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Establishment and Culture of Patient-Derived Breast Organoids

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Abstract

Breast cancer is a complex disease that has been classified into several different histological and molecular subtypes. Patient-derived breast tumor organoids developed in our laboratory consist of a mix of multiple tumor-derived cell populations, and thus represent a better approximation of tumor cell diversity and milieu than the established 2D cancer cell lines. Organoids serve as an ideal *in vitro* model, allowing for cell-extracellular matrix interactions, known to play an important role in cell-cell interactions and cancer progression. Patient-derived organoids also have advantages over mouse models as they are of human origin. Furthermore, they have been shown to recapitulate the genomic and transcriptomic—as well as metabolic—heterogeneity of patient tumors; thus, they are capable of representing tumor complexity as well as patient diversity. As a result, they are poised to provide more accurate insights into target discovery and validation and drug sensitivity assays. In this protocol, we provide a detailed demonstration of how patient-derived breast organoids are established from resected breast tumors (cancer organoids) or reductive mammoplasty-derived breast tissue (normal organoids). This is followed by a comprehensive account of 3D organoid culture, expansion, passaging, freezing, as well as thawing of patient-derived breast organoid cultures.

SUMMARY:

A detailed protocol is provided here for establishing human breast organoids from patient-derived breast tumor resections or normal breast tissue. The protocol provides comprehensive step-by-step instructions for culturing, freezing, and thawing human patient-derived breast organoids.

INTRODUCTION:

Breast cancer (BC) is the most commonly occurring malignancy in females, with 287,850 new cases estimated to be diagnosed in the United States in 2022[1]. Despite the recent advances in early detection with annual screenings, targeted therapies, and a better understanding of genetic predisposition, it prevails to be the second leading cause of cancer deaths in females in the United States, with >40,000 deaths attributed to breast cancer annually[1]. Breast cancer is currently classified into multiple subtypes

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DISCLOSURES:
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based on histopathological and molecular evaluation of the primary tumor. Better subtype stratification has improved patient outcomes with subtype-specific treatment options[2]. For instance, the identification of HER2 as a proto-oncogene[3] has led to the development of Trastuzumab, which has made this highly aggressive subtype manageable in most patients[4]. Further research into the genetics and transcriptomics of this complex disease in a patient-specific manner will aid in developing and predicting better patient-specific personalized treatment regimens[2, 5]. Patient-derived organoids (PDOs) are a promising new model to gain insights into cancer at the molecular level, identifying novel targets or biomarkers and designing new treatment strategies[6–8].

PDOs are multicellular, three-dimensional (3D) structures derived from freshly resected primary tissue samples[8, 9]. They are grown three-dimensionally by being embedded in a hydrogel matrix, typically composed of a combination of extracellular matrix (ECM) proteins, and hence can be used to study tumor cell-ECM interactions. PDOs represent patient diversity and recapitulate cellular heterogeneity and genetic features of the tumor[10–12]. Being *in vitro* models, they allow for genetic manipulation and high-throughput drug screens[13–15]. Further, PDOs can be plausibly utilized to evaluate patient drug sensitivity and treatment strategies in parallel to the clinic and help predict patient outcomes[16–18]. Besides chemotherapy, certain organoid models have also been used to examine individual patient responses to chemoradiation[19, 20]. Given the promising applicability of PDOs for research and clinical use, the National Cancer Institute has initiated an international consortium, The Human Cancer Models Initiative (HCMI)[21], to generate and provide these tumor-derived novel cancer models. Many of the organoid models of various cancer types developed through the HCMI are available *via* the American Type Culture Collection (ATCC)[22].

