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Reconstituting Breast Tissue with Organotypic Three-dimensional Co-culture of Epithelial and Stromal Cells in Discontinuous Extracellular Matrices

Gang Ren, Vandana Sharma, Joshua Letson, Yashna Walia,
Veani Fernando and Saori Furuta*

Department of Cancer Biology, College of Medicine and Life Sciences, University of Toledo Health Science Campus, 3000 Arlington Ave. Toledo, OH 43614, USA
*For correspondence: Saori.Furuta@utoledo.edu

[Abstract] Co-culture systems utilizing reconstituted or synthetic extracellular matrix (ECM) and micropatterning techniques have enabled the reconstruction of surface epithelial tissues. This technique has been utilized in the regeneration, disease modeling and drug screening of the surface epithelia, such as the skin and esophagus. On the other hand, the reconstruction of glandular epithelia would require more intricate ECM organizations. Here we describe a protocol for a novel three-dimensional organotypic co-culture system for the reconstruction of mammary glands that utilizes the discontinuous ECM. In this technique, primary mammary fibroblasts first establish a layer of the connective tissue rich in collagen I. Then, mammary epithelial cells form acinar structures, the functional glandular units, within the laminin-rich basement membrane embedded in the connective tissue. This method allows for the regeneration of the *in vivo*-like architecture of mammary glands and could be utilized for monitoring the real-time response of mammary glands to drug treatment.
Keywords: ECM, Organotypic 3D co-culture, Glandular epithelia, Mammary gland, Cell culture stamp

[Background] Three-dimensional (3D) co-culturing of cells in the extracellular matrix (ECM) substratum allows for the reconstruction of the *in vivo*-like tissue architecture and behavior, as well as the analysis of cellular interactions within the tissue microenvironment (Langhans, 2018). Furthermore, the advent of synthetic ECM materials and micropatterning techniques in the 2000s has enabled the regeneration of optimal ECM composition and spatial orientation for each tissue type in co-culture (Hotary *et al.*, 2000; Gudjonsson *et al.*, 2002; Carey *et al.*, 2017). In these 3D co-culture systems, fibroblasts form a layer of the connective tissue while epithelial cells grow on top of the connective tissue or on the plastic surfaces exposed by micropatterning the ECM (Stark *et al.*, 2004; Kalabis *et al.*, 2012; March *et al.*, 2015). This method enables the reconstruction of 3D architecture of surface (stratified) epithelia, such as skin and esophagus. Such 3D co-culture technique has been utilized in generation of the transplantable skin organoids (Kim *et al.*, 2018), disease modeling (*e.g.*, psoriasis, esophagitis) and drug screening (Klicks *et al.*, 2017; Whelan *et al.*, 2018; Sarkiri *et al.*, 2019). On the other hand, this technique is not suitable for the reconstruction of glandular epithelia that have more intricate ECM organizations. The glandular epithelium is enclosed by the basement membrane, which is, in turn, embedded in the connective tissue encompassing fibroblasts and other stromal cells.

Here we introduce a novel 3D organotypic ECM co-culture technique for reconstructing the 3D

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structure of the mammary gland. Mammary epithelial cells and mammary fibroblasts are individually cultured in the discrete, physiologically-relevant ECM. The discontinuous ECM is generated by micropatterning the fibroblast-containing collagen I-rich matrix and filling the grooves with the epithelial cell-containing laminin-rich basement membrane. This organotypic 3D ECM co-culture system allows for the growth of epithelial and stromal cells in the distinct locales, serving as a robust *in vitro* technique for modeling diseases and testing drug efficacy.

Materials and Reagents

A. Materials

Flasks, plates and insert

1. T-75 flasks, vent (Corning, catalog number: 08-772-1F)
2. Corning Cell Culture 12 well plates (Corning, catalog number: 07-200-82)
3. Corning 12 mm Transwell with 3.0 μm Pore Polycarbonate Membrane Insert (Corning, catalog number: 07-200-157)

Histology

1. Bio-wraps (Leica Biosystems, catalog number: 3801090)
2. Tissue and Biopsy Cassettes (Fisher Scientific, catalog number: 15-200-403J)

Tools for Constructing a Custom Stamp

1. 5-minute epoxy (JB Weld, ClearWeld # 50112, Home Depot)
2. Round wooden disks (25 mm in diameter, 3 mm in thickness, Hobby Lobby)
3. Standard 2 mm drill bit
4. Standard 5 mm drill bit

