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Mammary Epithelial and Endothelial Cell Spheroids as a Potential Functional In vitro Model for Breast Cancer Research

Azzarito, Giovanna ; Szutkowska, Marta Ewa ; Saltari, Annalisa ; Jackson, Edwin K ; Leeners, Brigitte
; Rosselli, Marinella ; Dubey, Raghvendra K

Abstract: Breast cancer is the leading cause of mortality in women. The growth of breast cancer cells and their subsequent metastasis is a key factor for its progression. Although the mechanisms involved in promoting breast cancer growth have been intensively studied using monocultures of breast cancer cells such as MCF-7 cells, the contribution of other cell types, such as vascular and lymphatic endothelial cells that are intimately involved in tumor growth, has not been investigated in depth. Cell-cell interaction plays a key role in tumor growth and progression. Neoangiogenesis, or the development of vessels, is essential for tumor growth, whereas the lymphatic system serves as a portal for cancer cell migration and subsequent metastasis. Recent studies provide evidence that vascular and lymphatic endothelial cells can significantly influence cancer cell growth. These observations imply a need for developing in vitro models that would more realistically reflect breast cancer growth processes in vivo. Moreover, restrictions in animal research require the development of ex vivo models to elucidate better the mechanisms involved. This article describes the development of breast cancer spheroids composed of both breast cancer cells (estrogen receptor-positive MCF-7 cells) and vascular and/or lymphatic endothelial cells. The protocol describes a detailed step-by-step approach in creating dual-cell spheroids using two different approaches, hanging drop (gold standard and cheap) and 96-well U-bottom plates (expensive). In-depth instructions are provided for how to delicately pick up the formed spheroids to monitor growth by microscopic sizing and assessing viability using dead and live cell staining. Moreover, procedures to fix the spheroids for sectioning and staining with growth-specific antibodies to differentiate growth patterns in spheroids are delineated. Additionally, details for preparing spheroids with transfected cells and methods to extract RNA for molecular analysis are provided. In conclusion, this article provides in-depth instructions for preparing multi-cell spheroids for breast cancer research.

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TITLE:

Mammary Epithelial and Endothelial Cell Spheroids as a Potential Functional *In Vitro* Model for Breast Cancer Research

AUTHORS AND AFFILIATIONS:

Giovanna Azzarito¹, Marta Ewa Szutkowska¹, Annalisa Saltari², Edwin K. Jackson³, Brigitte Leeners¹, Marinella Rosselli¹, Raghvendra K. Dubey^{1,3}

¹Department of Reproductive Endocrinology, Wagistrasse 14, University of Zurich and University Hospital Zurich, 8952 Schlieren, Switzerland

²Department of Dermatology, Wagistrasse 18, University of Zurich and University Hospital Zurich, 8952 Schlieren, Switzerland

³Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA, USA

E-mail address of co-authors:

Giovanna Azzarito (giovanna.azzarito@usz.ch)

Marta Ewa Szutkowska (marta.chou@wanadoo.fr)

Annalisa Saltari (annalisa.saltari@usz.ch)

Edwin K. Jackson (edj@pitt.edu)

Brigitte Leeners (brigitte.leeners@usz.ch)

Marinella Rosselli (marinella.rosselli@usz.ch)

Raghvendra K Dubey (raghvendra.dubey@usz.ch)

Corresponding author:

Raghvendra K. Dubey (raghvendra.dubey@usz.ch)

KEYWORDS:

breast cancer, tumor, spheroids, neovascularization, lymphatic vessels, tumor growth, endothelial cells, epithelial cells, cell proliferation.

SUMMARY:

Crosstalk between mammary epithelial cells and endothelial cells importantly contributes to breast cancer progression, tumor growth, and metastasis. In this study, spheroids have been made from breast cancer cells together with vascular and/or lymphatic endothelial cells and demonstrate their applicability as an *in vitro* system for breast cancer research.

ABSTRACT:

Breast cancer is the leading cause of mortality in women. The growth of breast cancer cells and their subsequent metastasis is a key factor for its progression. Although the mechanisms involved in promoting breast cancer growth have been intensively studied using monocultures of breast cancer cells such as MCF-7 cells, the contribution of other cell types, such as vascular and lymphatic endothelial cells that are intimately involved in tumor growth, has not been investigated in depth. Cell-cell interaction plays a key role in tumor growth and progression.

Neoangiogenesis, or the development of vessels, is essential for tumor growth, whereas the lymphatic system serves as a portal for cancer cell migration and subsequent metastasis. Recent studies provide evidence that vascular and lymphatic endothelial cells can significantly influence cancer cell growth. These observations imply a need for developing *in vitro* models that would more realistically reflect breast cancer growth processes *in vivo*. Moreover, restrictions in animal research require the development of *ex vivo* models to elucidate better the mechanisms involved.

This article describes the development of breast cancer spheroids composed of both breast cancer cells (estrogen receptor-positive MCF-7 cells) and vascular and/or lymphatic endothelial cells. The protocol describes a detailed step-by-step approach in creating dual-cell spheroids using two different approaches, hanging drop (gold standard and cheap) and 96-well U-bottom plates (expensive). In-depth instructions are provided for how to delicately pick up the formed spheroids to monitor growth by microscopic sizing and assessing viability using dead and live cell staining. Moreover, procedures to fix the spheroids for sectioning and staining with growth-specific antibodies to differentiate growth patterns in spheroids are delineated. Additionally, details for preparing spheroids with transfected cells and methods to extract RNA for molecular analysis are provided. In conclusion, this article provides in-depth instructions for preparing multi-cell spheroids for breast cancer research.

