** or 20x **(Figure 3C).** The images demonstrate that the BC-MPS contains the native collagen (purple), reticular fibers (blue), and the adipocytes large unilocular shape in between the connective tissue is preserved at 14 days in culture. To identify primary macrophages in the system, HBT was cultured for 0, 3 and 7 days and then fixed and stained with the anti-CD45, anti-CD68, and anti-CD206. CD45 was used as a general leukocyte identifier. CD68 was used as a monocyte stain to identify macrophages while CD206 identifies a common receptor present on macrophages. The staining of the macrophage markers was quantified using an imaging software **(Figure 3D)**. This demonstrates the preservation of primary macrophages after 3 and 7 days in culture. These data demonstrate that BC-MPS preserves the native elements of HBT in culture. Further experiments can be carried out to study how the BC cells remodel the native elements of HBT. This can include assessing changes in macrophage markers to determine if they are being altered by the presence of BC cells. Changes in the ECM can be further analyzed by comparing the Movats pentachrome staining of samples that were cultured with or without BC cells to determine if collagen content is changed and the size of the adipocytes can be compared using image analysis software such as Image J to determine if BC cells alter adipocyte size.

Metabolic crosstalk

Adipocyte-cancer metabolic crosstalk is an important pathway whereby the tumor microenvironment influences BC progression. BC cells that accumulate intracellular lipids demonstrate increased drug resistance and a more invasive phenotype[15]. While BC cells have been shown to induce lipolysis in immature, murine adipocytes, the physiological relevance of this has remained unclear because metabolic crosstalk between BC and primary human mammary adipocytes has never been shown. To demonstrate that BC-MPS can model this adipocyte-BC metabolic crosstalk RFP-labeled MDA-MB-231 cells were cultured alone or in BC-MPS for 14 days. The cells were enzymatically digested into single cell suspension, stained with the neutral lipid dye Bodipy, and analyzed by flow cytometry **(Figure 4A,B)**[16]. The proportion of lipid-positive MDA-MB-231 cells was 26.2-fold SEM +/- 2.5 greater in BC-MPS vs. 2D culture **(Figure 4C)**. This increase in lipid staining demonstrates that the cancer cells are interacting with the adipocytes and taking up lipids released from the mature adipocytes. Cell sorting was performed to select for RFP+ Bodipy+ cells. After sorting, the cells were plated on collagen I coated glass coverslips and allowed to attach overnight. The following day the cells were fixed in 4% paraformaldehyde for 30 min and then imaged on a fluorescent microscope at 100x

magnification. Cells cultured in BC-MPS **(Figure 4E)** displayed increased lipid droplets compared to cells cultured in standard 2D culture **(Figure 4D)**. This demonstrates metabolic crosstalk between the BC cells and the HBT and, also, shows how lipid accumulation can be analyzed by flow cytometry or by fluorescent microscopy. Further analysis of lipid accumulation in cancer cells can be done through quantifying the size of lipid droplets and the number of lipid droplets per cell.

Amoeboid movement

Metabolic cross talk between BC cells and adipocytes increases the invasive ability of the cancer. To assess the increased invasive ability of the cancer cells cultured with the HBT the migration of BC cells throughout HBT was assessed by fluorescence microscopy. The metastatic TNBC cell line MDA-MB-231 expressing RPF was cultured in BC-MPS for 4 days to allow the system to stabilize. Time-lapse fluorescence microscopy was performed after 4 days to image the MDA-MB-231 motility with one image captured every 20 min for 19 h at 37 °C and 5% CO_2. The resulting video demonstrated amoeboid movement patterns and a surprisingly high degree of motility for the MDA-MB-231 cells **(Figure 5** and **Video 1)**. This demonstrates an increase in invasive ability of the BC cells and the ability of the BC cells to invade the HBT. The migration of the BC cells in the HBT can be further analyzed using previously published methods for quantifying the velocity and directionality of migrating cells[17,18]. The MDA-MB-231 cells were also observed to appear to undergo mitosis. These behaviors are consistent with its aggressive phenotype, are important behaviors for cancer progression and metastasis, and highlight new BC behaviors that may be targeted for BC therapies.

FIGURE AND TABLE LEGENDS:

Figure 1: General workflow of BC-MPS setup. (**A**) Gelatin is solidified in an upright plunger. (**B**) The plunger with gelatin is placed onto the thermoresponsive upper ASC cell sheet. (**C**) A weight is used to force the gelatin to maintain contact with the cell sheet while the cells incubate at room temperature for 30 min followed by incubation at 4 °C for 30 min. (**D**) The plunger is removed from the cold dish removing the intact cell sheet with it. (**E**) The plunger with the upper cell sheet is transferred to a dish containing the bottom cell sheet and the minced HBT and BC cells. (**F**) The bottom cell dish with media, plunger, HBT, BC and both cell sheets are incubated at 37 °C for 30-60 min to allow the gelatin to melt and the cell sheets to from contacts. (**G**) Removal of the plunger leaves the HBT and BC sandwiched between two ASC cell sheets. A 3D printed plunger was designed using the free online software TinkerCad (https://www.tinkercad.com/) and printed on a 3D printer. The plunger is composed of polylactic acid. (**H**) The dimensions of a plunger for 1 well of a 6-well plate are indicated. The recess of the plunger is 2.5 mm. (**I**) An image of the actual 3D printed plunger is shown.