B. Cells

1. Primary human mammary epithelial cells (ScienCell, catalog number: 7610)
2. MCF10A human mammary epithelial cells (Barbara Ann Karmanos Cancer Institute)
3. HMT-3522-S1 human mammary epithelial cells (Mina Bissell Laboratory, Lawrence Berkeley National Laboratory, Berkeley, CA)
4. Primary human mammary fibroblasts (ScienCell, catalog number: 7630)

C. Media

For primary mammary epithelial cells

1. Mammary Epithelial Cell Medium (MEpiCM, ScienCell, catalog number: 7611) supplemented with 1% Mammary Epithelial Cell Growth Supplement (MEpiCGS, catalog number: 7652) and 1% penicillin/ streptomycin (Thermo Fisher, catalog number: MT30002CI)

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For MCF10A human mammary epithelial cells

1. DMEM/F12 (Thermo Fisher, catalog number: 11320033) supplemented with the MCF10A additives (Recipe 1) (Debnath *et al.*, 2003)

For HMT-3522-S1 human mammary epithelial cells

1. DMEM/F12 (Thermo Fisher, catalog number: 11320033) supplemented with the HMT-3522-S1 additives (Recipe 2) (Vidi et al., 2013)

For primary human mammary fibroblasts

1. Fibroblast Medium (FM, ScienCell, catalog number: 2301) supplemented with 1% Fibroblast Growth Supplement (FGS, catalog number: 2352) and 1% penicillin/streptomycin

D. Reagents

Reagents for culturing

1. 0.05% trypsin/EDTA solution (Fisher Scientific, catalog number: 25-300-062)
2. 0.25% trypsin/EDTA solution (Fisher Scientific, catalog number: 15-050-057)
3. Soybean trypsin inhibitor (10 mg/ml, Thermo Fisher, catalog number: 17075-029)
4. Dispase I (2mg [20U] Sigma-Aldrich, catalog number: D4818)
5. 0.5 M EDTA, pH 8.0 (Thermo Fisher, catalog number: AM9260G)
6. PBS (Thermo Fisher, catalog number: SH3037803)
7. EGF (Sigma-Aldrich, catalog number: E-9644)
8. Hydrocortisone (Sigma-Aldrich, catalog number: H-0888)
9. Cholera toxin (Sigma-Aldrich, catalog number: C-8052)
10. Horse serum (Thermo Fisher, catalog number: 16050122)
11. Prolactin (Sigma-Aldrich, catalog number: L6520)
12. β-estradiol (Sigma-Aldrich, catalog number: C-8052)
13. Sodium selenite (Sigma-Aldrich, catalog number: 214485-5G)
14. Transferrin (Sigma-Aldrich, catalog number: T2252)
15. HMT-3522-S1 additives (see Recipes)
16. MCF10A additives (see Recipes)
17. Phosphate buffered saline (PBS) (see Recipes)
18. Acellular layer matrix (see Recipes)
19. Cellular layer matrix (see Recipes)
20. Paraformaldehyde (see Recipes)

Reagents for ECM

1. Matrigel (~10 mg/ml, growth factor reduced, Corning, catalog number: 354230)
2. Corning Collagen I, Rat Tail (3-4 mg/ml, Corning, catalog number: CB-40236)
3. DMEM/F12 (10x), made from powder (Thermo Fisher, catalog number: 12500062)

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4. Sodium Bicarbonate 7.5% solution (50x, Thermo Fisher, catalog number: 25080094)
5. FBS (JR Scientific, catalog number: 43603-500 [Research grade])
6. L-Glutamine (200 mM [50x], Thermo Fisher catalog number: 25030081)

Reagents for Histology

1. Paraformaldehyde (Sigma-Aldrich, catalog number: P6148-500G)

Equipment

1. Hand drill (De Walt, model: #dcd7916 20v 1/2" chuck, Home Depot)
2. Stainless steel rods (Uxcell, 30 mm x 2.5 mm, Amazon.com)
3. 4" cast iron drill press vice (Irwin, model: #226340, Home Depot)
4. 37 °C water bath
5. 37 °C humidified incubator with 5% CO_2
6. Cell culture benchtop centrifuge
7. Hemocytometer
8. Micropipettes (P20, P200 and P1000)
9. Pipette aid
10. Microscope with a color CCD camera

Procedure

A. Cell culture and maintenance

Primary Human Mammary Epithelial cells

Primary human mammary epithelial cells (ScienCell) were isolated from human breast and cryopreserved at passage one.