INTRODUCTION:

The use of animals for experiments has limitations. Animal studies cannot accurately mimic disease progression in humans, and animals and humans do not have identical responses to pathogens. Additionally, restrictions in animal experimentation due to concerns for animal suffering and ethical problems^{1,2} increasingly constrain research programs. *In vitro* systems have been widely developed to circumvent the use of animals; moreover, the use of human cells has made *in vitro* models more relevant for the pathophysiological and therapeutic investigation. Conventional monolayer (2D) cell cultures are widely used because they mimic human tissues to some degree. However, 2D monocultures fail to mimic human organs, and 2D monocultures are unable to simulate the complex microenvironment of the original organs and mimic the *in vivo* situation³⁻⁶. Additionally, in monolayer cell cultures, drug treatments could easily destroy/damage all of the cells. Importantly, some of these limitations can be overcome by switching to multilayer three-dimensional (3D) cell cultures^{7,8}. In fact, 3D culture models have been shown to better reflect the cellular structure, layout, and function of cells in primary tissues. These 3D cultures can be formed using a variety of cell lines, similar to a functional organ. Indeed, there are two models of 3D cultures. One model produces spheroids of aggregated cells that form clusters and reorganize them into spheres (scaffold-free models). The second yields organoids, which have a more complex structure and consist of combinations of multiple organ-specific cells, which are considered as a miniature version of organs^{9,10}. Due to this, 3D culture systems represent an innovative technology with many biological and clinical applications. Thus, spheroids and organoids have numerous applications for disease modeling and studies related to regenerative medicine, drug screening, and toxicological studies^{6,11-15}. Carcinogenic spheroids, derived from 3D technology, recreate the morphology and phenotype of relevant cell types, mimic the *in vivo* tumor microenvironment, and model cell communications and signaling

pathways that are operational during tumor development^{16–18}. In addition, to improve cancer biology understanding, tumor spheroids/organoids can also be used to identify a potential patient-specific anti-cancer therapy (personalized) and assess its efficacy, toxicity, and long-term effects^{19–22}. Spheroids have opened prominent opportunities to investigate pathophysiology, disease modeling and drug screening because of their ability to preserve cellular and three-dimensional tissue architecture, the ability to mimic the *in vivo* situation, and the cell-cell interactions. However, one must also be aware of the limitations of this system, such as the lack of vascular/systemic component, functional immune or nervous system, and the system represents a reductionist approach as compared to animal models. Indeed, in contrast to animal models, 3D structures provide only an approximation of the biology within a human body. Understanding the limitations of the 3D method may help researchers to design more refined and valid processes for producing spheroids that better represent an organ at a larger scale^{23–25}.

Cancer is the leading cause of death worldwide, and breast cancer is the most common cancer in women^{26–28}. To mimic the complex microenvironment of breast cancer, breast cancer spheroids should be cultured using cells that play a prominent role in breast tumors, i.e., epithelial cells, endothelial cells, fibroblasts, and/or immune cells. Moreover, for a spheroid representing breast cancer, the expression of female hormone receptors (estrogen/progesterone receptors), ability to conserve the patient tumor histological status, and ability to mimic response to therapy should also be considered. Studies have shown that 3D co-culture systems have a cellular organization similar to that of the primary tissue *in vivo*, have the capability to react in real-time to stimuli, and have functional androgen receptors^{29–32}. Hence, a similar approach could be useful to mimic a breast tumor *in vitro*. The purpose of the current protocol is to establish a new method of generating breast cancer spheroids. This method utilizes estrogen receptor-positive MCF-7 cells (an immortalized human cell line of epithelial cells) and vascular endothelial cells (HUVECs) or lymphatic endothelial cells (HMVEC-DNeo) to create a model that mimics or closely reflects the interactions between these cells within a tumor. Although MCF-7 (estrogen-responsive) and endothelial cells have been used to develop spheroids in the present study, other cells such as fibroblasts which represent ~80% of the breast tumor mass, could also be combined in the future to better represent and mimic breast tumor.

There are several methods to form spheroids, such as: 1) the hanging droplet method that employs gravity^{33,34}; 2) the magnetic levitation method that uses magnetic nanoparticles exposed to an external magnet³⁵, and 3) the spheroid microplate method that is performed by seeding cells on low-attachment plates^{36,37}. On the basis of the existing methods, which use only one cell type, the present protocol has been optimized using epithelial and endothelial cells to better mimic the growth conditions of breast cancer tumors *in vivo*^{38–41}. This method can be easily achieved in the laboratory at a low cost and with minimal equipment requirements. Based on the need/goals of the lab, different approaches were used to form spheroids and gain relevant cellular material from these spheroids. In this context, for DNA, RNA, or protein analysis, the 3D spheroids are produced by co-culturing endothelial and epithelial cells with the hanging drop method. However, for functional studies, for example, to monitor cell growth after short interfering (siRNA) transfection and/or hormone treatment, the spheroids are generated using 96U-bottom plates.

