Title

Development of Organoid Models of Breast Cancer Residual Disease After Neoadjuvant Therapy

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Development of Organoid Models of Breast Cancer Residual Disease After Neoadjuvant Therapy

by
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of the
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Development of Organoid Models of Breast Cancer Residual Disease After Neoadjuvant Therapy

Sigal Eini

Abstract

Organoid culturing is a three-dimensional tissue culturing method with significant promise for increasing our ability to model cancer subtypes in the laboratory. We modified tissue processing methods and used a natural hydrogel (GrowDex) as well as basement membrane extract (BME) to optimize conditions for growth and immunostaining of breast cancer organoid cultures. We focused on breast cancers that were Estrogen Receptor (ER) and/or Progesterone Receptor (PR) positive, generating cultures of residual disease after treatment with neoadjuvant endocrine therapy in the Investigation of Serial studies to predict your therapeutic response with Imaging and Molecular AnaLysis 2 (NCT01042379, I-SPY2) clinical trial. Patients on an Endocrine Optimization Protocol (EOP) in I-SPY2 were treated with amcenestrant alone or in combination with an aromatase inhibitor or a CDK4/6 inhibitor prior to specimen collection and culturing. The goal of these studies was to generate conditions for successful propagation of residual breast cancer resistant to primary treatment, including verification of expected protein expression patterns, for future experiments to test new therapies for ER+ breast cancer resistant to endocrine therapy.

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List of Abbreviations

Basement membrane extract (BME)

Cell Titer Glo (CTG)

Ductal carcinoma in situ (DCIS)

Endocrine Optimization Pilot (EOP)

Extracellular matrix (ECM)

Estrogen Receptor (ER)

Estrogen Receptor 1 (ESR1)

Functional tumor volume (FTV)

GrowDex (GD)

Human Epidermal Growth Factor Receptor 2 (HER2)

Investigation of Serial studies to predict your therapeutic response with Imaging and Molecular AnaLysis 2 (I-SPY2)

Progesterone Receptor (PR)

Triple negative breast cancer (TNBC)

3-dimensional (3D)

Introduction

In 2020, there were 2.3 million women diagnosed with breast cancer and 685,000 deaths globally. As of the end of 2020, there were 7.8 million women alive who had been diagnosed with breast cancer in the past 5 years, making breast cancer the world's most prevalent cancer. (1)

Approximately 70% of fatal breast cancers express the luminal estrogen receptor (ER), a phenotype that is often initially responsive to therapies that target ER prior to the development of drug resistance. (2) In order to fully understand the ER pathway, a stable in vitro culture is necessary, but has been a particular challenge to the field. Recent 3-dimensional (3D) culture methods have been used to propagate tumors with high efficiency and are promising for culturing ER positive breast cancers. Sachs et al. was able to recapitulate histological and genetic breast cancer heterogeneity providing a more representative model of breast cancer compared to standard 2-dimensional cell lines using a technique called organoid culturing. (3) Rosenbluth et al. further used these methods to propagate hormone sensing cell types in normal breast tissues in culture, improving our ability to isolate and grow these cell types compared to prior culturing methods, but demonstrating persistent challenges with slow growth rates and variable loss of ER activity in the cultured cells. (4,5)

Most 3D culturing methods, including organoid culturing, rely on Matrigel, a practice which developed out of pioneering work in the 1970's and 1980's using collagen followed by laminin-rich hydrogels to advance tissue culture. (6) Matrigel is a

solubilized basement membrane matrix that is secreted from mouse sarcoma cells. It has an abundance of basement membrane proteins (laminin, collagen IV, heparan sulfate proteoglycan) and growth factors like TGF-beta and EGF. (7) Though it is widely used, Matrigel is not a well-defined matrix because it is produced by a cell line, and this and other features can cause variability and errors in experiments as well as practical handling issues. Matrigel is liquid at low temperatures and a gel at room temperature. Thus, it requires being kept on ice and quick action to prevent it from solidifying while it is being plated.

A recent study suggested that a novel hydrogel can be used as an alternative to Matrigel to culture ER-expressing breast cancers, although growth was only assessed in the short-term and the ability to successfully expand and propagate cultures was not assessed. Munne et al. found that ER alpha, one of two main types of ER that is encoded by the gene Estrogen Receptor 1 (ESR1), signaling is regulated by matrix stiffness in primary breast cultures. By utilizing an animal-free hydrogel (GrowDex) instead of Matrigel, in vitro ER alpha-expressing cultures were stably maintained. (3) Here I undertook efforts to optimize 3D culture methods to ensure ER alpha-expressing cultures are grown. I also identified additional benefits and challenges of using GrowDex for organoid culture.

In order to put these results in context, I will first provide background on breast cancer subtypes, stromal stiffness and a natural hydrogel alternative for extracellular matrix.

Breast cancer subtypes

Breast cancer is considered a heterogeneous disease that is comprised of multiple subtypes, with classification schema generally including histopathological type, grade, and the presence or absence of key proteins that define the major subtypes of breast cancer (ER, PR, and HER2). These protein markers roughly correlate with major subtypes of breast cancer that have been defined based on their gene expression signatures from genomic analyses (luminal A, luminal B, HER2-type, and basal-type), as shown in Table 1.1. (8)

There are also different histopathologic types of breast cancer, as well as preinvasive lesions of the breast, as depicted in Figure 1.1.

Table 1.1. Gene signature correlation with protein marker patterns:

Subtypes	ER	PR	HER2	Ki-67
Luminal A	+	+/–	–	<14%
Luminal B	+	+/–	+/–	≥14%
HER2+	–	–	+	≥14%
TNBC	–	–	–	≥14%

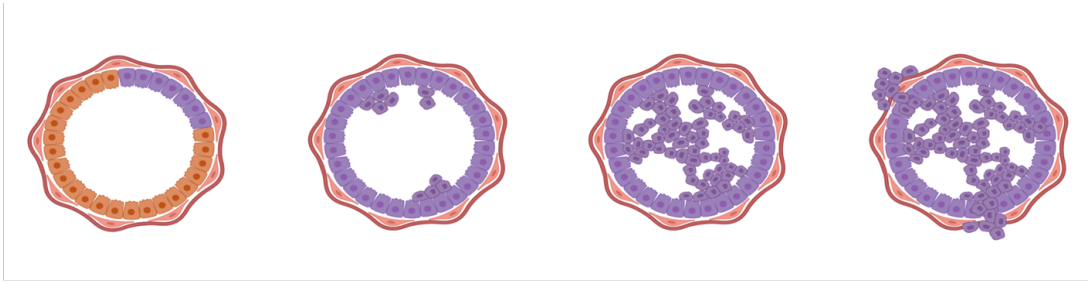


Figure 1.1. Invasive ductal carcinoma is cancer that happens when abnormal cells growing in the lining of the milk ducts change and invade breast tissue beyond the myoepithelial (basal) layer of the duct. The cells in the first two panels that are purple are destined to become carcinoma, and the purple cells in the last two panels are carcinoma cells. The luminal and basal (myoepithelial) layers are the interior and exterior cell layers, respectively.

Cancer tissue stiffness

Matrix stiffness can have a substantial impact on breast cancer phenotypes including ER expression and cancer progression (2). Matrix stiffening is caused by the accumulation, contraction, and crosslinking of the extracellular matrix by cancer and stromal cells. These cells in turn respond to matrix stiffness in myriad ways, which influences the phenotypes of the cells. In addition, matrix stiffness activates and/or inactivates specific transcription factors in cancer and stromal cells to regulate cancer progression.

Munne et al. found that in short-term cultures of freshly isolated fragments of breast tissue, the hormone receptor expression is lost and that by varying the stiffness/ identity of matrices (including GrowDex), ER expression is regulated by matrix-dependent mechanical forces. (2) Thus, using matrices with the optimal stiffness is an important factor to consider for modeling and sustaining ER-expressing organoids.

GrowDex as a potential substrate for organoid cultures

GrowDex is an animal-free hydrogel that mimics the extracellular matrix (ECM) and supports cell growth and differentiation. High water content ensures easy diffusion of nutrients and metabolites. (9) Unlike basement-membrane extract (BME) or matrigel, GrowDex is temperature-stable and can be stored and used at room temperature. Since GrowDex is made from Finnish birch pulp (nanofibrillar cellulose), there is reported to be notably less lot-to-lot variation as compared to animal-based hydrogels that have varying amounts of animal proteins. GrowDex is tunable in terms of stiffness (modulated

with dilution) and chemical composition through use of GrowDex-A. GrowDex-A consists of avidin conjugated nanofibrillar cellulose which can be customized by binding different biotinylated molecules such as proteins or peptides to create a cell-specific matrix.