Figure 2: Representative stability of BC-MPS in culture. (**A**) A brightfield image at 100x magnification of the confluent ASCs demonstrating the striated pattern of the confluent cells. Scale bar = 100 µm. (**B**) BC-MPS, containing 100mg of HBT, was cultured in a 6 well plate. After 14 days a color photograph was taken to demonstrate that the HBT is anchored to the bottom of the well of a 6 well plate. (**C**) The triple negative BC cell line MDA-MB-231 expressing RFP was cultured with HBT for 14 days and then imaged using fluorescent microscopy at 40x

magnification. The images demonstrate that the MDA-MB-231 cells were still viable and growing in the HBT. Scale bar = 200 µm D) PDX Tumors of triple negative breast cancer from mice were minced into 3 cm^2 pieces and labeled with Cell Tracker Red CMPTX. The pieces were cultured in HBT and the resulting BC-MPS was imaged using a fluorescent microscope at 40x magnification on day 6 demonstrating its stability in culture. Scale bar = 200 µm.

Figure 3: Representative preservation of native elements of HBT present in BC-MPS. BC-MPS was cultured for 14 days and then either imaged with bright field microscopy at 40x (**A**) and then fixed with 10% formalin and stained with Movats pentachrome stain. Images were taken at 100x (**B**) or 20x (**C**). Imaging of the HBT indicates the preservation of large uniloculor adipocytes clustered together and tissue slices indicates the presence of preservation of the native ECM such as collagen (yellow) and reticular fibers between the adipocytes (blue). To demonstrate the preservation of macrophages in the model HBT was cultured for 0, 3, or 7 days, stained for macrophage markers, and imaged using confocal microscopy (**D**). The fluorescent intensity of the staining of the macrophage markers was quantified using commercially available software demonstrating that macrophages are still present in the tissue after 3 and 7 days in culture. Scale bar = 200 µm.

Figure 4: Representative metabolic crosstalk between adipocytes and cancer cells in BC-MPS. RFP-labeled MDA-MB-231 cells were cultured in BC-MPS vs. 2D for 14 days, then stained with Bodipy (250 nM) and analyzed by flow cytometry. (**A**) Flow of Bodipy-stained MDA-MB-231 cells after 14 days in 2D culture. Cells in B2 were $Bodipy^+RFP^+$; cells in B1 were $Bodipy^-RFP^+$. B2/(B1+B2) = 3.26%, indicating minimal lipid accumulation. (**B**) Flow cytometry analysis of Bodipy-stained MDA-MB-231 cells after 14 days in BC-MPS. B2/(B1+B2) = 85.35%, indicating extensive lipid accumulation. (**C**) Lipid accumulation in MDA-MB- 231 cells after 14 days in 2D culture vs. BC-MPS. Fluorescent microscopy analysis of Bodipy staining of MDA-MB-231 cultured in 2D (**D**) vs BC-MPS (**E**) demonstrates increased lipid accumulation in cells grown in BC-MPS. Error bars indicated SEM. ** $p < 0.01$, n=2 biological replicates, Scale bar = 50 µm.

Figure 5: Representative migration of TNBC in BC-MPS. RFP-labeled MDA-MB-231 cells were cultured in BC-MPS for 4 days and then sequential time-lapse images of RFP-labeled BC-MPS + MDA-MB-231 cells were captured using a fluorescence microscope (One frame per 60 min, 100x magnification.) Yellow asterisks = mitotic event. Yellow box = cellular debris used for positional reference. Blue arrows = 231 cells showing amoeboid movement with pseudopods and high motility. Scale bar = 100 µm.

Video 1: Representative migration of TNBC in BC-MPS. BC-MPS containing RFP-labeled MDA-MB-231 cells were cultured for 4 days and the fluorescent microscopy was used to image the cells every 20 min at 19 hours at 37 ° C and 5% CO_2. The images were compiled into a video (One frame per 60 minutes, 100x magnification).

DISCUSSION

New systems for modeling human breast cancer are needed to develop a better understanding of the disease. Development of human microphysiological systems to model disease settings that

include native ECM and stromal cells will increase the predictive power of pre-clinical studies. The BC-MPS model presented here is a newly developed system that overcomes the limitations of previous models allows for the evaluation of BC in its native HBT environment. This system can be used with cancer cell lines or with tumor explants and is stabile for at least 14 days. Fluorescently labeling the BC cells allows their real time visualization of cancer cell motility and cell-cell interactions, migration, viability, or proliferation to be monitored either in real-time or at specific time points (**Figure 3** and **Figure 6**). This model system can be used to elucidate important aspects about cancer biology as it relates to the interactions between cancer cells and their microenvironment. Interrogation of this system can inform on TME regulation of cancer progression, drug response, and the initiation of metastasis.

To successfully set up the system, there are a few critical steps that need to be followed. The first critical step is the culturing and transfer of the ASC cells sheets. The pNIPAAm-coated plates are temperature sensitive. When the plates are above 32 °C the surface is hydrophobic, and the ASCs will adhere to the surface. The surface will become hydrophilic below this temperature and the cells will begin to detach[19]. Thus, it is necessary to determine how quickly a given cell type will begin to detach from these surfaces. For HBT-derived ASCs this will take > 30 min if the cells are confluent. To prevent the cells from detaching during normal maintenance the media should be pre-warmed to 37 °C and the tissue culture plates should not be kept at room temperature for too long to prevent the plates from cooling and the cells detaching. It is also important to determine the optimal time for the gelatin plungers to be on the pNIPAAm-coated plates to transfer the entire cell sheet. The ASCs need to be confluent and the entire sheet needs to be transferred. If the top cell sheet does not have time to completely detach and bind to the gelatin then the cell sheet will be broken during transfer. If the entire cell sheet is not completely transferred, then the ASCs will not be able to sandwich the buoyant breast tissue.