1. Prepare a poly-L-lysine (2 µg/cm^2)-coated T-75 flask. To obtain a 2 µg/cm^2 poly-L-lysine-coated culture vessel, add 10 ml of sterile water to a T-75 flask and then add 15 µl of poly-L-lysine stock solution (10 mg/ml). Leave the flask in a 37 °C incubator overnight. Rinse the flask.
2. Thaw a frozen vial of cells (> 5 x 10^5 cells) in a 37 °C water bath, and dispense the contents of the vial into the poly-L-lysine-coated flask.
   Note: Dilution and centrifugation of cells after thawing are not recommended.
3. Gently rock the flask to distribute cells evenly. Return the culture vessel to the incubator, and leave the culture undisturbed for at least 16 h after initiation.
4. Replace medium the next day to remove residual DMSO and unattached cells.
5. Thereafter, change the medium every three days, until the culture is approximately 70% confluent. Once the culture reaches 70% confluence, change the medium every other day until the culture is approximately 90% confluent.
6. Subculture when cells reach 90% confluence. Gently rinse the flask with PBS and add 4 ml 0.25%

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Trypsin/EDTA solution into the flask. Gently rock the flask to ensure complete coverage of cells by Trypsin/EDTA solution, return the flask to the 37 °C incubator and incubate it for 2-3 min or until cells have detached.

7. After cells have completely detached from the flask, add 180 µl of soybean trypsin inhibitor to the flask and transfer detached cells to a 15 ml centrifuge tube.
8. Centrifuge the tube at 400 *x g* for 5 min and discard the supernatant. Resuspend the cell pellet in culture medium and plate them in new poly-L-lysine-coated flasks at the seeding density of 5,000 cells/cm^2.
9. Cells can undergo ten population doublings (early passages are recommended).

MCF10A Human Mammary Epithelial cells

MCF10A cells were collected from human fibrocystic breast tissue and went through spontaneous immortalization. MCF10A cells maintain many characteristics of normal cells, including (a) the inability to form tumors in nude mice; (b) dependence on growth factors and hormones for growth and survival; and (c) lack of anchorage-independent growth (Soule *et al.*, 1990). Culturing of MCF10A cells follows the protocol described by Debnath *et al*. (2003) with slight modifications.

1. Thaw a frozen vial of cells (1 x 10^6 cells) in a 37 °C water bath and dispense the contents of the vial into a T-75 flask containing 10 ml culture medium.
2. Gently rock the flask to distribute cells evenly. Return the culture vessel to the incubator and leave the culture undisturbed for at least 16 h after initiation.
3. Replace medium the next day to remove residual DMSO and unattached cells.
4. Change the medium every three days until the culture is approximately 70% confluent. Once cells reach 70% confluence, change the medium every other day until the culture is approximately 90% confluent.
5. Subculture when cells reach 90% confluence. Gently rinse the flask with PBS and add 4 ml 0.25% Trypsin/EDTA solution into flask. Gently rock the flask to ensure complete coverage of cells by Trypsin/EDTA solution, return the flask to the 37 °C incubator and incubate it for 2-3 min or cells have completely detached.
6. Add 180 µl of soybean trypsin inhibitor to the flask and transfer detached cells to a 15 ml centrifuge tube.
7. Centrifuge the tube at 400 *x g* for 5 min and discard the supernatant. Resuspend cell pellets in culture medium and plate them in new flasks at a 1:3 ratio.
8. Cells can undergo 35 passages (early passages are recommended).

HMT-3522 S1 Human Mammary Epithelial cells

HMT-3522 S1 was derived from a benign mammary fibrocystic lesion and became spontaneously immortalized in culture (Briand *et al.*, 1987; Moyret *et al.*, 1994; Rizki *et al.*, 2008; Vidi *et al.*, 2013). S1 cells retain non-malignant characteristics, requiring EGF to grow in culture and being unable to form tumors in nude mice (Briand *et al.*, 1987; Rizki *et al.*, 2008; Vidi *et al.*, 2013).

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Note: The use of S1 cells is restricted to passages below 60 because of genotypic drift at higher passages (Rizki et al., 2008; Vidi et al., 2013). Culturing of HMT-3522 S1 cells follows the protocol described by Vidi et al. (2013) with slight modifications.