Notably, GrowDex hydrogels do not auto-fluoresce so imaging with any microscope is easier, especially for distinguishing low-intensity signals from background noise. Though there is a one-step recovery of cells and organoids using GrowDase, an enzyme that breaks down GrowDex hydrogel without impacting cell viability or functionality, organoids need to be processed further to ensure 3D growth, otherwise they grow in a planar fashion.

Overall, GrowDex properties are purported to represent an alternative hydrogel that is conducive to easy handling, minimal lot-to-lot variation, low fluorescence background, and optimal stiffness for maintained ER expression.

METHODS

Tumor direct chop/ enzymatic degradation

A biobank of organoid cultures was generated from surgical tissues obtained from patients who gave informed consent and were enrolled in the I-SPY2 Endocrine Optimizaton Pilot Protocol (EOP). (10, 11) These patients were treated with neoadjuvant systemic therapy (with an endocrine therapy backbone) and underwent surgery at UCSF. In most cases, each tissue sample was cut into smaller pieces, washed thoroughly, stored and/or fixed and embedded. Fresh tissue was viably frozen in 90% FBS (Cytiva, Cat. No. SH30910.02) and 10% DMSO for future organoid culture generation, with a small piece formalin fixed and paraffin embedded (FFPE) for histology. The remaining majority of tissue was used to generate organoid cultures by a direct chop or digestive method.

The direct chop method directly embedded tissue after a mincing process using opposing scapel movements in 50 µL of 4 ℃ 10 mg/mL Cultrex growth factor-reduced BME type 2 (Trevigen, Cat. No. 3533-010-02). The gel containing organoids was placed in a 24-well plate and incubated at 37 ℃ for 30 minutes to allow the BME to polymerize and mimic the extracellular matrix providing support for the 3D organoids. Then 500µL of warmed Type 1 and Type 2 organoid culture medium as described by Dekkers et al. was placed over the organoid dome. (11) Medium was changed every 2-3 days and organoids were passaged using TrypLE Express (Invitrogen, Cat. No. 12604-013) every time a culture reached a threshold growth.

In the digestive method, tissue was placed into a 50 mL conical tube containing 2 mL 1 mg/mL collagenase (Sigma, Cat. No. C9407), 18 mL AdDF+++ (Advanced DMEM/F12 (Thermo Fisher, Cat. No. 12634-028) containing 1x Glutamax (Thermo Fisher, Cat. No. 35050-061), 10 mM HEPES (Thermo Fisher, Cat. No. 15630-080, and penicillin-streptomycin (Thermo Fisher, Cat. No. 15140-122) and primocin (Invitrogen, Cat. No. Ant-pm-1)). The conical tube was wrapped in parafilm and placed in a shaker at 37 ℃ for 30 minutes. Subsequent shearing was achieved using sequential pipetting with 10, 5, and 1 mL pipette tips before a pellet of primary breast organoids was obtained by centrifuging at 1000 rpm for 3 minutes and finally embedded in BME and, after the BME solidifies as above, overlaid with organoid medium.

Splitting Organoids

To passage organoids, the media was aspirated, and 500 µL of 37 ℃ TrypLE was added with serial pipetting to break up and homogenize the gel dome, followed by incubation for 2 minutes then homogenized again and incubated for an additional 2 minutes. Contents were transferred from the well to a 15 mL tube with 200 µL of FBS. The well was washed 3 times with 500 µL of 4 ℃ AdDF+++ and the contents were added to the tube. The 15 mL tube was filled to 10 mL with AdDF+++ and centrifuged for 3 minutes at 900 rpm to create a pellet. Then the supernatant was aspirated while being careful not to disrupt the pellet/ lose cells and organoids. 50 µL of 4 ℃ BME per well was added to the pellet being careful not to introduce bubbles while pipetting. The gel/ organoids were dispensed to a plate and incubated for 30 minutes at 37 ℃ to allow the gel to polymerize followed by the addition of 500 µL or organoid medium.

Growth of Organoids in GrowDex

2.5 mL of organoid medium was added to a 15 mL tube. The 2.5mL GrowDex (GD) syringe (Perkin Elmer, Cat. No. 100103002) was sterilized with ethanol and then the entirety of the syringe was slowly dispensed. A low-retention tip was used to pipette up and down slowly to avoid bubble formation. A 0.25 % GD solution was made from the 0.50% GD stock and organoid medium in a one-to-one ratio. Single cells were prepared from organoids (refer to splitting organoid protocol). Instead of adding BME to the pellet, the pellet was diluted in 1 mL AdDF+++ and homogenized. To count the cells, 10 µL of cells in AdDF+++ and 10 µL of trypan blue were added to a 1.5 mL tube and quantified using a hemocytomer. 50 µL of homogenized GD was dispensed per well using a multichannel pipette and reservoir into optically clear luminescence 96-well plates (Fisher Scientific, Cat. No. 07-000-128). 50 µL of cells were dispensed per well on top of GD solutions. For the 10% BME wells, 50 µL of cells, 40 µL AdDF+++, and 10 µL of 4℃ BME were combined and dispensed per well. The plate was incubated at 37 ℃. Every 2-3 days, 30 µL of organoid medium were added per well while monitoring the cells/organoids growth with an Echo brightfield microscope.

Quantification of Cell Viability

Cell Titer Glo (CTG) (Promega, Cat. No. G7572) reagents were temperature equilibrated from -20 ℃ to 2 ℃ overnight and then to room temperature 30 minutes before the assay. CTG reagents (60% of media in well= CTG volume/well) were added using a multichannel while the tissue culture lights were kept off. The CTG and cell

solution was mixed vigorously for 5 minutes and shaken for 5 minutes. Luminescence was recorded with EnVision 2105 luminometer using a previously validated protocol that removed background cross-well signal spillover. This protocol was adapted from CellTiterGlo 3D Cell Viability Assay TM412.

Immunofluorescence Staining

Immunofluorescence antibody staining was performed on organoids cultured on 8-well glass chamber slides (Thermo Fisher, Cat. No. 12-565-8). Organoids were blocked in 10% goat serum in 1x TBST (TBS-Tween 20) with 1% bovine serum albumin (BSA) at room temperature. Conjugated and primary antibodies were diluted (1:200) in 1% goat serum in 1x TBST with 1% BSA and incubated overnight at 4 ℃. Slides were incubated with secondary antibodies, where needed (1:200), for 20 minutes at room temperature and incubated with 1 mg/mL DAPI (1:5000) in PBS for 1 hour at room temperature. Slides were mounted with Prolong anti-fade and coverslips sealed with nail polish. Confocal images were taken on a Leica SP8 LSM WLL at the Diabetes Imaging Center at UCSF.

Antibodies

The following antibodies, cell stains and dyes were used:

AlexaFluor 647 anti-estrogen receptor alpha antibody (EPR4097) –(AbCam, Cat. No. ab267512)

Progesterone receptor A/B (D8Q2J) XP rabbit mAb (AlexaFluor 488 conjugate)- (Cell Signaling Technology, Cat. No. 35591S)

Phalloidin conjugates, biotium- 00044-T, 50 U, Phalloidin, CF568 conjugate- (ThermoFisher, Cat. No. 89427-136)

DAPI (4',6 Diamidino-2-Phenylindole, Dihydrochloride) (ThermoFisher, Cat. No. D1306)

Single-cell RNA-sequencing

RNA-Sequencing was performed at UCSF Parnassus in the 10x Co-labs with the assistance of Shruti Warhadpande. Briefly, organoids were processed to the single cell level with TrypLE and serial pipetting, and filtered through a 40 micron filter, and loaded onto the 10x Chromium 3' platform immediately. An aliquot was stained for viability and counted just prior to loading. Libraries were sequenced at the Institute for Human Genetics, and paired-end reads were processed and mapped to the GRCh38 human genome using CellRanger. Data analysis was performed using Partek flow software by Jennifer Rosenbluth.