Normal breast organoids have been shown to be comprised of different epithelial cell populations present in the mammary gland[11, 23] and thus serve as great models to study basic biological processes, to analyze driver mutations causing tumorigenesis, and for cancer cell-of-origin lineage studies[6, 15]. Breast tumor organoid models have been used to identify novel targets that are encouraging prospects for developing new therapies, particularly for resistant tumors[24–26]. Using patient-derived xenograft (PDX) and matched PDX-derived organoid (PDxO) models of treatment-resistant breast tumors, Guillen et al. showed that organoids are powerful models for precision medicine, which can be leveraged to evaluate drug responses and direct therapy decisions parallelly[28]. Furthermore, the development of new co-culture methods for culturing PDOs with various immune cells[27–29], fibroblasts[30, 31] and microbes[32, 33] presents an opportunity to study the impact of the tumor microenvironment on cancer progression. While many such co-culture methods are actively being established for PDOs derived from pancreatic or colorectal tumors, similar established co-culture methods for breast PDOs have only been reported for natural killer cells[34] and fibroblasts[35].

The first biobank of >100 patient-derived organoids representing different breast cancer subtypes was developed by the Hans Clevers group[36, 37]. As part of this effort, the Clevers group also developed the first complex culture medium for breast organoid growth, which is currently widely used[36]. A follow-up study provided a comprehensive account

of the establishment and culture of breast PDOs and patient-derived organoid xenografts (PDOXs)[38]. The Welm lab developed a large collection of BC PDX models and PDxOs that are cultured in a comparatively simpler growth medium containing fetal bovine serum (FBS) and fewer growth factors[39, 40]. We have independently developed and characterized a large array of naïve patient-derived breast cancer organoid models[11], and participated in developing BC PDO models as part of the HCMI initiative[21]. Here, we aim to provide a practical guide detailing the methodology employed by us in generating patient-derived breast organoid model systems.

PROTOCOL:

Tumor resections from breast cancer patients, along with the distal and adjacent normal tissue, were obtained from Northwell Health in accordance with Institutional Review Board protocols IRB-03–012 and IRB 20–0150, and with written informed consent from the patients.

NOTE: All procedures mentioned below were performed in a mammalian tissue culture BSL2 room designated for patient samples upon approval of the biosafety committee. All procedures should be performed following safety protocols maintaining aseptic conditions in biosafety cabinets. Each centrifugation step is performed at room temperature (RT) unless stated otherwise. Tissue/organoids and basement membrane matrix stocks are always placed on ice unless stated otherwise. New plates are incubated overnight for pre-warming. Plating domes on pre-warmed plates ensures the best results to obtain rounded domes that don't flatten out while plating or lifting off later from the plate surface.

1. Medium preparation and recipes

1.1. Prepare R-spondin1 conditioned media as described below (alternatively can be bought commercially).

1.1.1. Prepare two separate growing media composed of Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 5% penicillin-streptomycin, and with or without 300 μg/mL zeocin supplementation.

1.1.2. Thaw a vial of HEK293T-HA-Rspondin1-Fc cells (obtained from Calvin Kuo's lab at Stanford University and also commercially available) in a 75 cm^2 tissue culture flask with 15 mL of medium.

1.1.3. Split the cells into 2 × 175 cm^2 culture flasks, each with 35 mL of medium containing zeocin.

1.1.4. Passage the cells again to obtain as many flasks as needed for generating the desired batch of R-spondin1 conditioned medium. Use growth medium without zeocin.

1.1.5. When the flasks containing medium without zeocin are confluent, remove and replace the growing medium with Ad-DF+++ (step 1.2;

Table 1). Place the cells in a 37 °C incubator with 5% CO_2 for 1 week.

1.1.6. Spin down the medium at 400 × *g* for 5 min to remove any unattached cells. Filter the medium through a 0.22 μm filter. Make 25 mL aliquots and store them at −20 °C (can be stored for up to 6 months).

1.1.7. Using HEK293T cells, perform a dual luciferase TOPFLASH[41, 42] assay using a commercial DNA transfection reagent at a 4.8:1 reagent to DNA ratio with the diluent DMEM (high glucose, pyruvate). Add 100 μL of R-spondin1 conditioned media (or basal media for the negative control) to 100 μL of transfected cells. Then, perform the dual luciferase assay as per the manufacturer's protocol to verify Wnt activity of the R-spondin1 conditioned medium.