The purpose of this technical protocol is to provide a detailed step-by-step description for 1) forming breast cancer multicellular-type spheroids, 2) preparing samples for histological staining, and 3) collection of cells for extraction of RNA, DNA, and proteins. Both the inexpensive hanging drop method and the more expensive U-bottom plates are used to form spheroids. Here, a protocol for preparing (fixing) spheroids for sectioning and subsequent immunostaining with markers to assess cell proliferation, apoptosis, and distribution of epithelial and endothelial cells within a spheroid is provided. Additionally, this protocol shows a complete step-by-step analysis of histological data using ImageJ software. Interpretation of biological data varies depending on the type of experiment and the antibodies used. Sectioning of fixed spheroids and subsequent staining of the sections was performed by a routine pathology lab (Sophistolab: info@sophistolab.ch)

PROTOCOL:

1. Cell culture

NOTE: Conduct cell handling under sterile conditions.

1.1. Human Umbilical Vein Endothelial Cells (HUVECs) subculture

1.1.1. Coat 75 cm² flasks with collagen (5 µg/cm²) (rat-tail) overnight (ON) at room temperature (RT); or 2–3 h at 37 °C, rinse with water, and allow the flask to dry.

1.1.2. Grow HUVECs in growth medium (EBM-2, Endothelial Basal Medium-2) supplemented with a commercial glutamine supplement (1x = 2 mM), antibiotic-antimycotic solution (AA; 100 µg/mL of streptomycin, 100 µg/mL of penicillin and 0.025 µg/mL of amphotericin B), LSGS (2% v/v FCS, 1 µg/mL of hydrocortisone, 10 ng/mL of human Epidermal Growth Factor, 3 ng/mL of human basic Fibroblast Growth Factor, 10 µg/mL of heparin) and 10% FCS (Fetal Calf Serum) under standard tissue culture conditions (37 °C, 5% CO₂). Change the medium every 2 days until the cells reach 70%–80% confluence.

1.1.3. Wash the sub-confluent cultures with 5 mL of HBSS (without Ca²⁺ and Mg²⁺) and add 3 mL of trypsin (0.25% diluted in HBSS (without Ca²⁺ and Mg²⁺)).

NOTE: HBSS can also be supplemented with PBS.

1.1.4. Incubate the cells at 37 °C for 2 min (microscopically check whether the cells are detached and rounded up) and stop the detaching reaction by adding 6 mL of 10% FCS medium (DMEM-F12 or EBM-2, 10% FCS).

1.1.5. Centrifuge the cell suspension at 250 x *g* for 5 min at RT and discard the supernatant.

1.1.6. Suspend the cells in growth medium and seed in 75 cm² flasks or culture dishes.

1.2. Michigan Cancer Foundation-7 (MCF-7, human breast adenocarcinoma cells) subculture

1.2.1. Grow human breast cancer cell lines in 75 cm² flasks in growth medium (DMEM/F12 medium supplemented with a commercial glutamine supplement (1x), antibiotic-antimycotic solution (AA; 100 µg/mL of streptomycin, 100 µg/mL of penicillin and 0.025 µg/mL of amphotericin B), and 10% FCS) under standard tissue culture conditions (37 °C, 5% CO₂). Change the medium every 2–3 days.

1.2.2. Wash the subconfluent cell cultures (70%–80% confluence) with 5 mL of HBSS (without Ca²⁺ and Mg²⁺) and add 3 mL of trypsin (0.5% diluted in HBSS (without Ca²⁺ and Mg²⁺) at 37 °C for 5 min (microscopically verify that the cells are detached).

NOTE: HBSS can also be supplemented with PBS.

1.2.3. Add 8 mL of 10% FCS medium to stop the detaching reaction, centrifuge at 250 x g for 5 min at RT and discard the supernatant.

1.2.4. Suspend the pellet in growth medium and plate in 75 cm² flasks or culture dishes.

2. Spheroid formation

2.1. Plate 5 x 10⁵ cells/mL (HUVECs and MCF-7) in a 3.5 cm round dish and culture for 48 h.

2.2. Treat or transfect the plated cells for 24 h or as desired.

2.3. Optional step performed in 96-well U-bottom plate: Wash the cells with 1 mL of HBSS (Ca²⁺ and Mg²⁺) and stain HUVECs using a blue dye (0.5 µg/mL) and MCF-7 cells using a green dye (0.5 µM) diluted in the respective growth medium and place into a 37 °C and 5% CO₂ incubator for 30–40 min.

2.4. Wash the cells with 1 mL of HBSS (without Ca²⁺ and Mg²⁺), add 1 mL of enzymatic detachment reagent (0.25% trypsin for HUVEC and 0.5% trypsin for MCF-7) to each round dish using a P1000 pipette, and then incubate for 2–5 min until the cells are detached.

2.5. Collect each cell type separately in a 5 mL round-bottom Polystyrene tube and add 1 mL of 10% FCS medium using a P1000 pipette to stop the enzymatic reaction. Centrifuge the cell suspensions at 250 x g for 5 min.

2.6. Carefully aspirate the supernatant and suspend the cells in 1 mL of steroid-free medium (EBM-2, glutamine supplement, antibiotic-antimycotic, 0.4% FCS sf (charcoal-stripped)).

NOTE: Steroid-free medium can be replaced with normal growth medium.