Graphs were made with GraphPad Prism or Microsoft Excel and schematics were made with BioRender. Brightfield images were taken with an Echo Revolve.

Results

Optimization of GrowDex for 3D breast culture

Although promising, many organoid cultures grow at slow rates with patient-to-patient heterogeneity and notable differences between breast cancer subtypes. Efforts in the Rosenbluth lab indicated that some luminal breast cancers grew slowly as organoids and were challenging to expand in the lab. Furthermore, immunofluorescent staining and imaging of organoids within a 3D gel resulted in significant background fluorescence which appeared to be due to the hydrogel. Therefore, we decided to assess different matrices in order to improve growth and imaging conditions. We decided to test GrowDex due to the ability to modulate stiffness and due to the described decrease in lot-to-lot variability.

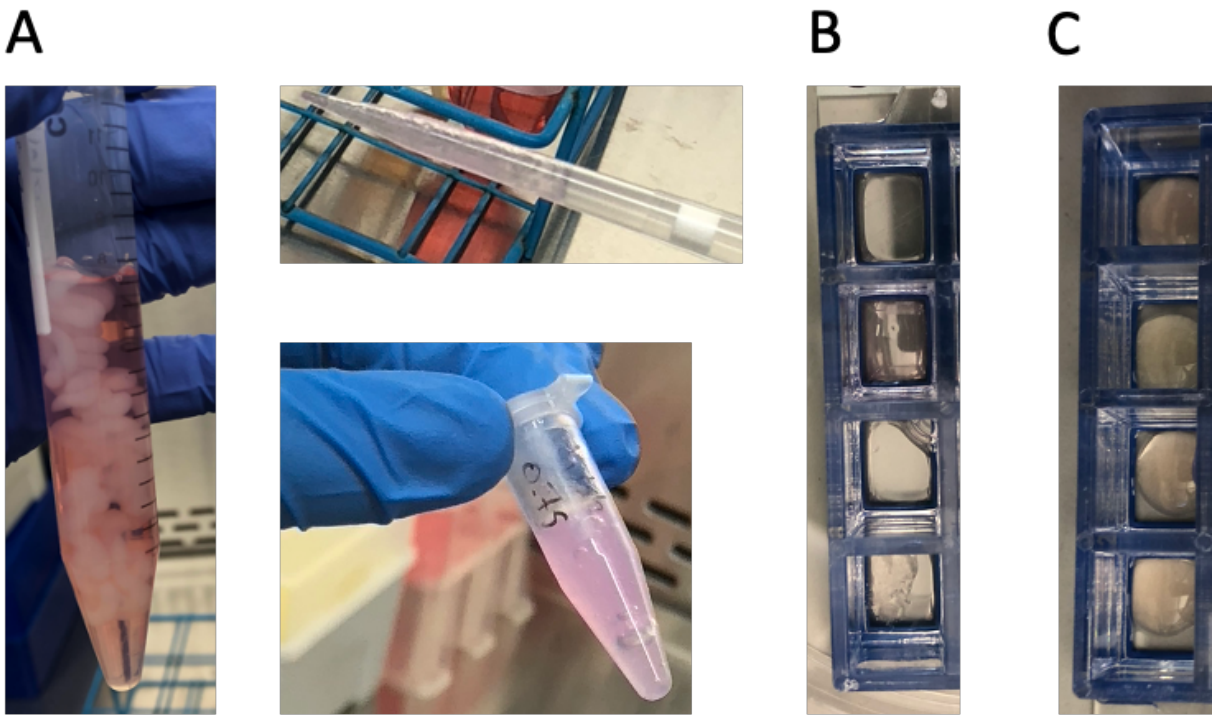
Before GrowDex could be assessed as a viable alternative to BME, it was necessary to optimize experimental conditions and protocols for this application. During these initial experiments utilizing GrowDex we identified several challenges that could be overcome with straightforward technical adaptations. An undiluted GrowDex syringe is at 1.5% whereas the suggested working concentrations are 0.75%-0.25%. To dilute the GrowDex, the syringe is ejected into cell media where it needs to be pipetted up and down slowly to create a homogenous solution preventing the formation of bubbles. Figure 3.1 panel A shows one potential pitfall; the pipette retained a significant portion of the hydrogel, suggesting the need to use low retention tips to prevent adhesion and to serially dilute the working solutions. Though GrowDex and alternatives like GrowDex-A are utilized for their optical/ imaging properties, the GrowDex-A (shown in Figure 3.1

panel B) washed off the glass chamber slide presumably because of the anionic group avidin, whereas regular GrowDex was able to retain its dome shape in culture over time (shown in Figure 3.1 panel C). Thus, we were not able to use GrowDex-A for microscopy methods that utilize organoids grown in 3-dimensional culture on a chamber slide prior to staining and imaging but were able to obtain sufficient optical properties with GrowDex, as described further below.

After implementation of these technical modifications, we were able to develop working solutions of GrowDex for subsequent optimization experiments. In addition, these protocols enabled use of GrowDex for the same set of applications routinely used for initial culture and characterization of organoid cultures using BME or Matrigel. Prior optimization studies in the Rosenbluth laboratory suggested that BME is not inferior and may be superior to Matrigel for some applications, therefore we proceeded with comparisons between GrowDex and BME.

Figure 3.1. Trouble shooting practical solutions for working with GrowDex

A. 0.75% GrowDex is extremely viscous and stuck to the pipette showing the need to use low retention tips, work within appropriate volumes by batching experiments and practice slow pipetting methods **B.** GrowDex-A washed off the glass chamber slide presumably because of the anionic group **C.** GrowDex was able to retain its dome over time demonstrating the need to use GrowDex for optical imaging rather than GrowDex-A which was recommended as the ideal hydrogel for imaging due to lower background noise.



We next optimized a protocol for mixing the breast cancer cells with the GrowDex working solutions to generate organoid cultures. UPM Biomedicals, the manufacturer and distributor of GrowDex suggests two orders of operations for cell/organoid cultures that would need to be assessed when working with a new cell or tissue type (Figure 3.2). In the “on top” method, GrowDex is plated first followed by the addition of cell/ media solution. This allows the cells to anchor onto the top of the dome structure, where they can grow maintaining a tether to this surface. In the “embedded” method, GrowDex and cells are mixed before they are added to the plate. This creates a homogenous mixture of GrowDex and cells after which organoids can grow within the gel.

These methods are similar to what has been previously used in the mammary biology field, for example methods to culture an immortalized MCF10A breast cell line in 3-dimensional culture using the “on top” method demonstrated successful growth of acinar structures across the top of the gel and tethered to the gel for two weeks. (4,12) Our lab standard had been to use basement membrane extract (BME, an alternative to Matrigel) using the “embedded” method for growing normal breast organoids. Though both methods were suggested by the manufacturer, during our protocol optimization we determined that the “on top” method is preferential when using GrowDex. In the embedded method, mixing the cells with the GrowDex invariably created bubbles that substantially disrupted the structure/integrity of the dome over time causing fewer organoids to grow and be retained in culture. This was true across all gel stiffnesses tested and across serial experiments. Thus, we proceeded with GrowDex using the “on top” method.

To assess GrowDex as an alternative to BME, organoids grown in BME were split into single cells and plated into different concentrations of GrowDex with different cell concentrations (Figure 3.3). After changing the culture medium, on the final day of the experiment the plate was imaged, and the cell viability quantified using a CellTiter-Glo (CTG) assay and a luminometer. The CTG assay determines the number of viable cells in culture based on the amount of ATP, an indicator of metabolically active cells, present. The lab previously used a standard curve to show that CTG correlates with organoid number in a linear fashion across working cell densities.

Figure 3.2. GrowDex workflow with different order of operations

Schematic showing comparison of two proposed protocols for generating organoid cultures using GrowDex, adapted from prior 3-dimensional culturing methods.