NOTE: The assay uses a TOP plasmid with Firefly luciferase activity read-out, and the FOP plasmid is used as a negative control.

1.2. Prepare basal medium (i.e., Ad-DF+++ media, as previously published by Sachs et al.[36]

1.3. Prepare complete medium for patient-derived breast organoids as previously published by Sachs et al.[36].

1.4. Prepare 2 mg/mL collagenase IV solution by weighing the required amount of collagenase IV powder and solubilizing it in Ad-DF+++ basal medium. Filter through a 0.22 μm filter before use.

1.5. Prepare 0.5% bovine serum albumin (BSA) solution from 7.5% stock solution using 1x Dulbecco's phosphate buffered saline (DPBS) as a diluent. Filter through a 0.22 μm filter before use.

2. Establishing breast tumor/normal organoids from resected tissue (Figure 1)

2.1 Transport a surgically resected breast tumor/normal tissue specimen to the lab in a 50 mL conical tube containing Ad-DF+++. Store the tube on ice until the start of isolation.

2.2. Thaw a bottle of basement membrane matrix on ice or overnight at 4 °C.

2.3. Transfer the resected tissue to a 10 cm sterile Petri dish. Examine the tissue macroscopically and make a note if it appears morphologically fatty, vascularized, or necrotic. Additionally, record the size and shape of the tissue. Ideally, take a picture of the tissue with a ruler in view (Figure 2).

2.4. Mince the tissue into very small pieces (~1 mm^3) with a sterile no. 10 scalpel, and transfer it into a 50 mL conical tube.

2.5. Add 10 mL of 2 mg/mL collagenase IV solution and seal the tube with a transparent film. Place the tube on an orbital shaker at 37 °C at 140 rpm for 30–90 min in an angled position (~30° angle).

2.6. During the incubation, place an aliquot of complete medium to pre-warm in a 37 °C bead/water bath.

2.7. Every 15 min, resuspend the tissue by mixing up and down vigorously using a 5 mL sterile serological pipette. Pre-coat the pipette with 0.5% BSA solution to prevent tissue loss caused by the sample sticking to the pipette. Monitor dissociation over time by observing the conical tube under the microscope at a 5x or higher magnification.

2.8. Once the tissue is dissociated, centrifuge at 400 × *g* for 5 min. Aspirate the supernatant, add 10 mL of Ad-DF+++, and centrifuge at 400 × *g* for 5 min.

2.9. Discard the supernatant carefully. Tissue pellets can occasionally be loose, so be careful when aspirating. Repeat the step once more.

2.10. If the tissue pellet is partially red, add 2 mL of red blood cell lysis buffer and incubate for 5 min at room temperature. Do not incubate for longer, as this will significantly reduce cell viability.

2.11. Add 10 mL of Ad-DF+++ to the tube, centrifuge at 400 × *g* for 5 min, and discard the supernatant. Resuspend the pellet in 50–300 μL of undiluted, cold basement membrane matrix and mix by pipetting carefully to avoid forming bubbles.

NOTE: The volume of the basement membrane matrix added depends on the pellet size. If unsure, plate a small dome of ~10 μL from the resuspended pellet and observe the confluency under the microscope. Adjust by adding more basement membrane matrix if needed. Refer to Figure 3 (day 1 image) for a reference seeding density.

2.12. Plate 300 μL of basement membrane matrix dome containing organoids in each well of a pre-warmed, labeled, 6-well tissue culture plate. Refer to Table 3 for recommended basement membrane matrix and medium volumes if using different plates.

2.13. Leave the plate undisturbed in the hood for 5 min and then carefully place in the incubator at 37 °C for 20–30 min for the basement membrane matrix dome to fully solidify.

2.14. Add 3 mL of pre-warmed complete medium dropwise to each well of a 6-well plate and place in the incubator at 37 °C and 5% CO_2. Take images of the organoids using a 5x objective on an inverted brightfield microscope.

2.15. Replenish the medium every 4–8 days based on the growth of organoids. Carefully aspirate the old medium without disturbing the dome, and add fresh, pre-warmed medium dropwise.