The ASCs used to sandwich the HBT are normal stromal cells of the breast microenvironment that secrete ECM, growth factors, and cytokines that influence BC[20]. To sandwich the HBT between two cell sheets, the ASC sheets are transferred using thermoresponsive tissue culture plates. This allows for the native ECM secreted by the ASCs to be intact and serves to anchor the buoyant adipocytes in tissue. By using ASCs derived from human breast tissue this ensures that the interactions that the ASCs have with the cancer cells will mimic what occurs in breast cancer as ASCs from different depots display different gene expression and ECM remodeling[21].

The second critical step is the processing of the HBT. It is necessary to use freshly isolated HBT to preserve the viability of the tissue. When processing the HBT it is critical to finely mince the tissue and to remove as much of the fascia as possible. If large pieces of fascia are left in the HBT, this will prevent the cell sheets from properly anchoring the buoyant tissue. In addition, fascia left in the tissue can dissociate from the plate and it will pull multiple clusters of the HBT with it making the well unusable for further experimentation. The HBT should be placed in the middle of the well before adding the top cell sheet otherwise the ASCs will not be able to adequately sandwich the HBT and the tissue on the edge of the wells will not be anchored. If these critical steps are followed, then the process of producing BC-MPS is straight forward to set up.

The model detailed here is an improved system for studying BC in its natural microenvironment of primary HBT that retains the mature adipocytes, ECM, and resident immune cells (**Figure 4**). Pre-clinical models that mimic the adipose microenvironment lack multiple elements of the microenvironment, such as mature adipocytes, immune cells, fibroblasts, and the ECM. Using fully mature HBT allows for the simultaneous and—reciprocal interrogation of this complex environment that includes multiple elements that influence cancer development, drug response, and invasiveness through paracrine and juxtacrine signaling[22–25]. Resident fibroblasts and ASCs influence cancer metastasis through remodeling of the ECM in tissue and through paracrine signaling[26,27]. The ECM and its remodeling are essential for the initiation of metastasis. Alterations in the ECM of the tumor microenvironment influence the migration and metastasis of BC, but most BC studies do not incorporate native ECM into their studies and instead use simplified models composed of collagen I or matrigel[28,29]. The ECM in BC-MPS is from the HBT and from the ASCs. Thus, the way that the native ECM is remodeled in the tumor microenvironment in breast tissue and influences metastasis can be studied. Thus, this BC-MPS model incorporates multiple elements of the tumor microenvironment that previous model systems did not have.

This model can be used to study metabolic crosstalk between fully mature adipocytes and BC cells. Previous models studying the interaction between adipocytes and cancer cells use pre-adipocytes that are differentiated in vitro. Pre-adipocytes that are differentiated in vitro do not fully mature to the same extent as adipocytes differentiated in vivo[30,31]. To understand how the adipocytes influence cancer progression and drug response it is vital to properly mimic the complex molecular and chemical composition of native adipocytes. Here we have demonstrated that our system allows for assessing the metabolic crosstalk that occurs between adipocytes and BC cells (**Figure 4**).

BC-MPS can also be used in performing in vitro study on how conditions such as obesity, which cannot be properly modeled using standard cell lines, influence the progression of cancer. Obesity is associated with alteration of the microenvironment of HBT and an increase in metastasis in BC although the exact mechanisms are not fully understood[32–34]. Fibroblasts, ASCs, adipocytes and ECM are altered during obesity and further promote progression and metastasis of cancer[25,35,36]. Thus, it is important to use native stromal cells and ECM from obese tissue to understand how these alterations in the stromal cells and ECM influences BC. By utilizing HBT and ASCs from obese or lean patients in the BC-MPS model the mechanisms by which obesity promote a metastatic phenotype can be elucidated.

The method presented here is for culturing breast cancer cells in a culture that encompasses ASCs, mature adipocytes, and native ECM. Previous models of co-cultures systems have studied how these individual different elements of the stromal environment influence cancer. As the ASCs and the adipocytes both influence BC progression it can be complicated to determine which cell type is responsible for influencing the BC. However, this can be determined by comparing BC-MPS to simpler co-culture models with one cell type. One of the advantages of this system compared to on-a-chip or bioprinting systems is that no special equipment is needed to set up the system beyond the pNIPAAm coated plates which are commercially available. Model systems

of breast tissue have been previously described using bioprinting to deposit ASCs into scaffolds that are then differentiated in culture[22]. This requires an additional 2-3 weeks for the cells to differentiate and ASCs differentiated in culture do not fully differentiate to mature adipocytes. The mature adipocytes from the HBT in BC-MPS are ready to be used the moment they are removed from a patient.

The model system described here is useful system for studying the crosstalk between cancer cells and the microenvironment, however, it does have some limitations. One of the major limitations is the availability of primary human breast tissue. Human adipose tissue cannot be cryopreserved without a loss of viability and thus the tissue used for making BC-MPS needs to be freshly obtained and used[37]. Another limitation is that the model as presented here is a static system that is cultured in regular 6 well plates. The system lacks the microfluidic flow that is present in organs-on-a-chip which can influence oxygen and nutrient availability, sheer stress, and drug distribution[38,39]. A third limitation is the need for the cells to be separated from each other at the end of the experiment if the different cell types need to be analyzed individually. This requires the need to label the cells so that they can be separated from each other.

The incorporation of many elements of the tumor microenvironment produces a physiological model to study cancer progression and the initiation of metastasis. In addition to being able to study cancer cells in a more physiological model, BC-MPS can also be used to study the individual elements of the microenvironment and how they are influenced by cancer. While the data represented here demonstrate how BC-MPS can be used with one cell line, BC-MPS can be readily adapted to suit a researcher's particular experimental need. For example, obese HBT can be compared to lean HBT to determine how obesity affects cancer metastasis and progression. Different subtypes of BC can be seeded into BC-MPS to study how subtypes of cancer interact differently with the microenvironment. Gene editing techniques can be employed to determine how specific genes affect the interaction between BC and the microenvironment. This model and its applications will allow for a more accurate understanding of how the stromal environment and breast cancer interact to promote cancer progression and metastasis.