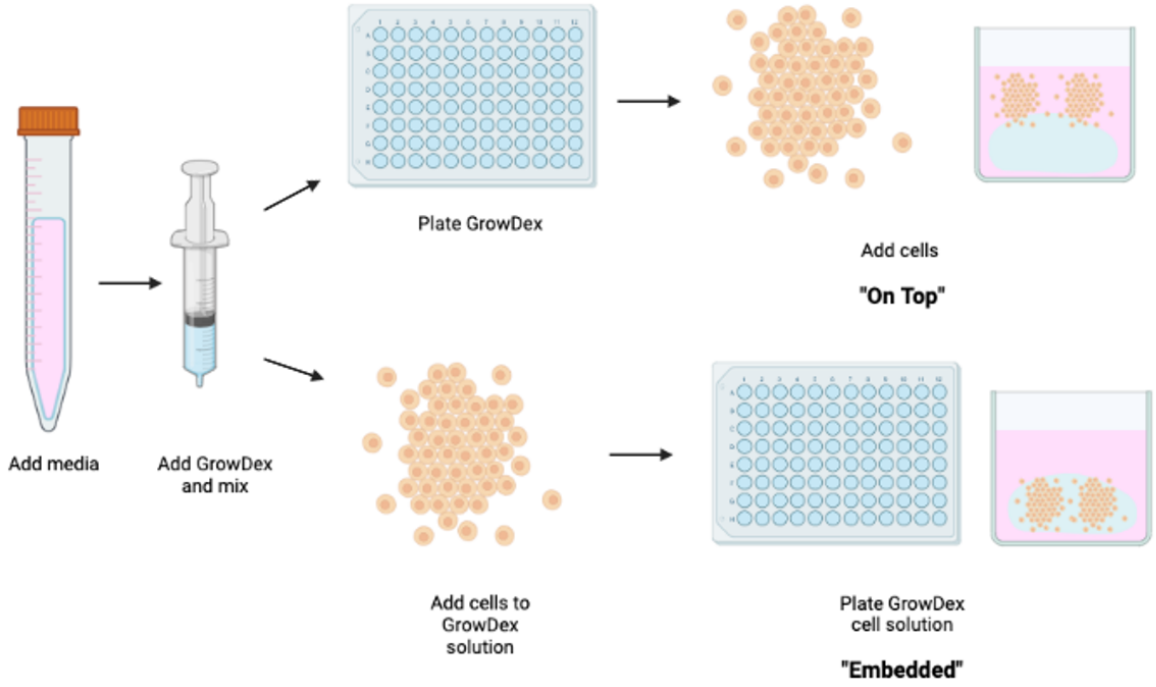
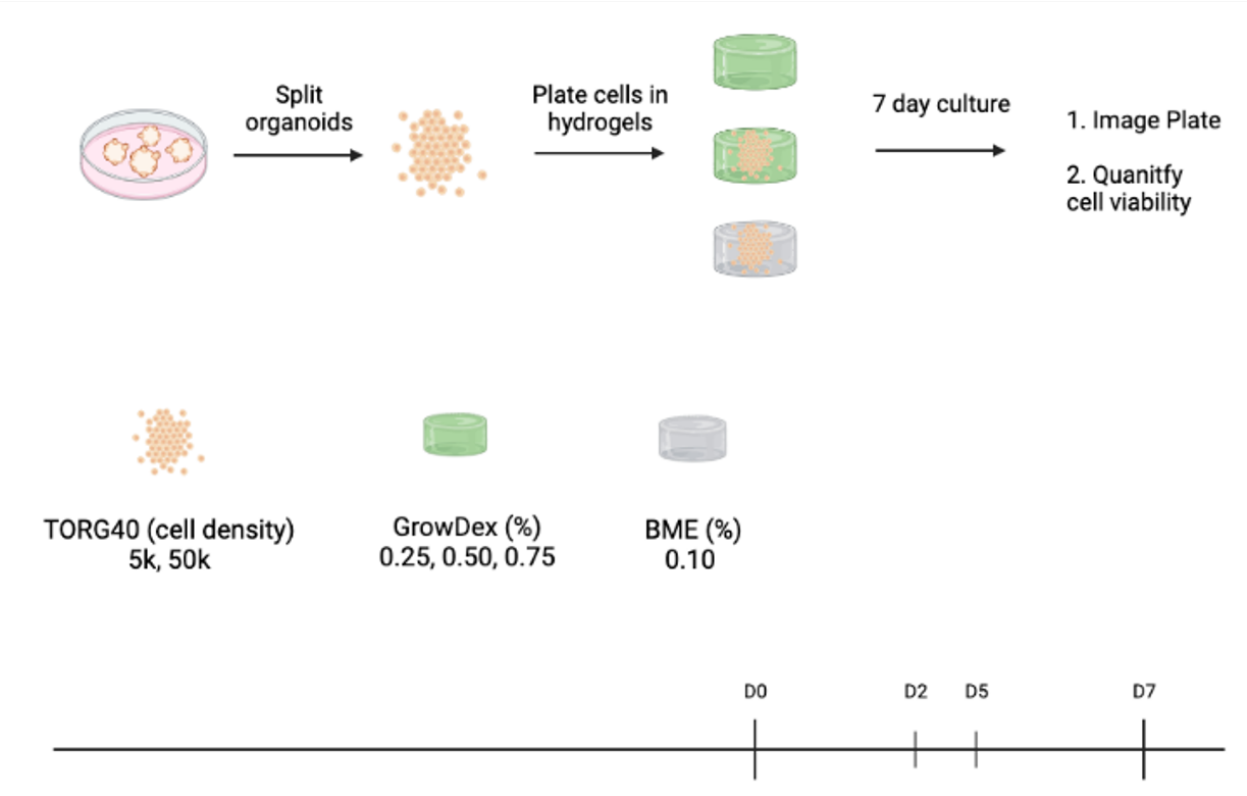


Figure 3.3. Experimental timeline

Schematic showing steps in optimization experiments to assess GrowDex as an alternative to BME for 3-dimensional organoid culturing.



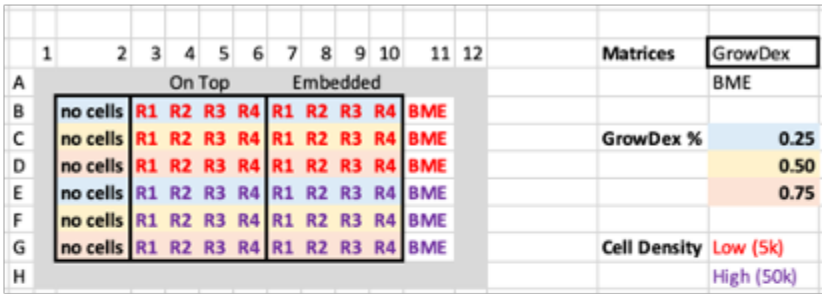
We set up repeated detailed experiments comparing low [5,000 (5K) cells] and high [50,000 (50K) cells] confluency for multiple hydrogel concentrations with four replicates per condition (R1, R2, R3, R4) given the potential for increased variability when plating organoids as opposed to single cells. The “no cells” condition provided a background measurement for the CTG assay while the 10% BME was our positive control for comparison of the culture conditions. The Rosenbluth lab had previously used 10% BME to conduct high-throughput compound screens using breast cancer organoids, so our initial comparison focused on this concentration of BME. Completion of these viability optimization experiments showed that 0.5% GD induced the greatest cell viability when compared to a 10% BME solution (Figure 3.4).

We proceeded with a “optimize-by-doing” phase for growth of breast organoid cultures in GrowDex versus BME using the ideal conditions for each hydrogel, assessing multiple human tissue types (normal breast, DCIS, and tumor) as well as mouse mammary tumors. For most of these comparisons we utilized 100% BME for the maintenance phase of organoid culture. After more than two weeks in culture, there was only a small difference in size using our standard growth conditions, although it was detected across multiple cultures (Figure 3.5 panels A and B). Initial growth differences (within the first three days) were more noticeable and were particularly helpful for one difficult-to-grow culture, ORG 9. After months of little to no growth in BME, there was a burst in growth and size when ORG 9 was cultured in GrowDex (Figure 3.5 panel C). Understanding the time dependency for the growth difference will be a focus for future studies to clarify when GrowDex should be used for optimal growth.

Figure 3.4. Cell viability for varying cell densities and GrowDex concentrations

The platemap in panel A displays different conditions used to compare GrowDex and BME as hydrogels for breast cancer organoid growth. The cell densities are 5,000 (5k) and 50,000 (50k) cells. R1-R4 refer to four replicates of the same condition. Though 0.75% GrowDex is listed, the solution was very viscous and was insufficient for the 50k cell concentration comparison. Panel B displays that at any cell concentration the highest cell viability achieved was using 0.50% GrowDex.