NOTE: The protocol is the same for establishing breast cancer organoids derived from the resected tumor and for normal organoids derived from resected healthy breast tissue (either from adjacent and normal breast tissue of the same patient or healthy breast tissue obtained from a reductive mammoplasty surgery).

3. Passaging and expanding patient-derived breast organoids in culture

3.1. Thaw a bottle of basement membrane matrix on ice or overnight at 4 °C.

3.2. Lift the basement membrane matrix dome into the medium in the well using a cell scraper or a 1 mL pipette tip.

3.3. Pre-coat the pipette tip with 0.5% BSA solution and then transfer the floating dome of the organoids with medium to a 15 mL or 50 mL conical tube, depending on the number of wells being harvested. For more than two wells containing 300 μL of basement membrane matrix domes, use a 50 mL conical tube. Add 1x DPBS to increase the volume to at least 5 mL.

3.4. Spin the tubes at 400 × *g* for 5 min. The basement membrane matrix with organoids forms a layer at the bottom. Carefully aspirate to remove the supernatant.

3.5. Add 5–20 mL of 1x DPBS, depending on the number of wells pooled in a tube. Pre-coat a sterile disposable pipette with 0.5% BSA and mix the organoid-basement membrane matrix pellet in DPBS uniformly by pipetting up and down.

3.6. Spin the tubes at 400 × *g* for 5 min. Carefully aspirate and discard the supernatant.

3.7. Add cell dissociation reagent at three times the volume of the basement membrane matrix to the tube. Using a 0.5% BSA-coated pipette tip, resuspend the organoids in the cell dissociation reagent.

3.8. Place the tube on an orbital shaker at 37 °C at 140 rpm for 8–15 min in an angled position. Monitor the tube by observing it under the microscope every 5 min to ensure the organoids are broken down into smaller clusters.

3.9. Add basal medium (i.e., Ad-DF+++) at a volume equal to or greater than the cell dissociation reagent, and pipette to mix the organoids. Spin at 400 × *g* for 5 min at RT to obtain an organoid pellet.

3.10. If the pellet contains organoids still embedded within the undissolved basement membrane matrix, repeat steps 3.7–3.9 for an additional 5–8 min of trypsinization.

3.11. Once a white organoid pellet with no undissolved basement membrane matrix is obtained, discard the supernatant, and add 1 mL of Ad-DF+++ to resuspend the pellet by pipetting. Then add more Ad-DF+++ up to 10 mL.

3.12. Spin at 400 × *g* for 5 min at RT for the wash step. Aspirate and discard the supernatant.

3.13. Add the required amount of basement membrane matrix to the digested organoids based on the appropriate split ratio (this will vary widely for each organoid line based on growth rate). Refer to Figure 3 (day 1 image) for an example of seeding density. Mix by gently pipetting up and down to avoid creating bubbles. Immediately place on ice.

Open and label a pre-warmed 6-well plate. It is recommended to plate a 10–20 μL dome in the corner of a well to observe the confluency under the microscope. If the organoids are confluent, more basement membrane matrix can be added to increase the split ratio.

3.15. Plate 300 μL domes of organoids resuspended in basement membrane matrix. Make sure to keep the tube on ice while plating multiple wells so that the basement membrane matrix remains in solution.

3.16. Leave the plate undisturbed in the hood for 5 min and then carefully place in a 37 °C incubator with 5% CO_2 without disturbing the domes.

3.17. After 20–30 min, the domes solidify. Add 3 mL of pre-warmed complete medium to each well and place the plate back in the incubator. Add fresh complete medium every 5–7 days.

NOTE: The protocol for the culture of breast tumors and normal organoids is the same. However, in our experience, normal organoids tend to grow well up to passage 6–8 before slowing down. It is advisable to make as many early passage stocks as possible for future research.