ACKNOWLEGDMENT:
We would like to thank the Tulane Flow Cytometry and Cell Sorting Core as well as the Tulane Histology Core for their technical support. This work was supported by the Southeastern Society of Plastic & Reconstructive Surgeons 2019 Research Grant and the National Science Foundation (EPSCoR Track 2 RII, OIA 1632854).

DISCLOSURES:
The authors declare that they have no competing interests.

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Figure 1 Workflow of BC-MPS

Click here to access/download;Figure;Figure 1.pdf

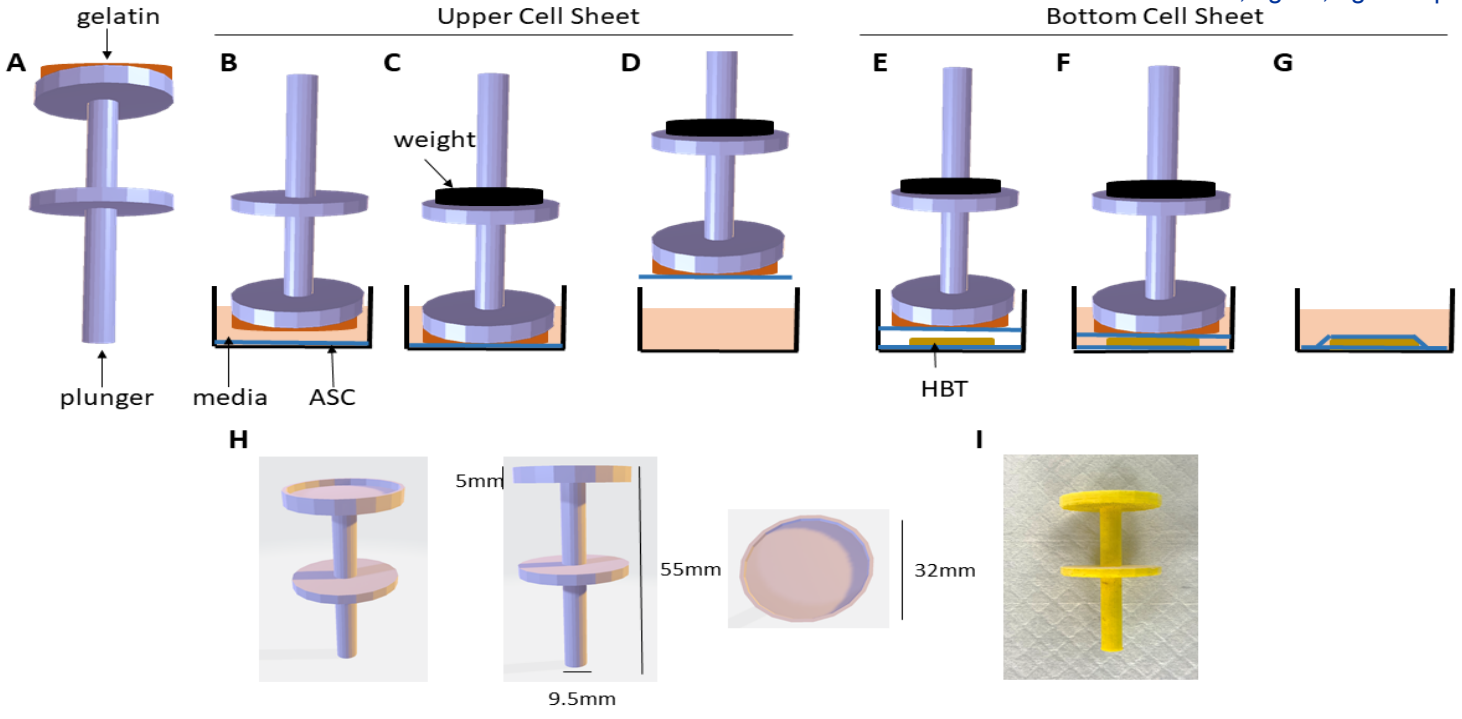

Figure 2 Stability in culture

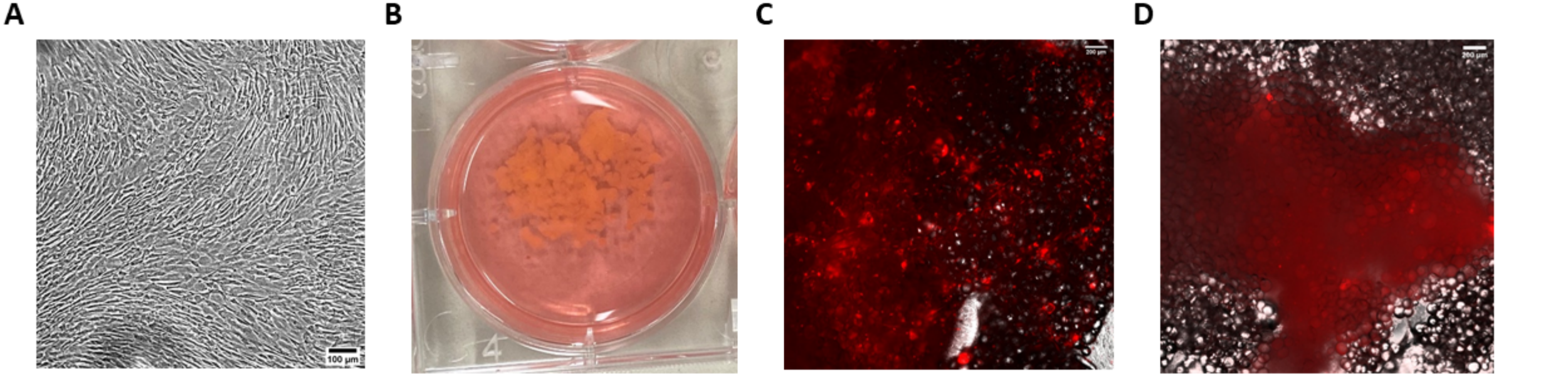

Figure 3 Native Elements of HBT

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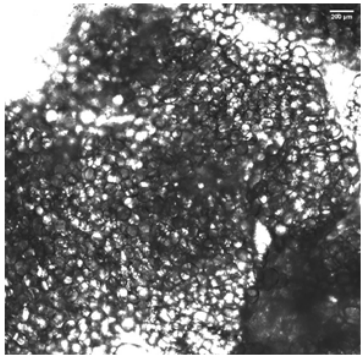
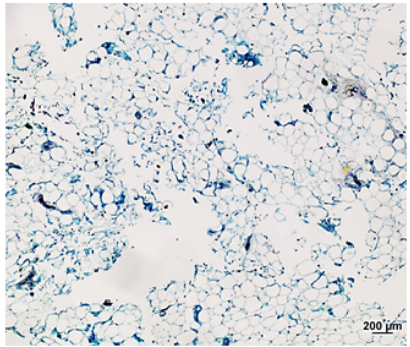
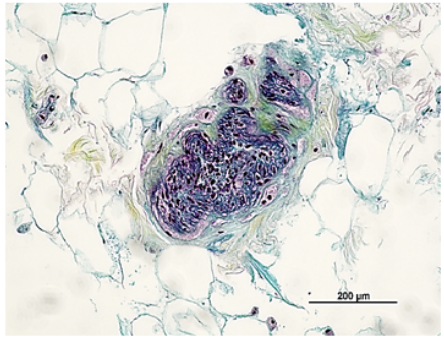
A



B



C



D

Sample	CD45 Intensity	CD 68 Intensity	CD206 Intensity
Native breast tissue	9508	5818	1207
HBT (day 3)	1510	729	682
HBT (Day 7)	1327	2400	266