A.



	1	2	3	4	5	6	7	8	9	10	11	12		
A			On Top				Embedded						Matrices	GrowDex
B		no cells	R1	R2	R3	R4	R1	R2	R3	R4	BME			BME
C		no cells	R1	R2	R3	R4	R1	R2	R3	R4	BME		GrowDex %	0.25
D		no cells	R1	R2	R3	R4	R1	R2	R3	R4	BME			0.50
E		no cells	R1	R2	R3	R4	R1	R2	R3	R4	BME			0.75
F		no cells	R1	R2	R3	R4	R1	R2	R3	R4	BME			
G		no cells	R1	R2	R3	R4	R1	R2	R3	R4	BME		Cell Density	Low (5k)
H														High (50k)

B.

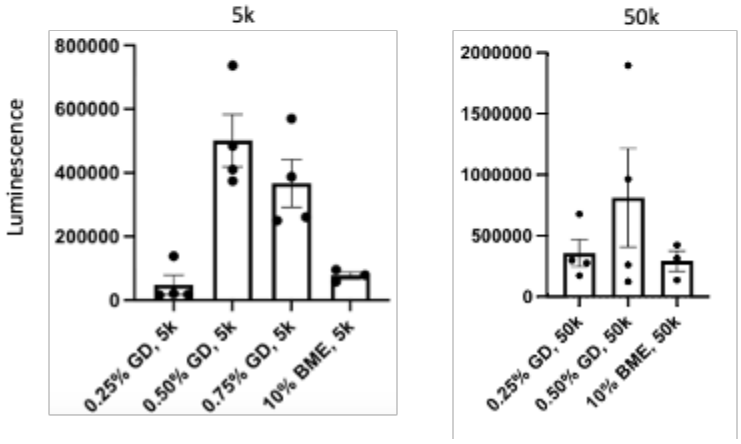
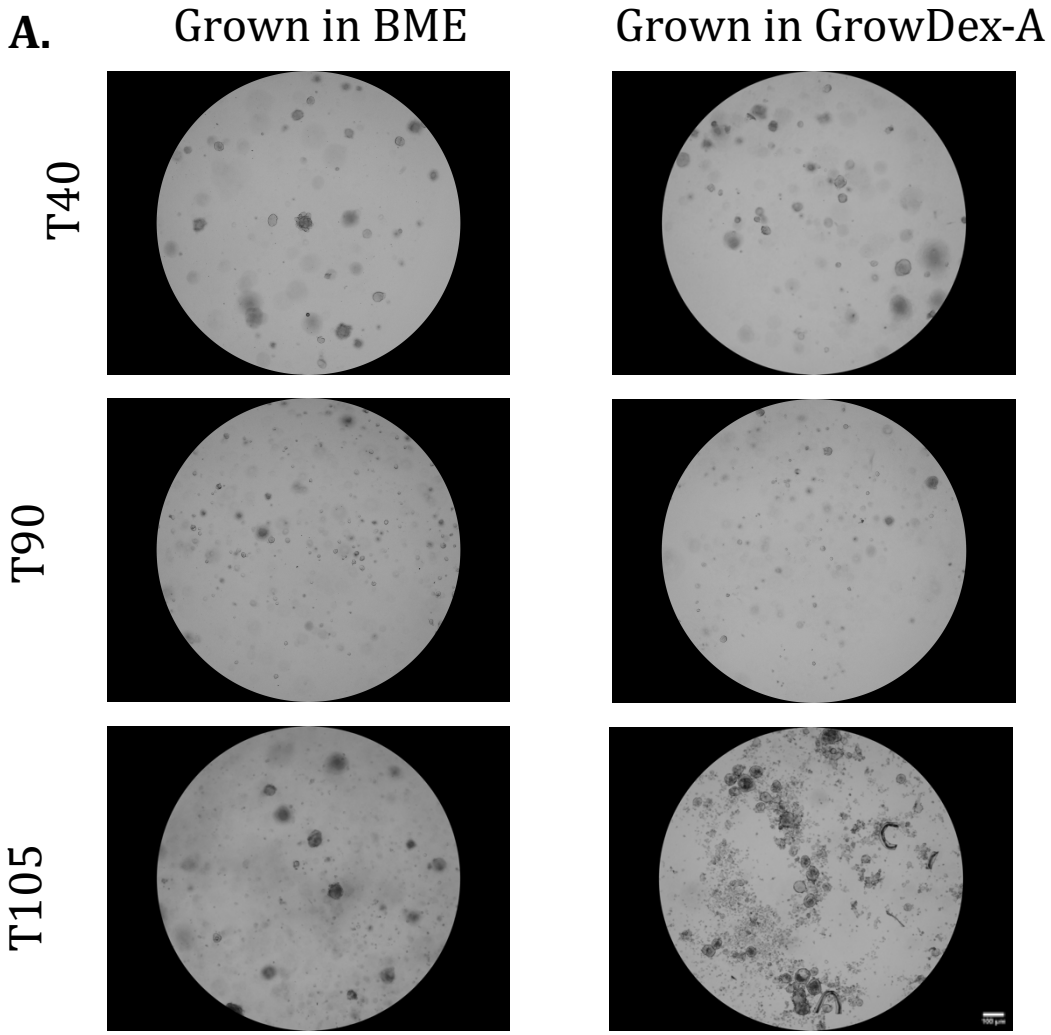
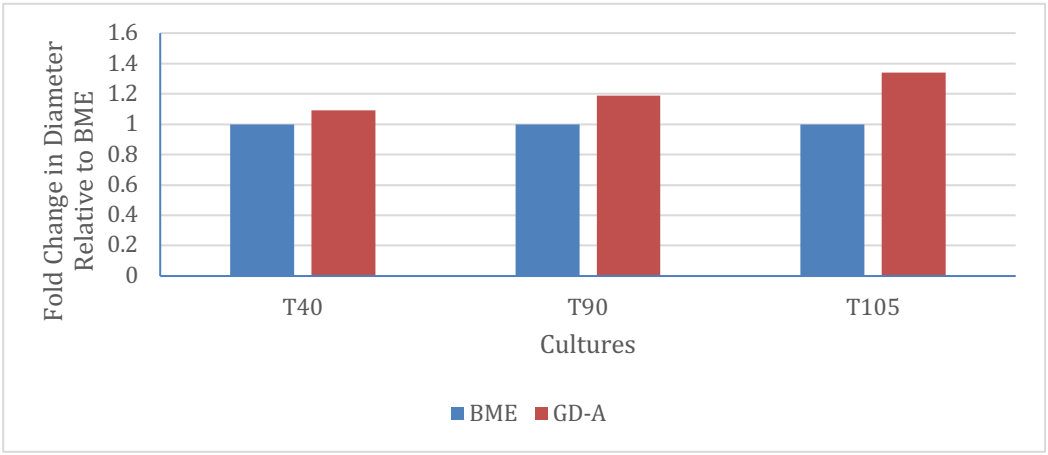


Figure 3.5. Brightfield images of organoids grown under the different conditions

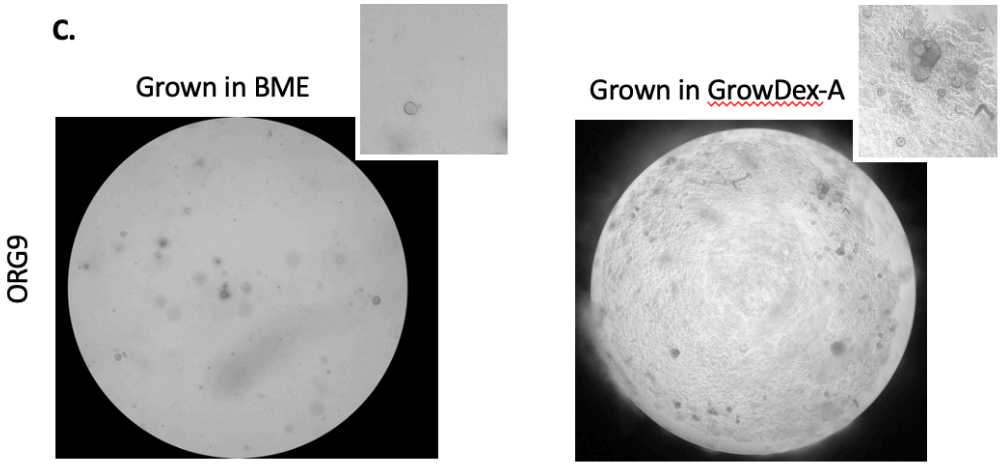
Panel A displays brightfield images of three different breast cancer organoid cultures grown in 100% BME and 0.5% GrowDex-A for two weeks. Panel B has the quantified fold change in organoid diameter (grown in GrowDex-A) relative to BME for all organoids in each image. Panel C and D show two examples of a normal breast organoid culture that was not growing over multiple passage attempts over several months but showed an increase in organoid number and size three days after transition from BME to GrowDex. Microscopy images in all panels were taken with low power (4x) magnification.