4. Freezing patient-derived breast organoids

4.1. Passage confluent wells of organoids to remove any basement membrane matrix, as stated in steps 3.1–3.12.

4.2. Resuspend the pellet of organoids in cell freezing medium with 1 mL of medium per 200–300 μL of basement membrane matrix volume before passaging. Perform a cell count before freezing for reference. Aliquot a small volume (at least 100 μL) of cells to undergo further trypsinization, if needed, to get single cells, and then count them using a cell counter machine.

4.3. Transfer 1 mL of cells into 2 mL cryovials labeled with organoid line number, date, and passage number. Be sure to label the tubes with a permanent marker or use cryolabels.

4.4. Move the vials to a cell freezing container and transfer the container to a −80 °C freezer. After incubation overnight, the vials are ready to be moved to a liquid nitrogen storage freezer for long-term storage.

5. Thawing patient-derived breast organoids

5.1. Thaw a bottle of basement membrane matrix on ice or overnight at 4 °C. Pre-warm Ad-DF+++ medium at 37 °C. Aliquot 9 mL of pre-warmed medium into 15 mL conical tubes for each frozen vial.

5.2. Rapidly thaw the frozen vial of organoids by placing it in a 37 °C bead bath. Spray 70% ethanol and transfer the tube to the biosafety cabinet.

5.3. Rinse the pipette tip with 0.5% BSA solution to avoid organoid loss. Mix the thawed organoids gently by pipetting up and down to mix any settled organoids in the tube.

5.4. Gently transfer 1 mL of frozen organoids to 9 mL of pre-warmed Ad-DF+++ in a dropwise manner. Spin the cells at 400 × *g* for 5 min and then carefully discard the supernatant.

5.5. Wash the pellet by adding 10 mL of fresh pre-warmed Ad-DF+++ to the organoid pellet, and mix gently with a pipette pre-coated with 0.5% BSA to resuspend the pellet. Spin the cells at 400 × *g* for 5 min and carefully discard the supernatant.

5.6. Place the organoid pellet on ice and remove any remnant Ad-DF+++ carefully using a smaller volume pipette tip. Resuspend the pellet in 300 µL of basement membrane matrix and plate in a pre-warmed 6-well plate. Place the plate in a 37 °C incubator with 5% CO_2.

NOTE: It is recommended to plate a small 10 µL dome from the resuspended pellet in a corner of the well to discern the confluency under the microscope. If the organoids look confluent, dilute by adding 300 µL of basement membrane matrix to plate into two wells of a 6-well plate.

5.7. After a 20–30 min incubation, add 3 mL of pre-warmed complete medium to the solidified dome.

REPRESENTATIVE RESULTS:

We have established a biobank of patient-derived breast tumor organoids comprising various subtypes[11]. Additionally, we have established multiple normal breast organoid lines derived from reductive mammoplasty tissue samples or adjacent/distal normal breast from BC patients using the approach outlined in Figure 1.

The various patient-derived breast tumor organoid lines differ in their morphology (Figure 2) and growth rate (Figure 3). Normal breast organoids, and the few early ductal carcinoma *in situ* (DCIS)-derived organoids we have established, resemble the normal breast structure with a central lumen surrounded by ductal cells (Figure 2A,B). Organoids derived from an invasive lobular carcinoma (Figure 2C) tend to form loosely attached grape bunch-like structures, as reported previously by other labs[36, 43]. Meanwhile, organoids derived from invasive ductal carcinomas (hormone receptor-positive and triple negative breast cancer) tend to form dense, large, and round organoids (Figure 2D,E). Organoids were fixed with 4% paraformaldehyde followed by being embedded in 2% agarose molds. They were then were paraffin embedded, and 10 µm sections were stained with hematoxylin and eosin (as described in Bhatia et al.[11]) to observe the morphology.

To demonstrate the differences in the rate of growth of different patient-derived breast tumor organoid lines, 1,000 viable single cells were plated in 10% basement membrane matrix suspension solution per 96-well plate, with six replicates each to determine cell viability at different time points to monitor growth over a period of 12 days (Figure 3B). The organoid formation was measured using a luminescent cell viability assay on days 3, 6, 9, and 12, with a baseline reading on day 1 after plating. Figure 3A shows bright-field images of the

same organoids expanded in 6-well plates over time. Some patient-derived organoid lines have a doubling time of 2 days, while some take 5 days (Figure 3B).