Figure 4 Metabolic Cross-talk

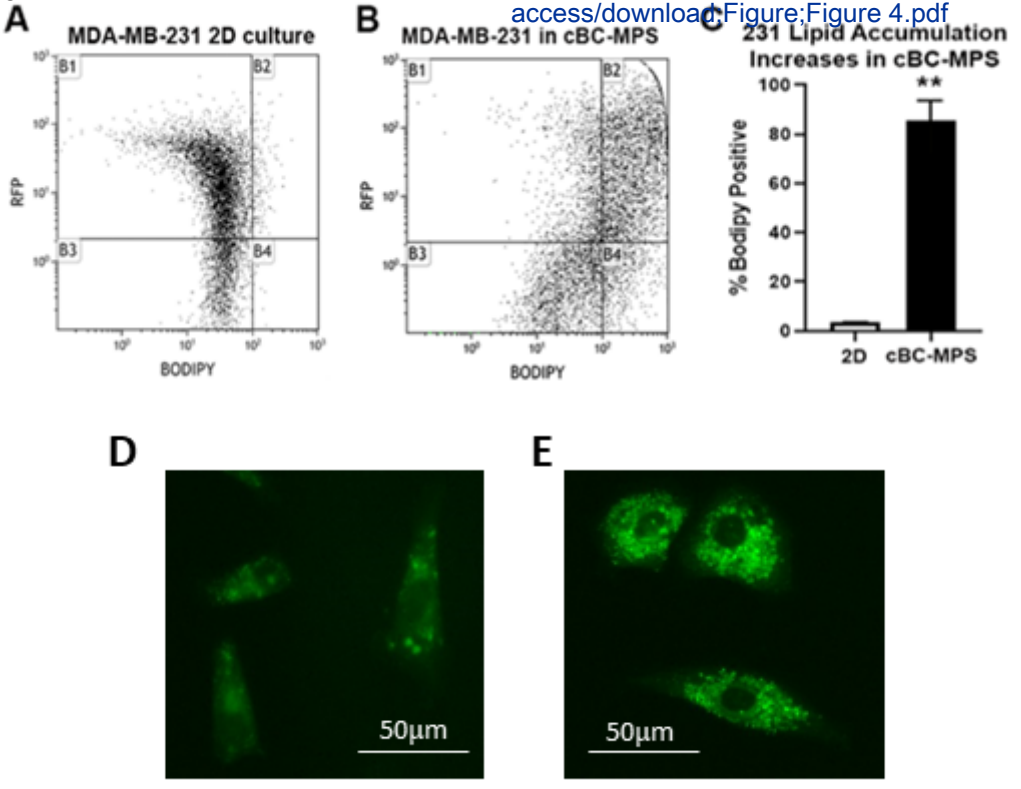


Figure 5 Migration

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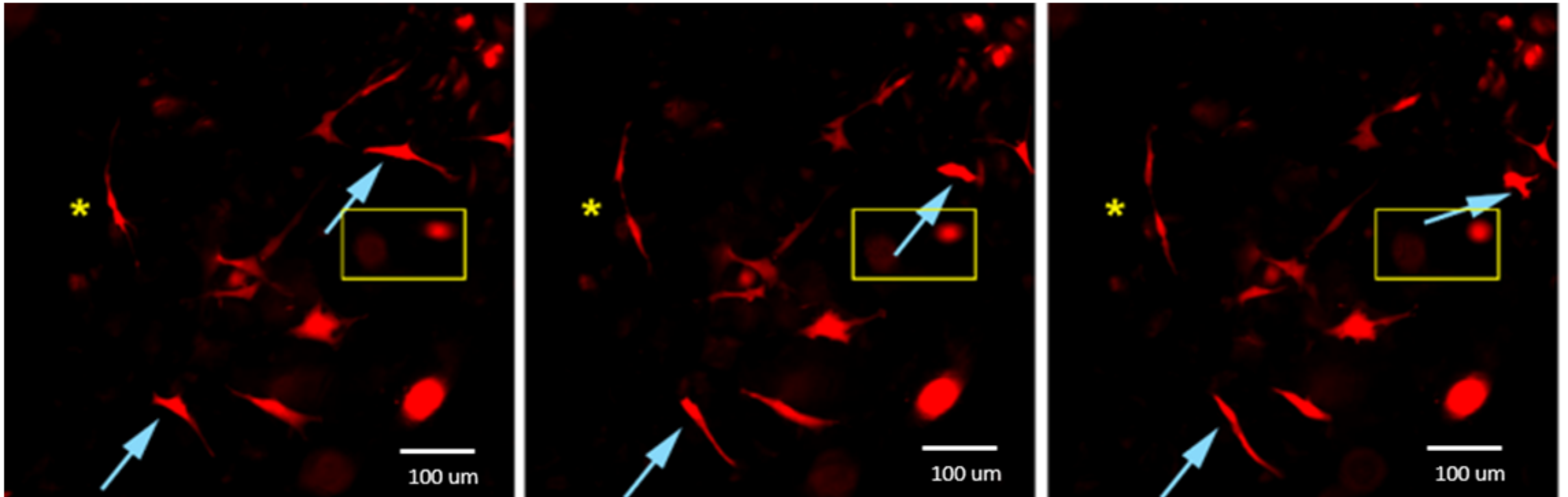
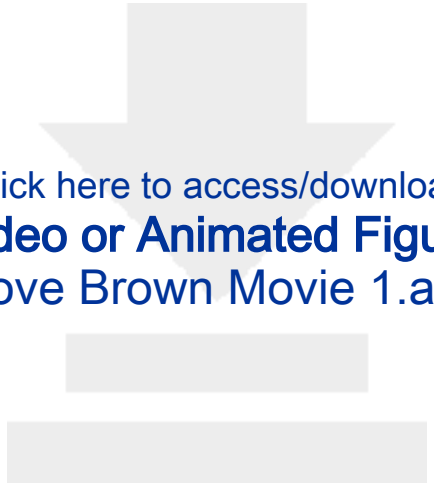


Video or Animated Figure



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Video or Animated Figure

Jove Brown Movie 1.avi

Table of materials

Click here to access/download;Table of Materials;Supplies for BC-MPS.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Accumax	Innovative Cell Technologies	1333	Cell disassoication solution for separation of BC-MPS
Accutase	Corning	25-058-CI	Cell detachment solution for passaging of cells
BioStor Container 16oz	National Scientific Supply Co	MPCE-T016	For Transport of sterile tissue
Cell Culture 75 cm flasks	Corning	430641U	For culturing ASCs
Conical Tubes 15mL	ThermoScientific	339650	
Curved Forceps	ThermoScientific	1631T5	For maneuvering tissue while mincing
DMEM low glucose, w/ Glutamax	Gibco	10567-014	For culturing ASCs and BC-MPS
FBS Qualified	Gibco	26140-079	
Gelatin	Sigma	G9391	
HBSS 10x	Gibco	14185-052	
NaOH	Sigma	221465	
Nunc UpCell 6 well plates	ThermoScientific	174901	Top ASC cell sheet
PBS	Gibco	10010-023	
Pen/Strep 5,000U	Gibco	15070-063	
Petri Dish 150 cm	FisherBrand	FB0875714	For holding tissue while mincing
Razor Blades	VWR	55411-055	Single Edge for mincing tissue
Strainer 250um	ThermoScientific	87791	For separation of BC-MPS
Tissue Culture 6 well plates	Corning	3506	Bottom ASC cell Sheet
Weights/Washers	BCP Fasteners	BCP672	For weighing plungers down 1/2" inner diameter

Hello,

We are resubmitting our manuscript **"**A Microphysiological System for Modeling Breast Cancer in Human Breast Tissue". After taking into consideration the comments from the editor and the reviewers, we have updated the figures as well as the manuscript itself. We thank the editor and the reviewers for their helpful suggestions. We have resubmitted our manuscript with one copy displaying the changes that were made as well as a supplemental file without the changes displayed to make it easier for the reviewers to read. We look forward to working with you further on this manuscript. Thank you for your time and consideration.