2.7. Use 100 μL of each cell suspension diluted with 10 mL of Diluent II (see **Table of Materials**) to determine the total cell number and calculate the amount of treatment medium (EBM-2, Glutamax, antibiotic-antimycotic, 0.4% FCS sf, vehicle/Treatment) needed to obtain a cell suspension of 5×10^3 cells/mL or 3.4×10^5 cells/mL per cell type.

2.7.1. Cell count

2.7.1.1. Turn on the machine and flush by pressing the **Function** and **Start** buttons (set-up S3), with Diluent II solution in a cell counter vial.

2.7.1.2. Use set up S4 and press **Start** to measure the blank with fresh Diluent II solution.

2.7.1.3. Change the scintillation vial with 100 μL of cell suspension in 10 mL of Diluent II solution and measure the samples: set-up S4 and press **Start**.

2.7.1.4. Note the number of cells per mL on the digital display.

NOTE: Cell counting can also be performed using other manual or automatic systems: Hemocytometer gridlines, light-scatter cell-counting technology, electrical impedance, imaging-based systems using cell staining, e.g., trypan blue.

2.7.2. 96-well U-bottom plates

2.7.2.1. Prepare 5 mL of each cell suspension (5×10^3 cells/mL) and mix them to get a final volume of 10 mL in the ratio of 1:1.

2.7.2.2. Use a manual repeating pipette to pipette out 100 μL of the cell suspension into each well of the 96-well U-bottom plates.

2.7.2.3. Place the plate into an incubator under standard tissue culture conditions and check for spheroids formation after 48 h.

2.7.2.4. Optional step: Take pictures of the spheroids (40x) using a fluorescence stereo microscope.

NOTE: This method generates 96 spheroids (one spheroid/well) after 48 h (area 1–2 pixel²), and it is usually used for growth studies.

2.7.3. Hanging drop

2.7.3.1. Mix the cell suspensions (3.4×10^5 cells/mL) in a 1:1 ratio to get a final volume of 2 mL.

2.7.3.2. Use a P20 pipette to seed the cell mixture in the form of 15 μL drops on the

inverted lid of a 10 cm Petri dish.

2.7.3.3. Invert the lid with the drops and add 5 mL of base medium (EBM-2) at the bottom of the dish to avoid evaporation of the drops.

NOTE: This method generates one spheroid/drop after 48 h. Ensure the drops are sufficiently apart from each other, so that they do not merge while switching the lid. Use more than one 10 cm Petri dish if necessary.

3. Spheroid growth study

3.1. Plate 100 μ L of HUVECs and MCF-7 cell mixture (5×10^2 cells/well) in 96-well U-bottom plates and place them in the incubator.

3.2. 48 h after plating, check for spheroids formation with an inverted microscope (Bright field). Take pictures (at least six pictures per treatment, 40x) at 96 h of culture.

3.3. Open the pictures using an image processing software and process the image to 300 pixel/in and save the image as a tiff file.

3.4. Download the ImageJ software and open it. Click on the **File** option and go to **Open**.

3.5. Convert the areas of interest to saturated black areas in a uniform manner to have a binary image (black and white). For this, select the **Image** option, choose **Type | 8-bit**. Now, the image is black and white.

3.6. Use the Freehand Selection Tool to draw the border of the spheroids.

3.7. To calculate the area of interest and to separate the object or the foreground pixels from the background pixels, use the threshold function: Select the **Image** option, choose **Adjust** and click on **Threshold**.

3.8. Now a Threshold pop-up window will open. The top bar indicates the minimum threshold value: set the value at zero. The bottom bar indicates the maximum threshold value: move the bar until the area of interest becomes completely red.

NOTE: If the color is not red, select **Red** from the Threshold window.

3.9. Go to **Analyze | Set Measurements** and select **Area** and **Perimeter**.

3.10. To measure the area of interest, select the **Analyze** option and use the **Analyze Particles** tool. In the **Analyze Particles** window, set the **Size** to measure (0.00003-Infinity or 3-Infinity, depending on the size of the spheroids), select **Summarize** and **Display Results**. Then, click on **OK**.

3.11. The results will show up in a chart. Copy the measurements into a spreadsheet to analyze the data.

NOTE: The area is calculated as the number of total pixels; use the Total Area for calculation.

4. Spheroid immunohistochemistry study

4.1. Sample preparation

4.1.1. Plate the cell mixture of HUVECs and MCF-7 (5×10^3 cells/well) in 96-well U-bottom plates in HUVEC growth medium for 96 h.

4.1.2. Collect approximately 50 spheroids into a 1.5 mL tube with a cut tip of a P200 μ L pipette; allow them to settle to the bottom of the tube by gravity, and then discard the supernatant.

4.1.3. Fix the spheroids with 500 μ L of 4% PFA for 1 h, RT.

4.1.4. Boil 2% Nobel Agar solution in PBS using a hot plate with a magnetic stirrer for 3–5 min to dissolve the agarose powder completely and cool down to around 60 °C.

4.1.5. Remove the PFA with a P1000 pipette, wash the spheroids with 500 μ L of PBS, and allow them to sediment. Discard the supernatant.

4.1.6. Carefully pipette 600 μ L of agarose solution into the 1.5 mL tube with spheroids and place the tube immediately in a centrifuge with a horizontal rotor at $177 \times g$ for 2 min.

4.1.7. Add a short string in the middle of the agarose solution to easily remove the plug from the tube.

4.1.8. Solidify agarose plug on ice or at 4 °C. Add 500 μ L of PBS into the 1.5 mL tube to avoid drying out of the pellet.