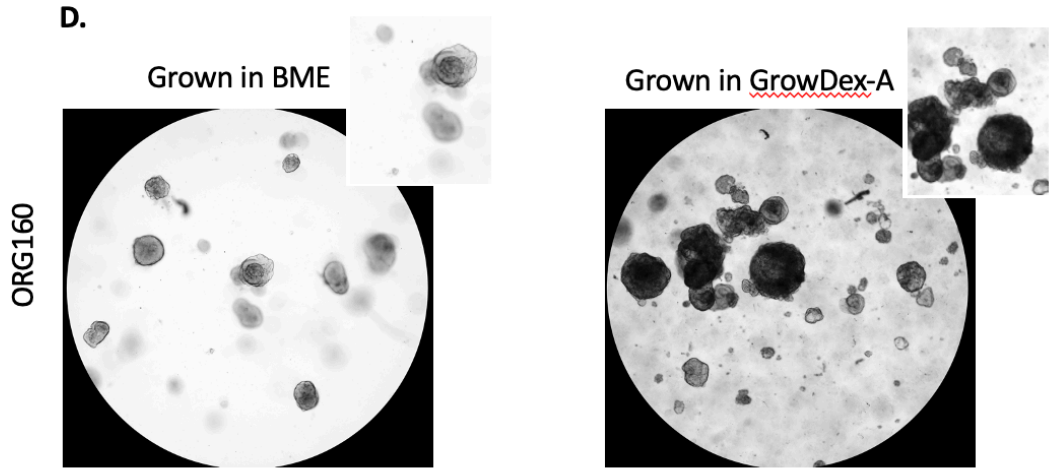
B.


Fold Change in Diameter Relative to BME
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
T40
T90
T105
Cultures
BME
GD-A

C.


Grown in BME
Grown in GrowDex-A
ORG9

D.

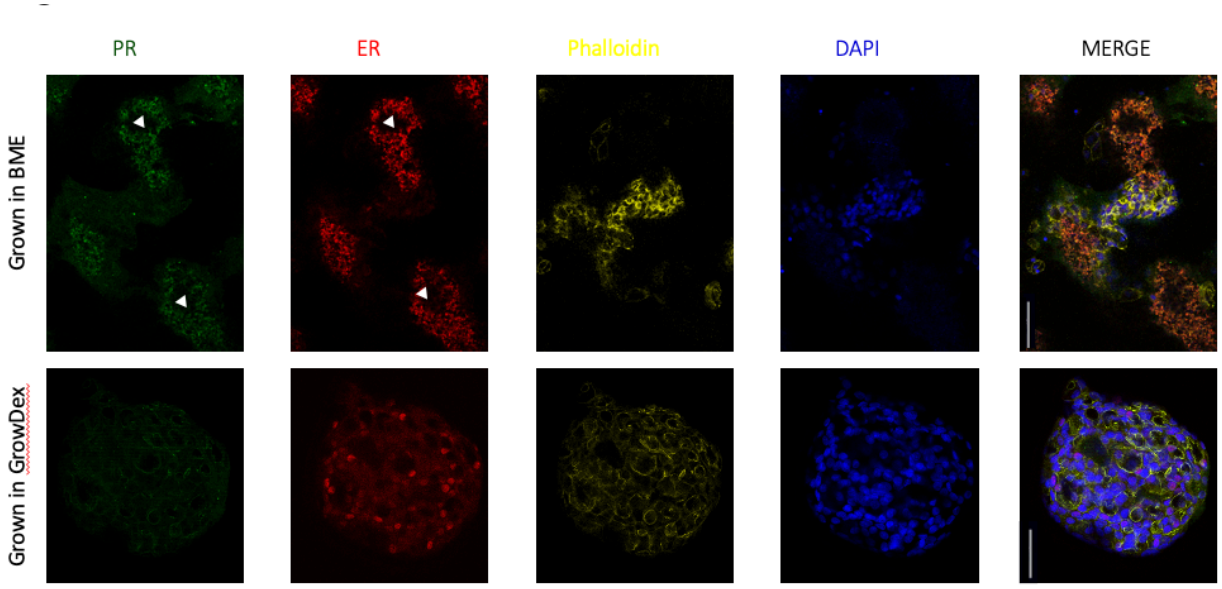

Grown in BME
Grown in GrowDex-A
ORG160

We next assessed the utility of GrowDex for microscopy experiments, including standard assessments to validate organoid cultures by expected protein expression patterns. We were particularly interested in whether we could detect ER expression, given challenges with maintenance of ER activity in organoid culture.

Prior experience with organoid cultures has shown that using Matrigel or BME can result in significant artifacts due to regions of autofluorescence within the hydrogel as well as autofluorescence from the organoids themselves. We found these artifacts to be greatly disruptive to qualitative and quantitative measurements of protein expression in organoid cultures when determining ER expression. We assessed the microscopy properties of GrowDex versus BME (Figure 3.6). These results showed a substantial reduction in artifacts produced within the BME and in organoids grown in BME, including autofluorescence of organoids and of apparent deposits within the hydrogel.

Figure 3.6. Immunofluorescence properties of GrowDex versus BME

TORG104, an organoid culture derived from an ER-expressing breast cancer, was grown and imaged in BME and GrowDex. The background autofluorescence, indicated with arrowheads (identified by the incongruence with the DAPI stain) was eliminated by using GrowDex to obtain the immunofluorescent (IF) images. The scale bar is equal to 100 microns (top) and 50 microns (bottom).



Sample collection from breast cancers resistant to amcenestrant on the I-SPY2 endocrine optimization protocol (EOP)

The I-SPY2 trial is an adaptive platform multi-center clinical trial for breast cancer patients. The EOP is an arm for luminal breast cancer patients in which they are treated with amcenestrant, an investigational Selective Estrogen Receptor Degrader (SERD). SERDs are drugs that bind to ER and in the process of doing so cause ER to be degraded and downregulated.

Patients on the EOP received amcenestrant either alone, or in combination with letrozole (an aromatase inhibitor) or abemaciclib (a CDK4/6 inhibitor). Patients on trial received pre-treatment biopsies in addition to tissue that was collected after 6 months of therapy at the time of surgery. We received core biopsies of tissue, taken at the time of surgery, from post-treatment tumors for generation of organoid cultures. The purpose of these organoids was to model clinically resistant disease, and also assess ER expression and activity in these samples to determine whether clinical resistance was associated with maintenance of luminal/ER characteristics, or an induced change in breast cancer subtype. First, we optimized conditions for growth of EOP breast cancer organoids.

Development of methodologies to establish EOP Organoids

Earlier studies in the Rosenbluth lab suggested that EOP organoid cultures grew very slowly (doubling time ~1 month) and were particularly challenging to establish.

Digestion steps with collagenase were noted to be particularly harsh to ER-expressing cells, resulting in low cell output at the end of initial processing steps. Early studies in the Rosenbluth lab proposed “direct chop” methods for organoid culturing and queried whether these could be a viable alternative to methods that utilized collagenase to digest tissues prior to culturing.

Direct chop is a mechanical digestion that breaks down tissue into smaller pieces exposing epithelial cells that eventually form organoids. Comparing methodologies, the direct chop method grew organoids faster and in greater number compared to the enzymatic digestion for the majority of ER positive tumor tissues. This was true of the tumor and preliminarily was also effective for normal breast tissue (although for normal tissues there was not as clear of an advantage over the collagenase-based method). Figure 3.5 shows representative organoid cultures grown using the direct chop methodology, including TORG90 (primary ER+ breast cancer) and TORG105 (metastatic ER+ breast cancer), as well as two cultures (not ER+ breast cancers) that were established using collagenase digestion (TORG40, a triple-negative breast cancer, and ORG9, a normal breast tissue).