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Abbreviations have been defined at the first use and the manuscript has been proofread to correct spelling.

2. Please provide an email address for each author.

The authors and their emails are as follows: Loren M Brown – lbro44@lsuhsc.edu; C. Ethan Byrne "Charles Byrne" - cbyrne4@tulane.edu ; Elizabeth C. Martin - emart93@lsu.edu ; Frank H. Lau - flau@lsuhsc.edu

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: glutamax, Accutase, Accumax,

Commercial language has been removed from the manuscript and replaced with generic terms.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The use of safety procedures such as use of hoods has been updated. The language has been corrected to remove phrases such as "should be" from the protocol.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps.

Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

More details have been added to the protocol and references to published material have been added detailing how to perform specific protocols.

6. 2.4: If you don’t plan to include the 3D printing in the video, please cite a reference to help readers replicate the protocol.

References have been added to help readers replicate the protocol. We can also upload the 3D design file of the plunger as a supplementary file if the editor thinks this will be useful to the reader.

7. 4.5, 6.9: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Centrifuge speeds have been converted to centrifugal force instead of rpm

8. As Figure 5 and Figure 6 actually describe the proof-of-concept of why this system works, please briefly include the analyses to be done to confirm that your system works (metabolic cross-talk, migration, etc). If this will not be filmed, please cite references to allow readers to replicate these methods.