DISCUSSION:

Our lab has successfully employed the above protocols to establish organoids from naïve tumor resections or scrapings. We have also utilized this protocol to develop normal organoids from breast tissue obtained *via* reductive mammoplasties or from cancer patients' adjacent or distal normal breast tissue. About 30%–40% of the resected primary tumors resulted in successful long-term (>passage 8) tumor organoid cultures. The tumor organoid lines that tapered off after a few passages either had an outgrowth of normal organoids or stromal cells, or consisted majorly of non-proliferative tumor cells[11]. Further evaluation and modification of the medium components which currently favor epithelial cell outgrowth, or other means of pre-selecting the tumor cell populations, should improve the success rate for the establishment and long-term culture of PDOs. Additionally, it is highly recommended to freeze down several vials of successfully expanded organoids and perform thawing tests on frozen vials for each established organoid line to authenticate their long-term success in culture.

Some successful organoid lines that slow down at times in culture or struggle to recover from a thaw often benefit from increasing the seeding density. Higher confluency is observed to aid in stimulating faster growth for most lines, probably as a result of cell-cell interactions or secreted factors. Additionally, filtering the organoids (after trypsinization) through a 70–100 μm filter can help to remove debris accumulated from dead organoids that sometimes appear after thawing some organoid lines and tend to hinder their growth. However, it must be noted that some healthy organoid material is also lost in the process of filtering. Certain organoid lines expressing estrogen receptor (ER+) can be slow growers compared to TNBC, and could potentially benefit from the addition of estradiol to the medium. We recommend that the established patient-derived breast tumor organoid lines be characterized genomically and transcriptomically. This can be done by utilizing several techniques, such as immunohistochemistry evaluation for the pathological and genetic features of patient tumors (expression of estrogen receptor, progesterone receptor, human epidermal growth factor 2, Cadherin, Ki-67, etc.), RNA-sequencing, single-cell RNA-sequencing, DNA sequencing for cancer driver genes, copy number variations, etc. For mechanistic and drug response studies, one must consider the specific inhibitors that are components of the medium and any prior treatments received by the patient before surgery.

Tumor organoid lines derived from different patients vary in genomic mutations, transcriptomics, morphology, growth rate, capacity to form large organoids, presence of heterogeneous cell populations, etc. These differences pose challenges in establishing and culturing them, however, they also make organoids robust models representing both patient diversity and tumor complexity. Breast tumor-derived organoids have been shown to recapitulate the original patient tumors at the genomic, transcriptomic[11, 36] and metabolomic levels[44]. All these factors make them powerful tools for drug screening and precision oncology[39]. Patient-derived organoids are an exciting new model system that will serve as three-dimensional *in vitro* models for studying cancer biology, as well

as examining therapeutically important questions for advancing the field of personalized medicine. Normal and tumor breast organoids can be genetically modified *via* CRISPR engineering[15, 38], prompting their favorable use for mechanistic genetic studies for tumorigenesis and cancer progression. Patient-derived breast tumor organoids have also been used to develop xenograft models[11, 38, 39] that can potentially mimic tumor growth in patients. Furthermore, patient-derived breast tumor organoid co-culture systems remain an under-explored area of research that can provide powerful insights into the importance of crosstalk between breast cancer cells and other cells, such as stromal cancer-associated fibroblasts, immune cells, adipocytes, etc. in tumor progression.