NOTE: The samples are ready for subsequent dehydration, paraffin embedding, sectioning, transfer onto the microscope slides, and IHC staining (collaboration with Sophistolab).

4.2. Image acquisition and processing

4.2.1. Acquire images of spheroid sections using a stereomicroscope.

4.2.2. Save three images for each sample as .jpg files.

4.2.3. Open the ImageJ software. To open the JPEG image, click on **File** and then on **Open**.

4.2.4. Select **Color** and **Color Deconvolution** in the **Image** option.

4.2.5. Select **H DAB** vectors in the Color Deconvolution 1.7 window, and the three images appear.

NOTE: The three images are: **Color 1** represents only the Hematoxylin staining (blue/purple), and **Color 2** represents only the DAB staining (brown). **Color 3** is not needed for the image analysis with two stainings.

4.2.6. Select the **Color 1** window and set the Threshold: go to **Image | Adjust** and click on **Threshold**. The **Threshold** window appears.

4.2.7. Leave the minimum threshold value set at zero and adjust the maximum threshold value to remove the background signal without influencing the true signal. Click on **Apply**.

4.2.7.1. Area measurement

4.2.7.1.1. Click on **Analyze**, choose **Set Measurement**. Select **Area**, **Mean grey value**, and **Min and Max grey value** and click on **OK** to confirm.

4.2.7.1.2. Measure the size of the nucleus using the **Analyze** option, and then select **Measure**.

NOTE: The **Results** window gives the name of the image (Label), size of the image (Area), the average pixel intensity of the IHC image (Mean), and the minimum and maximum gray values (Min, Max). Use the Mean value for calculation.

4.2.7.1.3. Repeat steps 4.2.6–4.7.1.2 for the **Color 2** (and **Color 3** if there is dual staining) window.

4.2.7.2. Cell quantification

4.2.7.2.1. Click on **Process**, choose the option **Binary** and select **Watershed** to separate cells.

4.2.7.2.2. Go to **Analyze** and click on **Analyze Particles**. On the **Analyze Particles** pop-up window, set the size of the cells to exclude unspecific particles. Next, on the **Show** option, click on the drop-down box and select **Outlines**. Then, click on **OK**.

4.2.7.2.3. Repeat steps 4.2.6–4.2.7 and cell quantification steps (section 4.2.7.2) for the **Color 2** (and **Color 3** if there is dual staining) window.

NOTE: Now three windows will appear: 1) Summary results (number of cells counted, total area, average size, % of area and mean), 2) Results (respective to the cells that are counted), and 3)

Drawing window (depicted image of the cells that are counted).

5. Live/dead staining in Spheroids

5.1. Prepare 4 mM stock solutions of Calcein AM in DMSO (stains live cells green) and 2 mM of Ethidium homodimer in DMSO (stains dead cells red) in 1.5 mL tubes.

5.2. Calculate the amount of solution necessary to add 10 μ L/well of a mixture of Calcein AM and Ethidium homodimer diluted 1:50 in EBM-2.

5.3. Add the staining mixture to the spheroids and place the plate in the incubator under standard tissue culture conditions for 30–60 min.

5.4. Acquire pictures (40x) using the Fluorescence stereo microscope.

6. Spheroid's protein and RNA isolation

6.1. Prepare cell suspensions at 3.4×10^5 cells/mL per cell type, mix them at a ratio of 1:1, and seed the cell mixture using a P20 pipette in the form of 15 μ L drops on the lid of a 10 cm Petri dish. Invert the lid and place it in the incubator under standard tissue culture conditions for 96 h.

6.2. Use 5–6 mL of PBS to collect spheroids in a 15 mL round-bottom Polystyrene tube using 5 mL sterile polystyrene pipettes.

NOTE: It is also possible to use 15 mL conical tubes.

6.3. Centrifuge the spheroids suspension at $250 \times g$ for 5 min, and then carefully aspirate the supernatant.

6.4. Add 500 μ L of trypsin (100%) and pipette up and down using a P1000 pipette for 30 s to disaggregate spheroids, achieving a cell suspension.

6.5. Neutralize trypsin with 10% FCS medium, centrifuge the tubes at $250 \times g$ for 5 min, and aspirate the supernatant.

6.6. According to the manufacturer's protocol (specific kit for DNA-free RNA isolation), use 300 μ L of RNA lysis buffer to lyse the cell pellet.

NOTE: Lysis buffer can also be directly added to the intact spheroids (no trypsin step required) to extract RNA. Add 300 μ L of the lysis buffer to the pelleted spheroids, place tubes in ice, and triturate 10 times using a P200 pipette. Subsequently, isolate RNA as above. Spheroid dissociation may be needed if RNA recovery is low due to matrix interference.

6.7. Alternatively, use 70 μ L of the Lysis buffer for protein isolation. Homogenize for 2–4 s by

sonication and determine the protein concentration according to the manufacturer's protocol using a BCA Assay Kit.

NOTE: For protein isolation, pelleted spheroids can be lysed directly in lysis buffer followed by sonication. Use 25 µg of total protein to perform western Blot.