1. Thaw a frozen vial of cells (1 x 10^6 cells) in a 37 °C water bath, and dispense the contents of the vial into a centrifuge tube with 5 ml of growth medium.
2. Centrifuge the tube at 400 *x g* for 5 min.
3. Remove the supernatant and resuspend the cell pellet in 3 ml of fresh growth medium.
4. Add 7 ml fresh growth medium into a T-75 flask and transfer the whole cell suspension.
5. Return the culture vessel to the incubator and leave the culture undisturbed for two days after initiation.
6. Change the medium every other day until colonies start forming rounded islands (the edges of the colonies become smooth).
   Note: This phenomenon usually takes place when the culture reaches ~60% confluence (6~10 days after plating).
7. Subculture when cells reach 60% confluence. Rinse the cells with 1 ml 0.25% Trypsin/EDTA solution and add 1 ml 0.25% Trypsin/EDTA solution into the flask. Gently rock the flask to ensure complete coverage of cells by Trypsin/EDTA solution, return the flask to the incubator and incubate it at 37 °C for 2-3 min.
8. Add 180 µl of soybean trypsin inhibitor to the flask and transfer detached cells to a 15 ml centrifuge tube.
9. Centrifuge the tube at 400 *x g* for 5 min and then discard the supernatant.
10. Resuspend cells in 3 ml culture medium and count cell density.
11. Place 10 ml growth medium into a T-75 flask.
12. Seed 1.5 x 10^6 cells in a T-75 flask (2 x 10^4 cells/cm^2).
13. Cells can be used until 60 passages (early passages are recommended).

Primary Human Mammary Fibroblasts

Primary human mammary fibroblasts (ScienCell) were isolated from human breast and cryopreserved at passage one.

1. Prepare a poly-L-lysine (2 µg/cm^2)-coated T-75 flask as mentioned above.
2. Thaw a frozen vial of cells (> 5 x 10^5 cells) in a 37 °C water bath, and dispense the contents of the vial into the poly-L-lysine-coated flask. Gently rock the flask to distribute cells evenly. Return the culture vessel to the incubator and leave the culture undisturbed for at least 16 h after initiation.
3. Replace medium the next day to remove residual DMSO and unattached cells.
4. Change the medium every three days thereafter, until the culture is approximately 70% confluent. Once cells reach 70% confluence, change the medium every other day until the culture is approximately 90% confluent.

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5. Subculture when cells reach 90% confluence. Rinse the cells with PBS and add 10 ml of 0.05% Trypsin/EDTA solution into flask. Gently rock the flask to ensure complete coverage of cells by Trypsin/EDTA solution, return the flask to the incubator and incubate it at 37 °C for 2-3 min. Add 180 µl of soybean trypsin inhibitor to the flask and transfer detached cells to a 15 ml centrifuge tube.
6. Centrifuge the tube at 400 *x g* for 5 min and discard the supernatant. Resuspend the cells in culture medium and plate them in new poly-L-lysine-coated flasks at the seeding density of 5,000 cells/cm^2.
7. Cells can undergo 15 population doublings.

B. Construction of a micropatterning stamp (Figure 1)

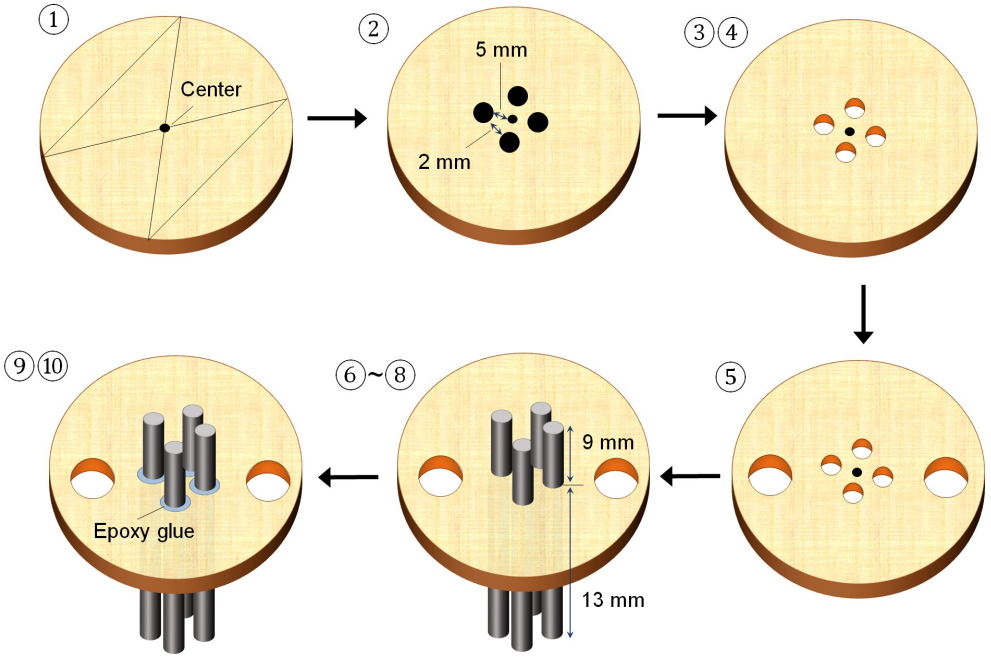

Figure 1. Construction of micropatterning stamp

A custom stamp for micropatterning the ECM in a 12-well plate insert is constructed as follows:

1. To find the center of a round wooden disk, draw two parallel chords with equal length across the circle with a pencil. Draw an “X” that links the ends of the chords diagonally. Mark the intersection of the “X” as the center of the circle.
2. Using a pencil, mark four 2 mm-spots on the disk at 5 mm from the center, 2 mm apart from each other.
3. Secure the disk horizontally in a drill press vice.
4. Using a hand drill in conjunction with 2 mm-drill bit, drill four holes through the spots marked on the disk.
5. Using a hand drill in conjunction with 5 mm-drill bit, drill two access holes on the opposite ends of the disk.

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6. Remove the disk from the drill press vice.
7. Insert 2.5-mm stainless steel rods into the 2 mm-holes on the disk. This provides a press fit for rods to hold them in place until they are secured with epoxy.
8. Adjust the rods so that the 9 mm-ends of the rods remain above the top surface of the disk and 13 mm-ends of the rods protrude from the bottom surface of the disk.
9. Mix and apply a small amount of epoxy to the perimeter of the rods on the top side of the disk where they meet the disk to secure them in place. Air-dry the stamp as recommended on the package of the epoxy.
10. Clean the metal rods of the stamp lightly with 70% alcohol, and UV-irradiate the stamp for 10-20 min in a tissue culture hood for sterilization before use.

C. Preparation of 3D organotypic co-culture (Figure 2)

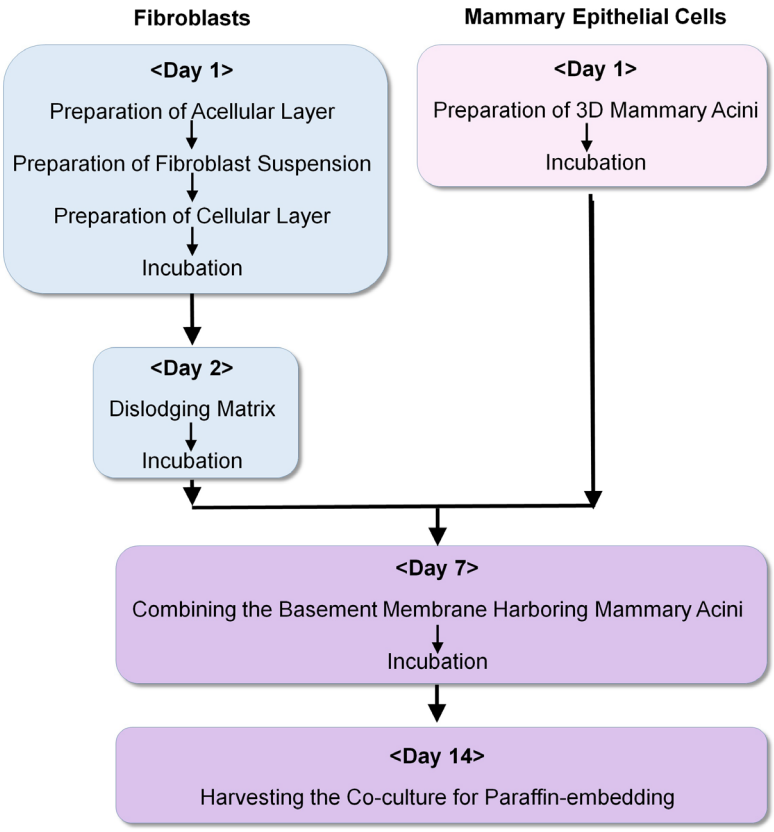

Figure 2. Flow Chart of 3D Organotypic Co-culture of mammary epithelial cells and fibroblasts

Day 1

1. Preparation of acellular layer
   a. Prepare a 12-well plate.
   b. Place a transwell insert into each well.
   c. Adjust the concentration of type I collagen to 2 mg/ml in PBS and the pH to 7.4 with sterile

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1 M NaOH.

d. To make the acellular layer, add 10x DMEM/F12, FBS, L-Glutamine, N-bicarbonate and type I collagen (2 mg/ml) in this order into a centrifuge tube on ice (Recipe 4).
e. Mix gently and readjust the pH to 7.4 with sterile 1 M NaOH.
f. Place 200 µl of gel into each insert.
g. Leave the plate undisturbed at room temperature in a tissue culture hood for 30 min during polymerization of collagen.