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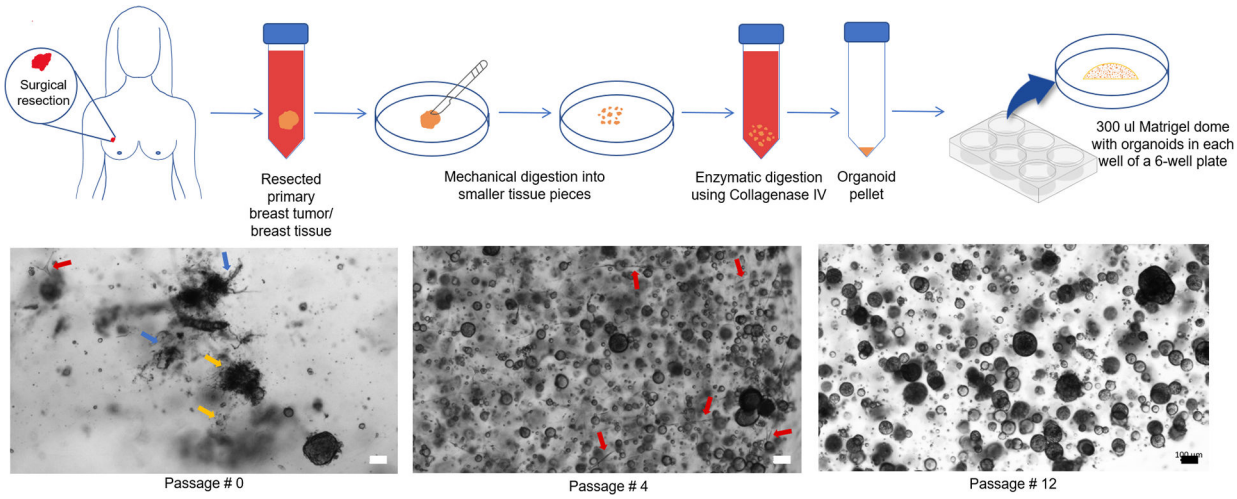

Figure 1: Establishing patient-derived breast organoids.

(**A**) Schematic representation of the major steps in establishing patient-derived breast tumors or normal organoids. (**B**) Representative images showing growth of DS117T patient-derived breast tumor organoids from establishment (passage 0) to long-term culture (passage 12). Blue arrows = fibrous material, yellow arrows = debris/dead cells, and red arrows = supporting cell types from the microenvironment (mainly fibroblasts). Scale bar = 100 µm.

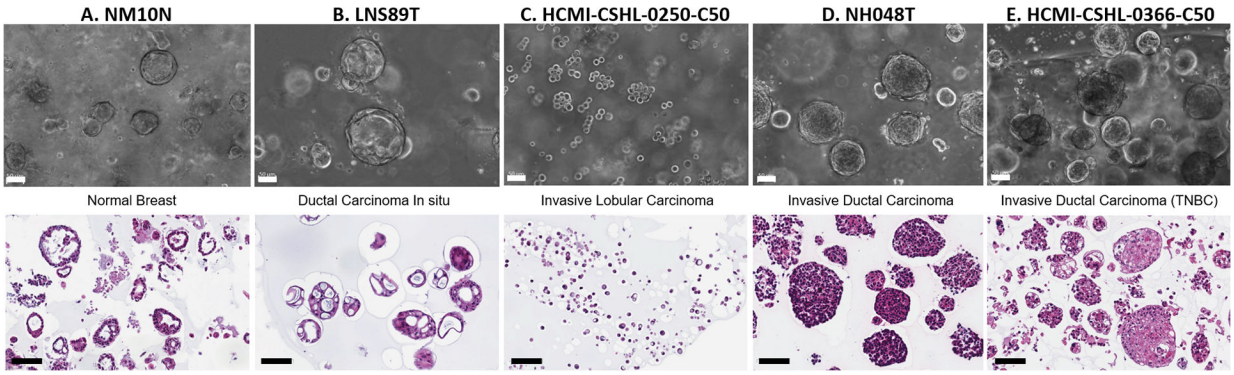

Figure 2: Patient-derived breast organoids representing different subtypes.

Top panel: morphological differences between organoid lines derived from patient breast tumors (**B–E**) of different histopathological and molecular subtypes. (**A**) The figure represents organoids derived from normal human breast tissue obtained post-reductive mammoplasty surgery. Scale bar = 50 μm. Bottom panel: H&E stained images of the same organoid lines. Scale bar = 100 μm. Abbreviation: TNBC = triple negative breast cancer.