REPRESENTATIVE RESULTS:

The spheroids model using epithelial and endothelial co-cultures is required to closely mimic *in vivo* conditions of breast tumors for *in vitro* experiments. The scheme in **Figure 1** depicts the protocol to form spheroids with breast cancer epithelial cells and vascular or lymphatic endothelial cells (**Figure 1**). Each cell type is seeded separately in a 3.5 cm round dish and treated with growth stimulators/inhibitors or transfected with oligonucleotides using Lipofectamine. The confluent monolayers of epithelial or endothelial cells are harvested following trypsinization, washed, and mixed at a 1:1 ratio. The cells are subsequently plated in 96-well U-bottom plates (**Figure 1A**) or on the inverted lid of a 10 cm diameter round culture dish to facilitate hanging drop culture (**Figure 1B**). Soon after seeding, the cells appear as flat, dense sheets of cells at the bottom of each well or drop, and, subsequently, they aggregate with time (24–48 h), thus forming intact spheroids at 48 h. In order to prevent any damage to the loosely formed cell aggregates, the plates must not be disturbed for at least 24 h.

In U-bottom plates, seeding of MCF-7 cells, but not endothelial cells or lymphatic cells, resulted in spheroid formation after 48 h (**Figure 2A,B**, respectively). Moreover, seeding of MCF-7 plus endothelial cells or lymphatic cells at a 1:1 ratio also resulted in spheroid formation (**Figure 2C,D**, respectively). To validate spheroids as a model to study tumor growth, time-dependent changes in spheroid size in response to growth-stimulating solution was measured/quantified and compared with the untreated controls (**Figure 2**). Photomicrographs in **Figure 2E** show time-dependent (24–120 h) sequential growth of a representative MCF-7 spheroid. The line graph in **Figure 2F** depicts the change in the area of MCF-7 spheroids over time when treated with or without a growth stimulator. The spheroid size increased from ~100 µm to ~200 µm over 5 days; moreover, an increased growth rate was observed in spheroids treated with the growth stimulator (**Figure 2F**). Because long-term treatment (168 h) resulted in reduced MCF-7 viability, potentially due to hypoxic conditions in the center of the spheroids (**Figure 3**), 3D structure growth was studied till 96 h. Spheroids formed with MCF-7 cells and HUVECs show a lower number of dead cells at 168 h compared to spheroids formed with MCF-7 alone.

In the initial experiments, only a small growth in spheroids' size was observed after 4 days of culture. It was hypothesized that this might be due to the high number of cells seeded to form spheroids. In the U-bottom culture plates, the growth of spheroids could have been limited by the concave shape of the well. Since growth was dependent on the initial number of cells seeded, different cell numbers were used to form spheroids to test and establish conditions that would be optimal to monitor spheroid growth. As shown in **Figure 4**, the findings suggest that for spheroid growth studies, seeding 500 cells/well to form spheroids was reliable and reproducible for monitoring of spheroids' growth for 4–6 days in response to growth modulatory factors. Spheroids that were most suitable for histological or immune-histological observations were

obtained on day 4, after seeding of 5×10^3 cells/well. This same initial concentration was used for cell protein or RNA extractions. Increasing the initial cell concentration by more than 7.5×10^3 cells/well did not improve spheroid formation, and the cells did not aggregate properly and assumed irregular structures (**Figure 4**).

In order to evaluate cell composition, proliferation, and apoptosis status, histological sections of spheroids formed with breast cancer cells plus endothelial cells were examined using H&E and Ki67 (dilution 1:300) and Cleaved Caspase-3 (dilution 1:500) antibodies. The process for final sample preparation requires care/attention and precision. **Figure 5A** depicts the workflow for the collection and incorporation of spheroids into agarose for sectioning. Photomicrographs in **Figure 5B–C** demonstrate the difference in MCF-7 spheroid formation in the presence (**Figure 5B**) and absence (**Figure 5C**) of Lipofectamine (0.17% final concentration), a commonly used transfection agent. Bright-field microscopic images of histological sections show a circular, compact structure of a homogenous mixture of two cell types (**Figure 5D–G**). The structure of the spheroids and the shape of their cells showed no abnormalities following transfection with control oligonucleotides in the presence of Lipofectamine (**Figure 5D**). Moreover, cells expressing the proliferative marker, Ki67, were distributed homogeneously in the spheroids; however, a ring of proliferative cells was also observed on the surface of the spheroids (**Figure 5E**). Cleaved Caspase-3 staining showed apoptotic cells after 4 days of culture (**Figure 5F**). Histological sections are useful to evaluate the distribution of epithelial and endothelial cells in spheroids using specific markers. Using CD31 as a specific marker for endothelial cells, it is possible to determine their distribution within the 3D structure. Since all the cells are stained with Hematoxylin (blue staining of the nucleus of the cell), the calculation of CD31 expression could provide the area of the spheroids containing endothelial cells following treatment or transfection (protocol section 4.2.7.1.). Moreover, by performing a dual staining assay, it is possible to have even more information, for example, as shown in **Figure 5G**, CD31 stains endothelial cells (red) and Ki67 stains proliferative cells (brown). Following the protocol section 4.2.7.2, **Figure 5G** revealed that 55% are epithelial cells, 45% are endothelial cells, and 25% of cells are proliferating.