Figure 3.7 shows the chronological development of human and mouse organoids grown from the direct chop method. Over time the tissue formed epithelial budding structures that led to the formation of organoids. The organoids grew in size and occasionally underwent morphology changes. For example, over the course of culture

development TORG104 ER+ breast cancer organoids went from a solid to a cystic morphology as shown in the mature culture panel in the top row of Figure 3.7.

Since this methodology was yielding robust organoid cultures, it was applied to the EOP cases that the lab received from the I-SPY 2 trial. Figure 3.8 shows eight EOP organoid cultures at various stages of development that contain heterogenous morphologies like differences in size, growth rate and structure (solid v. cystic, simple cyst v. complex cyst).

Figure 3.7. Direct Chop Methodology

Figure 3.7 displays the chronological development of direct chop tumor tissues (human and mouse) in organoid culture. The tumors initially contain adipocytes that are lost with serial passaging, and later have epithelial budding structures extending off the tissue chunks with fibroblasts growing in the background. The organoids continue to grow in size and eventually reach maturity. Top row TORG104, bottom row mTORG100 (mouse tumor tissue obtained from Shuting Li from the Goga Lab). The scale bar is equal to 100 microns.

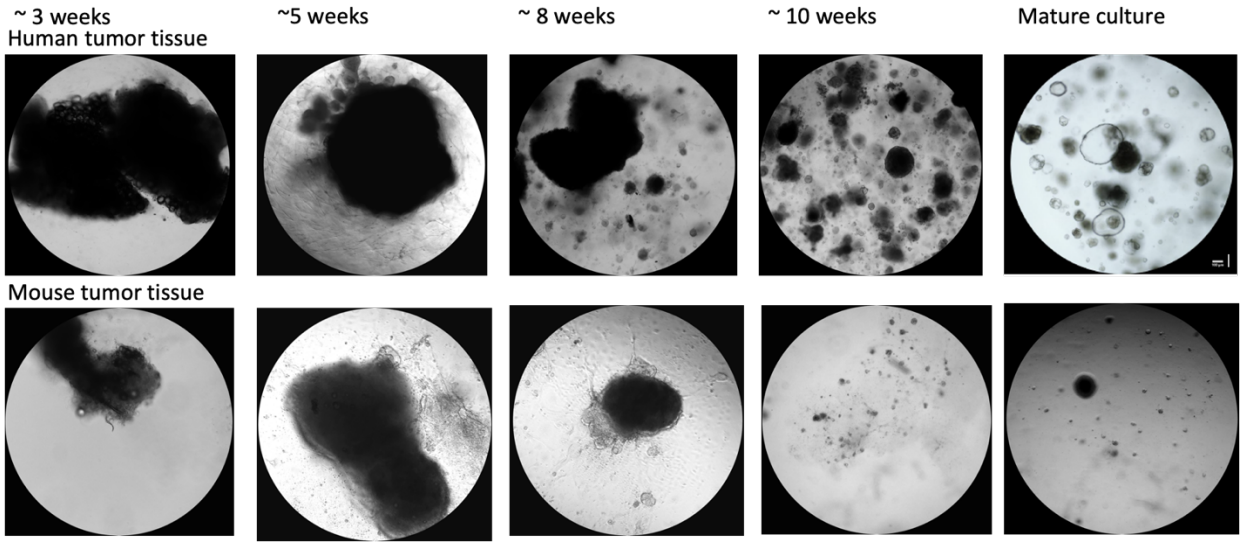
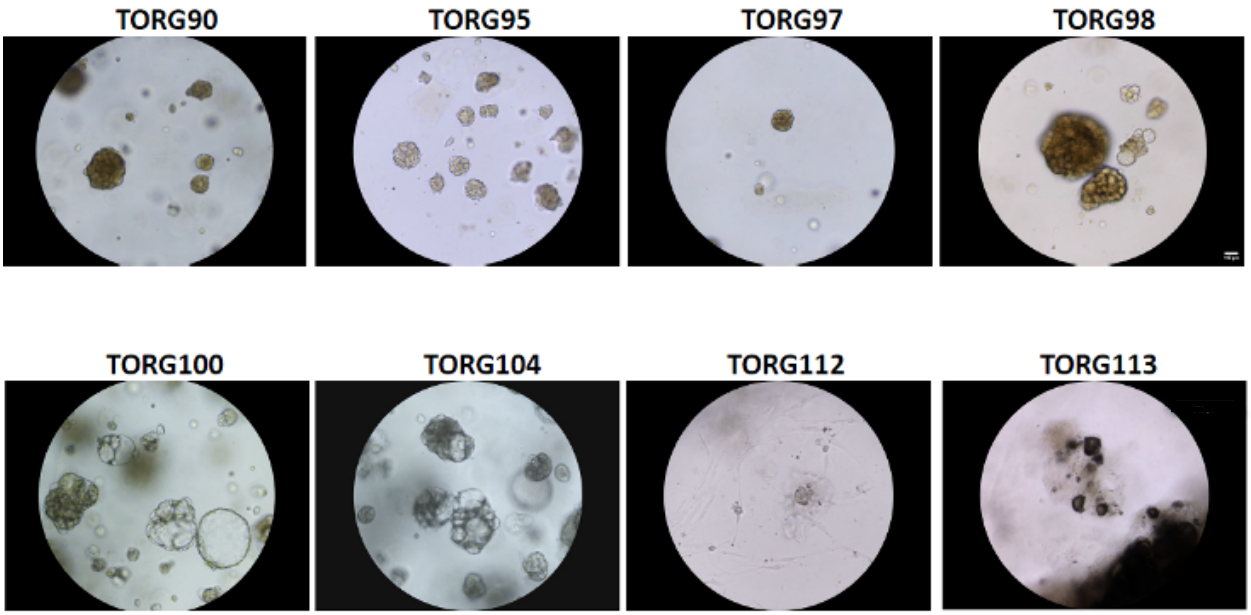


Figure 3.8. EOP organoids generated from direct chop

Bright field microscopy images of eight organoid cultures derived from residual disease tissues on the I-SPY2 EOP are shown at different stages of culture generation. The scale bar is equal to 100 microns.



Proof-of-principle preliminary findings using EOP organoids

The samples used to generate these organoid cultures were annotated with clinical features in collaboration with Drs. Jo Chien and Laura Esserman. These annotations were performed by our collaborator Kami Pullakhandam.

Interestingly, because amcenestrant is a SERD and is thought to lead to the degradation of ER, this was one of the first markers assessed in clinical samples. Additional clinical features were determined including PR (an indicator of breast cancer subtype and a target gene of ER) and Ki67 (a marker of proliferation) staining as well as functional tumor volume on MRI. These assessments were performed on tissue biopsies prior to treatment with amcenestrant, and again at the conclusion of treatment prior to surgery. There were a range of values pre- versus post-therapy for of these metrics across cases (Figure 3.9).

The first six derived organoid cultures from these cases were subjected to single-cell RNA-sequencing to determine if heterogenous cancerous cell types are present in each tumor and identify associations between intra-tumoral heterogeneity and metric of response. A total of 10,081 cells were analyzed by single-cell RNA-sequencing after completion of quality control measures including removal of potential doublets using Partek Flow. Unsupervised graph-based clustering (Leiden clustering) of these results confirmed that multiple tumor cell types were identified in this preliminary initial dataset, with most expressing Estrogen receptor (ESR1, shown in Figure 3.10A). Interestingly, each tumor organoid was comprised of multiple tumor cell types (Figure 3.10B),

suggesting that tumor organoids were preserving cell heterogeneity and could be a tractable model system for future studies to understand the role of heterogeneity in determining tumor response to neoadjuvant endocrine therapy.

Figure 3.9. Clinical Characteristics of EOP cases

Shown are levels of ER, PR, and Ki67 on the I-SPY 2 EOP trial for patients prior to neoadjuvant therapy (baseline), and at the completion of neoadjuvant endocrine therapy (surgery). Change in Functional Tumor Volume (FTV) on MRI is also shown. Clinical data was collected by Kami Pullakhandam. Each line is a separate patient.