More details have been included to cover the analyses that the system works and references have been added to allow readers to replicate these methods.

9. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. Please make this look different from Figure legend description.

More details have been added to the representative results section suggesting different ways to analyze the outcomes and how the results show the technique.

10. Please move figure legends (title and description) to appear after the Representative Results, before the Discussion. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

The figure legends have been moved to appear after the representative results.

11. As we are a methods journal, please add to the Discussion with citations:
a) Any limitations of the technique
b) The significance with respect to existing methods

The discussion has been expanded to include limitation of the technique as well the significance to existing methods.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This describes a organoid culture for breast cancer in a 3D sandwich that used breast cancer adipose cells.

Major Concerns:
The discussion needs to include key limitations. First, the situation described herein is a static culture organoid, not having fluid flow that can affect mass transfer. This needs to be discussed, and the ease of the current protocol versus the limitations compared to 'organs on a chip'. Second, the inclusion of the ASC leaves stem cells in the organoid. This needs to be discussed as stem cells impact the phenotype and behavior of cancer nodules.

The discussion has been expanded to include limitations of the model as well as discussing how this model compares to other co-culture models and organs on a chip. We have also expanded the discussion to highlight that the ASCs and the adipocytes will both influence the behavior of the cancer cells.

Minor Concerns:
The source or process for isolating the ASC needs to be described in a greater detail.

References for methods for isolating the ASCs have been added to the manuscript to assist the reader in isolating their own ASCs.

Reviewer #2:

Manuscript Summary:
The manuscript describes a microphysiological system that models the human breast, which is based on the sandwiching of adipose tissue between two adipose-derived stem cell sheets. The manuscript further shows how this model can be seeded with breast cancer cells, to form a microphysiological system of breast cancer.

The manuscript is in general clear and of interest. However, several points should be addressed:

Major Concerns:
- Page 7 line 224-226: more information is needed here. What is meant with 'intensity' as mentioned in the table and how is this scored? Also, I think the authors should be more critical regarding the

'preservation' of macrophages in the system. CD206 drops from 682 to 266 between day3 and day7, while CD68 drops from 729 to non-detectable. This is a significant reduction in macrophage markers, which does not show the 'preservation' of primary macrophages

The wrong intensity was included for the CD68 at day 7. The figure has been updated to include the correct intensity of the CD68 marker which displays better preservation of the primary macrophages in the system. Further work will be carried out analyzing how the cancer cells influence the immune cells in the system including their polarization but this is out of the scope of this manuscript.

Minor Concerns:
- Page 3 line 74: please state a seeding density in terms of ##cells per square cm or mm

This has been updated in the manuscript to include the number of cells per square cm.

- Page 4 line 85: including an image displaying the mentioned 'striated pattern' would be useful

An image of the ASCs demonstrating the striated pattern has been added to figure 2.

- Page 4 line 111-112: please use cm or mm as the standard scientific unit for length

The units for the box for transporting the BC-MPS have been changed from inches to cm.

- Page 5 line 143: what is meant with 'ready to use'? How is this determined?

The language in this section has been updated to clarify this part of the protocol.

- Page 6 line 181: please add the duration of incubation

The duration of the incubation has been added.

- Page 7 line 187: please add what type of shaker is used (rotary shaker? rocking plate?)

The step in the protocol has been updated to indicate that an orbital shaker should be used for this step.

- Page 7 line 213-214: why were these images taken after 6 days instead of 14 days? Does this mean that the system does not remain patent for 14 days for all applications?

These cultures were done for experiments where we were comparing different culture methods of PDX explants. The PDX explants cultured without BC-MPS deteriorate quickly so the experiment could only be carried out for 6 days to allow for a worthwhile comparison between the two culture methods. These images were taken to demonstrate the ability to culture PDX explants in the breast tissue and not to suggest that the culture is only viable for 6 days.

- Page 8 line 237 and 238: the text should refer to figure 5 and 5C instead of 4 and 4C

The text has been updated to refer to the correct figures.

- Page 9 line 267: it would be good to add a reference regarding the functioning of the pNIPAAm-coated surface.

References have been added regarding the functioning of the pNIPAAm-coated surface.

- Figure 2 legend line 362: should be polylactic acid instead of polysactic. Please also add the depth of the recess of the plunger. Also, I think it would be good to include a picture of the actual plunger.

The spelling mistake has been corrected, the depth of the recess of the plunger has been added to the figure legend and a picture of the actual plunger has been added.

- Figure 5 legend line 398: should be $p<0.01$ instead of $p>0.01$

The p value has been corrected.

- The last column of the table of materials should be included on the same page and not be transferred to the next page

The table of materials has been reformatted so that the last column is on the same page.

Reviewer #3:

Manuscript Summary:

This protocol describes a method for generating breast cancer mimetics, with malignant cells sandwiched between non-diseased human breast tissue (HBT) containing native
23 extracellular matrix, mature adipocytes, resident fibroblasts, and immune cells, and 2) sandwiching
24 the BC-HBT admixture between HBT-derived ASC sheets. While I am always excited to see new techniques for generating improved pathomimetics, the fidelity of this technique to primary breast cancers needs to be better described and compared to existing methods. Additionally, the authors have demonstrated similar sandwiching techniques in recent papers for encapsulating adipose tissue, raising questions of how this technique differs from their previously published work. If the novelty of this work arises exclusively from encapsulating breast tissue and not adipose, and the usefulness of this technique in regulating breast cancer phenotype has not been established, I cannot recommend this technique for publication.