The structure of spheroids may also be studied using immunofluorescence analysis following the labeling of cells with live dyes. Confluent 2D cultures of HUVEC and MCF-7 cells were separately stained with blue (HUVEC) and green (MCF-7) dyes. Subsequently, the stained cells were collected and mixed at a 1:1 ratio and seeded in wells for spheroid formation. Images of fluorescent cells stained were taken immediately after seeding (**Figure 6A**) and after 3 days of culture (**Figure 6B**). With the aim of assessing the percentage of live and dead cells, 4-day old spheroids were stained with calcein-AM (green) and with ethidium homodimer (red) (**Figure 6C**). Spheroids formed with MCF-7 and HUVECs could not be successfully frozen/preserved using the standard protocols for freezing cells (growth medium supplemented with 10% DMSO and 10% FCS) for freezing cells. In frozen and thawed spheroids, rupturing of cell aggregates and increased loss of cell viability were evident. The representative image in **Figure 6D** shows a freshly thawed spheroid stained for dead and live cells. It shows that spheroids are very sensitive to the freezing conditions.

After 5 days of culture in experimental conditions, lysis of around 100 spheroids was necessary

to collect enough protein (~3 µg/mL) or RNA (~60 ng/µL) for analysis. **Figure 7** represents a workflow from spheroids collection (generated with the hanging drops method) to cell lysis for protein extraction to perform western blotting or RNA isolation for future Microarray assays or analysis by RT-PCR. Isolation of RNA/DNA from a heterogeneous cell population in a spheroid for microarray analysis can bring some useful information. However, to identify key cell-specific mechanisms or cell-cell interactions, the spheroid cells can be separated after enzymatic (trypsin or accutase) dissociation, using FACS analysis and RNA from specific cells used for microarray analysis. Moreover, antibiotic-resistant cell lines (e.g., by using EGFP and/or puromycin resistance cell lines) could be used in spheroids to address the role of a specific cell type of cell-cell interaction. Additionally, gene silencing tools can be used to engineer the cells to address specific cell-cell interaction issues.

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow of spheroids formation. MCF-7 and HUVEC or LEC cells in 2D cultures are grown separately and collected after various desired treatments. Spheroids are subsequently generated by direct mixing of cells in **(A)** 96-well U-bottom plates, which promote the formation of 3D structure by cell-to-cell aggregation, or by using **(B)** hanging drop culture that supports spheroids formation via force of gravity.

Figure 2: Spheroid formation and development over time. Images depict the formation of a spheroid by MCF-7 cells (**Panel A**), the lack of spheroid formation by endothelial or lymphatic cells (HUVECs; **Panel B**) plated at the same density, and spheroid formation by MCF-7 cells plus HUVECs or LECs mixed at a 1:1 ratio (**Panels C,D**). Also depicted is the growth in size of a spheroid over time (24 h, 48 h, 72 h, 96 h, and 120 h). This spheroid was formed from a mixture of MCF-7 plus HUVECs in a 1:1 ratio and seeded at a density of 500 cells/well of a 96-well U-bottom plate (**Panel E**), (scale bar = 200 µm, 40x). Line graph (**Panel F**) shows the time-dependent growth of control (CTR) versus growth-stimulated (Treatment) spheroids. Spheroid's areas were measured and compared between untreated and treated spheroids. Results represent ± SEM, *** p < 0.001 compared to the respective control.

Figure 3: Spheroids long-term culture and viability. Photomicrograph shows cell viability in spheroids formed with MCF-7 cells alone at 96 h (**Panel A**) and 168 h (**Panel C**), whereas **Panels B** and **D** depict cell viability in spheroids made with MCF-7 plus HUVECs (1:1 ratio) at 96 h (**Panel B**) and 168 h (**Panel D**) (scale bar = 100 µm). Cells were stained with Calcein (red, dead cells) and Ethidium homodimer (green, live cells).

Figure 4: Cell seeding density and time-dependent growth profile of MCF-7 plus HUVEC spheroid. HUVECs and MCF-7 cells mixed at a 1:1 ratio were seeded at increasing densities (10^2 – 10^4 cells/well), and the growth of spheroids was monitored over 24–96 h. Microscopic bright-field images were taken (scale bar = 200 µm, 40x) to assess spheroid growth over time. Cells at a density of 500 cells/well provided optimal size to study spheroid growth.

Figure 5: Spheroid embedding for sectioning and histological staining. Panel A: Cartoon depicting the workflow for collection and incorporation of HUVECs/MCF-7 spheroids into

agarose. After paraffin embedding of the spheroids in an agarose plug and sectioning of paraffin blocks, the samples were used for standard histological staining. **Panels B and C** show representative MCF-7 plus HUVEC spheroids treated without and with Lipofectamine 2000, respectively (scale bar = 200 μ m, 40x). **Panels D–G** show representative images of spheroid sections stained with specific markers. **Panel D** shows spheroids stained with hematoxylin-eosin to assess spheroid structure (scale bar = 100 μ m). Staining in **Panel E** depicts proliferating cells positively stained with Ki67. **Panel F** shows apoptotic cells in a spheroid positively stained with Cleaved Caspase-3 (scale bar = 50 μ m). **Panel G** depicts spheroid sections with dual staining: CD31 (endothelial cells marker) and Ki67 (proliferative marker) (scale bar = 100 μ m). The sectioning of spheroids and staining were performed by Sophistolab (info@sophistolab.ch).