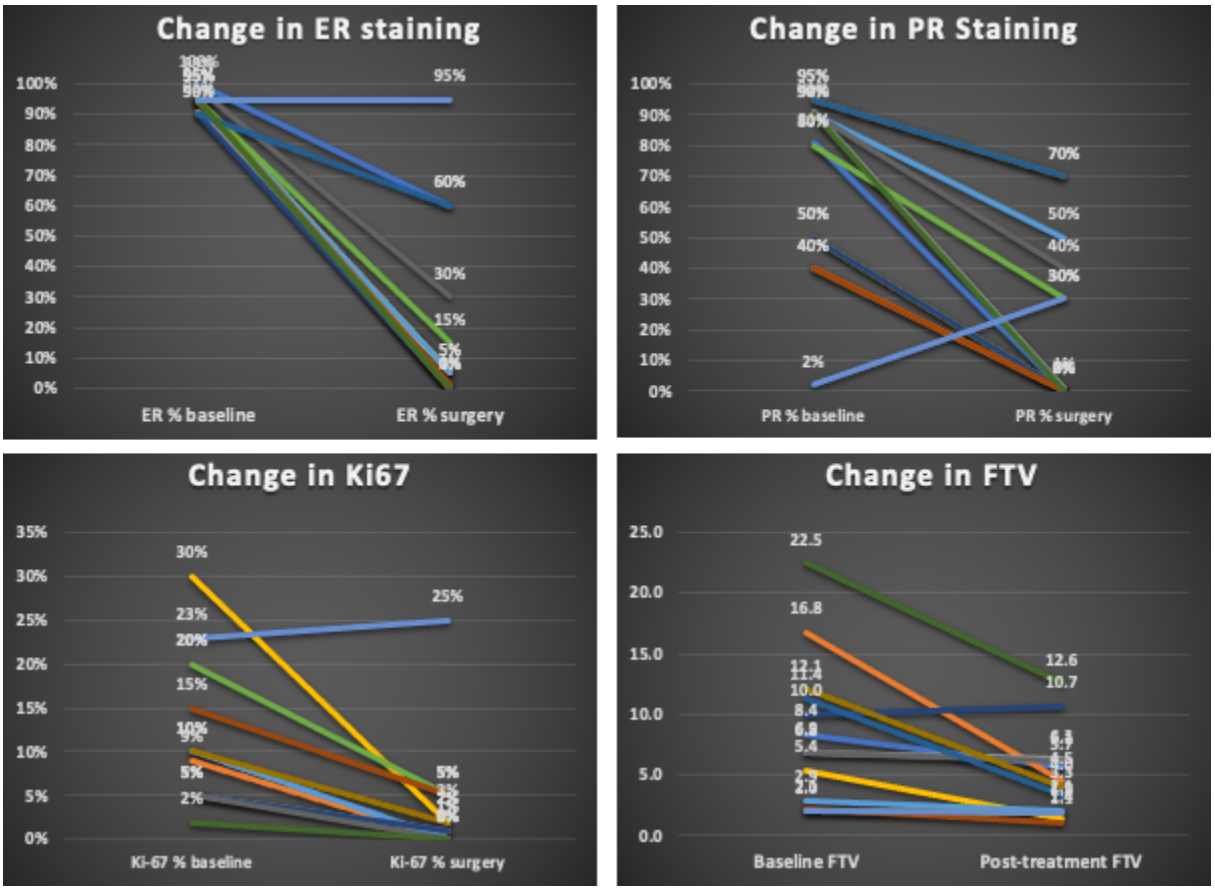
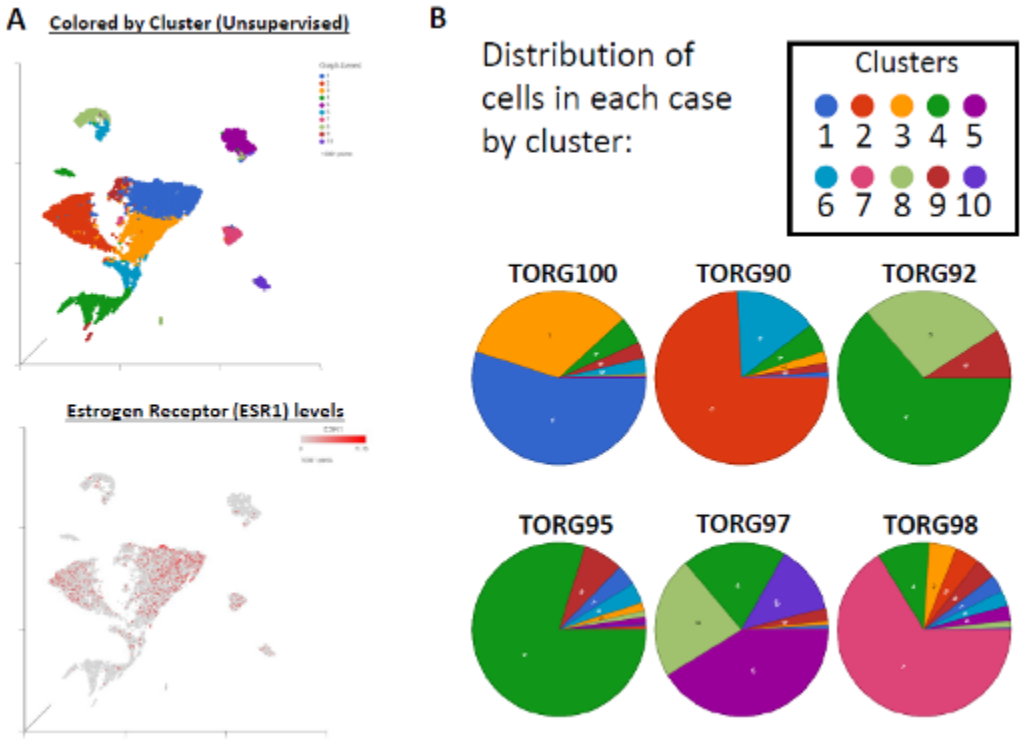


Figure 3.10. Heterogeneity of tumor cell types present

Single-cell RNA-sequencing on the first six organoid cultures from the I-SPY 2 EOP was performed by Shruti Warhadpande. Preliminary analyses performed in Partek Flow are shown below. The cluster types identified by unsupervised Leiden-based clustering are shown by UMAP (A top panel), and for each of the six clusters by distribution out of total (B). Levels of ESR1 are also displayed by UMAP for comparison (B bottom panel).



Discussion

We demonstrate herein that GrowDex is a viable hydrogel alternative to BME for breast cancer organoid culturing. By determining a workable protocol with modified lab supplies, order of operations and concentration, GrowDex can be used for several breast organoid applications due its modifiable stiffness and chemical composition and practical handling and imaging properties. GrowDex can be used for challenging cultures that show little to no growth over time in BME. This provides a critical option to salvage challenging-to-grow cultures from critical tissues. GrowDex can also be used for its improved optical qualities (lower autofluorescence) when compared to BME, because it makes for better receptor quantification measurements in immunofluorescence experiments. This was especially important when modeling organoids that were clinically resistant to amcenestrant and assessing ER and PR expression.

We also developed and performed a pilot experiment to assess a new direct chop methodology for generation of breast cancer organoids, under the assumption that collagenase-based protocols had demonstrated lower efficacy due to apparent loss of ER+ breast cells. We tested this method and demonstrated improved efficacy, generating a bank of EOP cultures from this traditionally challenging-to-grow tissue type. Preliminary studies using these organoid cultures showed that heterogeneous populations of tumor cells can be present in organoid cultures derived from a single tissue. In addition, ER expression is preserved in a subset of organoid cultures derived from tumors clinically resistant to amcenestrant, suggesting that the tumors may retain sensitivity to endocrine therapy. These studies will be expanded in future experiments

in the lab to understand the nature of these heterogeneous cell subtypes, ER signaling activity in the cell subtypes, and to expand the EOP organoid biobank.

This work highlights the importance of hydrogel and tissue digestion optimization for three-dimensional culturing of breast cancers and breast tissues. The organoid culturing methodology and established cultures from this work can be grown and analyzed for multiple future studies including investigations of cancer resistance to endocrine therapy and intra- and inter-tumoral heterogeneity using single-cell RNA sequencing.

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