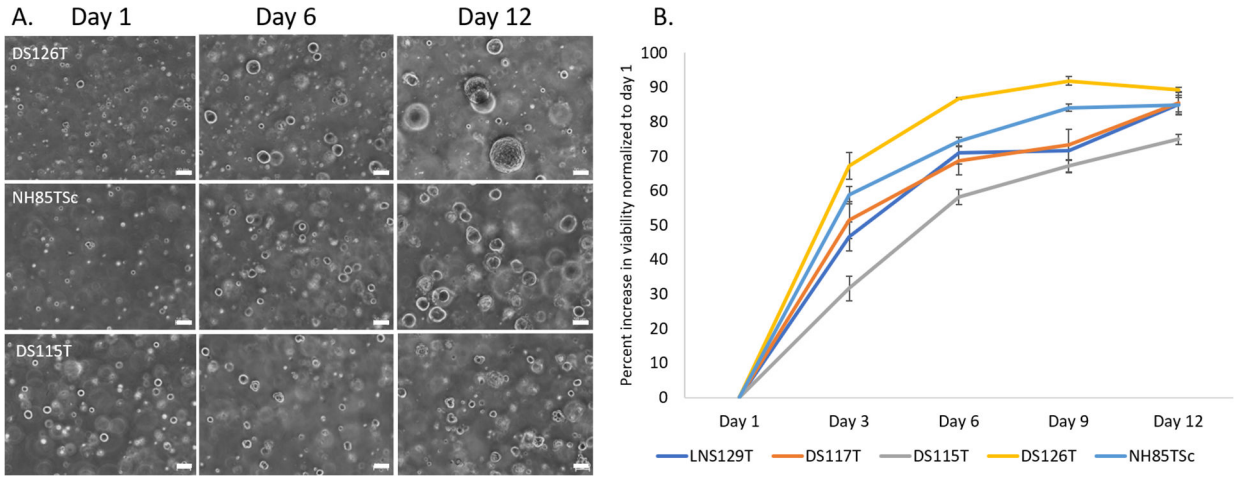

Figure 3: Growth in culture of different patient-derived breast tumor organoids.

Representative bright-field images and cell viability curves (**A** and **B**), as measured *via* a luminescent cell viability assay showing the growth in culture of different patient-derived breast tumor organoid lines over 12 days. Scale bar = 50 μm. Error bars represent SEM for n = 6.

Table 1:

Basal Ad-DF+++ media composition

Reagent	Stock Concentration	Final Concentration
Advanced DMEM/F12	1 x	1 x
GlutaMax	100 x	1 x
HEPES	1 M	10 mM
Penicillin-Streptomycin	10,000 U/ml; 10,000 μg/ml	100 U/ml; 100 μg/ml

Table 2:

Complete culture media composition for patient-derived breast organoids:

Reagent	Stock Concentration	Final Concentration
Ad-DF+++ media	1 x	1 x
R-spondin in-house	100%	10%
B-27 supplement	50 x	1 x
Nicotinamide	1 M	5 mM
NAC	500 mM	1.25 mM
Primocin	50 mg/ml	50 μg/ml
Noggin	100 μg/ml	100 ng/ml
Human EGF	5 μg/ml	5 ng/ml
Human Heregulin β1/Neuregulin1	75 μg/ml	37.5 ng/ml
Y-27632 Dihydrochloride (RhoKi)	100 mM	5 μM
A83–01	5 mM	500 nM
Human FGF-7	100 μg/ml	5 ng/ml
Human FGF-10	1 mg/ml	20 ng/ml
p38i	30 mM	498 nM

Table 3:

Amount of basement membrane matrix and growth medium recommended per well based on plate size.

Number of wells per plate	Matrigel per dome (μl)	Media per well (μl)
6	300	3000
12	100	1000
24	50	500
48	25	250
96	10 (suspension instead of dome)	100