Figure 6: Fluorescence images of MCF-7/HUVEC spheroids showing cell distribution and viability. **Panels A and B** depict representative spheroids with HUVECs stained with a blue dye and MCF-7 cells stained with a green dye. The localization of HUVEC and MCF-7 within the spheroid varies immediately after seeding (**A**) and after spheroid formation (**B**) (scale bar = 250 μ m). **Panels C–D** show spheroids stained with Calcein/Ethidium Homodimer to identify live (green) and dead cells (red) in the 3D structures in normal culture condition (**C**) and after freezing (**D**) (Scale bar = 100 μ m).

Figure 7: Schematic representation of spheroid collection for biochemical analysis. Cartoon showing the scheme to harvest spheroids, separate or release cells, and suspend them in lysis solution for protein analysis or RNA extraction.

DISCUSSION:

Compared to 2D cell cultures, revolutionary 3D spheroid culture technology is a better and more powerful tool to reconstruct an organ's microenvironment, cell-cell interactions, and drug responses *in vitro*. This is the first protocol describing the formation of spheroids from multicellular (epithelial and endothelial) cell lines for breast cancer research. This protocol ensures spheroidal 3D growth of spheroids for up to 5 days, and spheroids can be examined after paraffin embedding, sectioning, and histological staining. Interestingly, the cellular elements within the spheroid still express receptors that promote cell growth and are responsive to proliferative and apoptotic stimuli. The described protocol generates sufficient biological material for protein or RNA/DNA analysis. The above co-culture system, easily achieved in laboratory conditions and at low cost, could be an advantage for future applications, especially in breast cancer therapy and for drug sensitivity testing. In addition, this protocol can be applied to different epithelial and endothelial cell types.

It is extremely difficult to mimic *in vitro* a complex tissue that exists *in vivo*. This is because *in vivo* tissues consist of many interacting components, including nerves, blood vessels, mesenchyme, and immune cells^{30,42}. The aim of this protocol is to overcome some of these limitations by using a 3D co-culture system with two different cell types to approach the actual tumor state. Here, spheroids have been formed with epithelial tumor cells in combination with endothelial cells or lymphatic cells to mimic the *in vivo* situation as much as possible, including cell-cell interactions, susceptibility to apoptotic factors released into the intercellular spaces by dying cells, and hypoxic

conditions in the center of the spheroids. This protocol has been optimized for testing of cellular responses to growth factors, signaling factors, and hormones, which rapidly activate signaling cascades and target gene transcription and stimulate protein expression and transport^{30,31}. In spheroids, but not *in vivo*, responses of tumor cells to stimuli can be assessed rapidly and frequently.

In the present study, endothelial cells in the spheroids do not seem to form capillary-like structures. Since endothelial cells have been shown to form capillaries when plated at low density and monolayers at high density, it is feasible that lowering the MCF-7 to endothelial cells ratio may result in capillary formation within the spheroids. Alternatively, the addition of specific matrix proteins or matrigel may promote capillary formation. The lack of capillary-like structure in the spheroid does not dilute the advantage of MCF-7 plus endothelial cells versus MCF-7 cells per se, as the factors released by MCF-7 cells could promote endothelial cell proliferation and vice versa; such interactions would remain undetected in MCF-7 only spheroids.

Several studies have shown that 5×10^3 cells/well was the appropriate concentration to seed cells to form spheroids^{43–47}; however, in 96-well U-bottom plates, this number of cells limited the coordinated growth of spheroids in experimental conditions. Therefore, in each new study, for each new cell type, it is important to monitor the growth of the 3D system after drug treatment, to determine the effects of the treatment on spheroid size.

The limitation of the current protocol is that the culture of previously formed spheroids cannot be continued after freezing and thawing by the classical DMSO method. In fact, when thawed, most of the cells were dead. Vitrification may prove to be an alternative to keeping the cells alive⁴⁸.

Careful attention to technical details is necessary to avoid errors and produce and maintain well-formed spheroids. One challenge is the sample preparation for immunostaining (section 4.1). To facilitate the transfer by pipetting the spheroids into the 1.5 mL tube, it is important to use a P200 tip cut with scissors such that the area of the hole is larger than the spheroids themselves. Otherwise, the transfer could destroy the spheres. Another challenge is how to aspirate the medium after the spheroids have settled to the bottom of a test tube. In this regard, it is better to use a P1000 pipette instead of a vacuum pump to avoid the aspiration of spheroids. An important and delicate step in preparing the spheroids for sectioning is to add the agarose to the pelleted spheroids. The spheroids, once suspended in agarose, must be pelleted quickly to the bottom of the microfuge tube using horizontal centrifugation at room temperature and before the agarose polymerizes.

Overall, the two-cell spheroid made of MCF-7 and endothelial cells provides a viable alternative to investigate cell-cell interactions and mechanisms that drive the growth of either cancer cells or endothelial cells within a tumor. The spheroids could serve as a model to assess the efficacy of therapeutic agents targeting tumor or cancer growth. Importantly, this two-cell model could be further improvised to include other cell types relevant in tumor growth. In this context, fibroblasts that constitute 80% of the breast tumor mass may be included to form an MCF-7,

endothelial cells, and fibroblast spheroid. Moreover, gene silencing and genetically engineered cells could be used to address the role of cell-cell interaction, hormone receptors (estrogen/progesterone), growth factors, and signaling pathways on tumor/cancer growth as well as to test the efficacy of new anti-cancer molecules.

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The authors have nothing to disclose.

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