Major Concerns:

*How does this method differ from your previously published adipose sandwich method? (PMID 30176000)

*You claim that this method includes adipocytes, ECM, endothelium, & immune cells. To my read, you

incorporate human breast adipose derived stem cells (but no transdifferentiation to mature adipocytes), gelatin, and chunks of human breast tissue. Can you provide evidence that the current technique better mimics behavior of in vivo breast cancer or what this technique offers over mixed cocultures? That this work does not cite published results is very concerning.

This work builds upon our previously published adipose sandwhich method by incorporating the use of cancer cell lines and pdx tumor explants to understand how human breast tissue influences cancer progression and to understand how cancer cells remodel human breast tissue. This technique offers the ability to culture breast cancer cells with mature differentiated adipocytes as opposed to ASCs differentiated in culture which do not fully mature. This technique also allows the breast cancer cells to interact with the native ECM of the breast tissue instead of simplified scaffolds such as those composed of purely collagen I or murine scaffolds such as Matrigel. In addition to being able to study how the native human breast tissue influence breast cancer this model also allows for understanding how breast cancer can remodel human breast tissue. We are in the process of writing multiple manuscripts demonstrating the usefulness of this technique for studying cancer.

Reviewer #4:

Manuscript Summary:

The authors show a method to biofabricate a breast cancer in vitro model using primary cells with several phenotypes of physiological relevance.

Major Concerns:

* The paper is clear, althouth it would benefit from an english-editing service.

The language in the manuscript has been cleaned up to make it more readable and clear to the reader.

* The authors highlight that current systems do not meet the NIH criteria. However, the evidence shown in their manuscript do not meet criteria neither.

o No tight control in the architecture is evidenced.

The tissue is minced before placing the tissue samples between the ASC sheets. This mincing can alter the architecture of the tissue but once the BC-MPS has been made the overall structure is conserved.

o Is the architecture shown in Figure 4 reproducible? Are those dimensions relevant or similar to real BC histologies?

Figure 4 has now been made figure 3. The architecture shown in figure 3 is highly reproducible when the samples are processed the same. Further studies are being conducted to analyze how the breast cancer cells remodel the extracellular matrix and this will be compared to primary BC histologies. However, this is outside of the scope of this manuscript. Since the tissue is minced and large pieces of ECM are removed from the tissue before it is cultured this can result in changes compare to primary BC histologies.

o The survival time shown is shorter than the one stated by the NIH (2 weeks vs 4 weeks).

Our previous work demonstrated that the adipose tissue is stable for up to 8 weeks when cultured ex vivo. The culture time of 2 weeks was chosen for these experiments to allow time for the cancer cells to remodel the normal human breast tissue. We did not need to extend these experiments out to 4 weeks to see remodeling. The system is still viable at 2 weeks and can be further cultured if longer time points are needed.

o The physiological response is not proven with a single staining charaterization with Bodipy.

Previous work done by other groups have demonstrated that increased lipid uptake by cancer cells remodels their physiological response and that this correlates with increased Bodipy staining of the cancer cells. The staining with Bodipy is meant to demonstrate that the cancer cells are engaged in metabolic crosstalk with the adipose tissue and are taking up lipids released from the adipose tissue. Further experiments demonstrating the mechanism by which this changes the cancer cells physiological response are ongoing and are out of the scope of this protocol manuscript.

o The presence of immunce cells in the biofabricated construct is not evidenced.

Figure 3 has been updated with the correct immune cell intensity to better demonstrate the preservation of macrophages in the system. Further experiments will be conducted to determine how the cancer cells affect the polarization of the macrophages but this is out of the scope of this manuscript.

Minor Concerns:
* Do adipose-derived stromal cells (Line 62) and adipose-derived stem cells (Line 73) share the same abbreviation (ASCs)?

The language has been changed to be more consistent.

* The authors could improve the figures. They should be more informative. Specially Figures 3 and 4.

The figure legends and representative results have been updated to be more informative.

* Figure 6. Information is missing. What day of culture is this?

The information of the day of the culture has been included in the figure legend for figure 5 (previously figure 6).

* Can figures 1 and 2 be merged?

Figures 1 and 2 have been merged.

* Compared to extrusion bioprinting, what are the advantages of this biofabrication method in terms of time, control, resolution, and cost?

The discussion has been expanded to include comparisons to bioprinting methods as well as other co-culture methods including organ on a chip system.

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September 1, 2020

Ronald Myers, Ph.D.
Director of Editorial
Journal of Visualized Experiments

Dear Dr. Myers,

I am pleased to submit an Original Article entitled, "A Microphysiological System for Modeling Breast Cancer in Human Breast Tissue" for consideration in *Journal of Visualized Experiments*. In this manuscript, we describe a new protocol for setting up an *in vitro* microphysiological system for studying breast cancer using healthy primary human breast tissue. The key highlights of our system are:

- Inclusion of native elements of the breast tissue such as mature human primary adipocytes, stromal cells, and extracellular matrix
- Recapitulates the crosstalk between breast cancer cells and the adipocytes such as metabolic crosstalk
- Stability of the in vitro model system up to 2 weeks in culture

We believe this manuscript is an excellent fit for *Journal of Visualized Experiments* because it introduces a novel method for studying breast cancer in its native tissue microenvironment. Furthermore, this system includes several key features that previous model systems lacked such as inclusion of mature adipocytes, native extracellular matrix, immune cells, and stability in culture for weeks at a time. As such, we believe this manuscript will be of great interest to your readership.

Our manuscript has not been previously published and is not under consideration for publication elsewhere, and we have no conflicts of interest to disclose. If you feel that our manuscript is appropriate for your journal, we look forward to working with your editorial team to prepare this work for publication. Thank you for your time and consideration.

Sincerely,

Frank H. Lau, MD, FACS

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