2. Preparation of fibroblast suspension
   a. Grow primary mammary fibroblasts in three T-175 flasks.
   b. Trypsinize and combine all dissociated cells in a 50 ml centrifuge tube.
   c. Centrifuge, wash and resuspend the pellet in 1 ml of fibroblast growth medium.
   d. Count the cell density and adjust it to 1 x 10^8/ml with fibroblast medium.
3. Preparation of cellular layer
   a. Adjust the concentration of type I collagen to 2 mg/ml in PBS and the pH to 7.4 with sterile 1 M NaOH.
   b. Add 10x DMEM/F12, FBS, L-Glutamine, N-bicarbonate and type I collagen (2 mg/ml) in this order into a centrifuge tube on ice (Recipe 4).
   c. Mix gently and adjust the pH to 7.4 with sterile 1 M NaOH.
   d. Add Matrigel and fibroblasts.
   e. Place 1.0 ml gel into each insert (1x10^7 fibroblasts/sample).
   f. Place a custom stamp into the gel (Figure 3, top left)

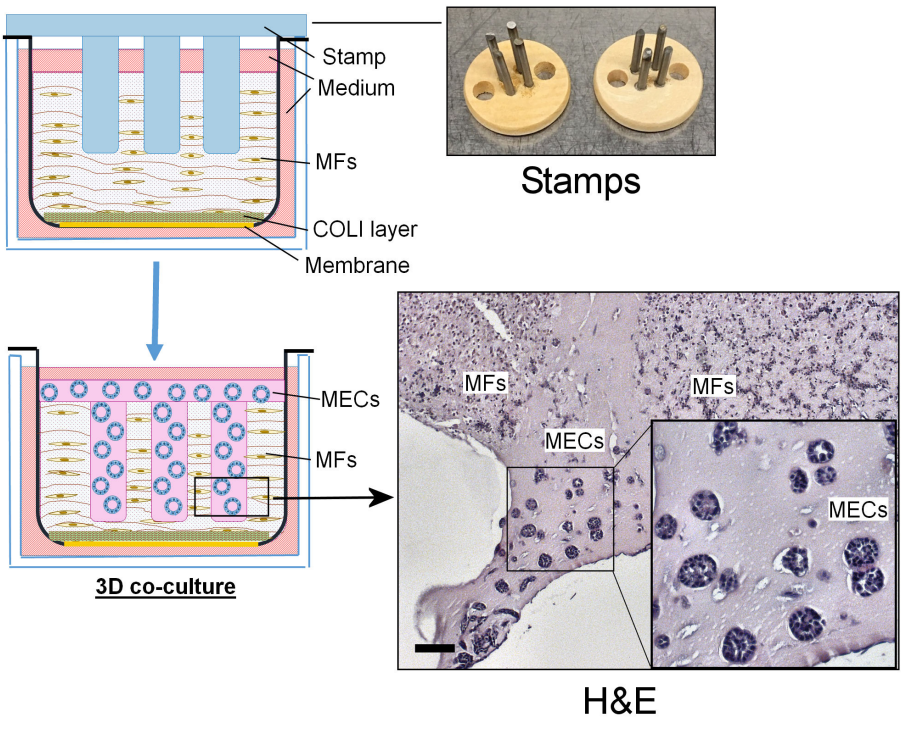

Figure 3. Scheme of organotypic 3D ECM co-culture of primary mammary fibroblasts

(MFs) and mammary epithelial cells (MECs). (Left) Schematic representation of experimental design of organotypic 3D ECM co-culture, where MECs and MFs are grown in distinct locales of the discontinuous ECM with the help of a micropatterning stamp on top of Collagen I matrix. (Right top) Image of the custom-made stamps. (Right bottom) Representative image of Hematoxylin and Eosin stained paraffin-embedded section of a co-culture showing the histomorphology of MFs and MECs at 40x and 200x magnifications. Scale bar: 50 µm.

g. Leave the plate undisturbed for 30-45 min in the tissue culture incubator (37 °C, 5% CO_2) during polymerization of the matrix.
h. Add the fibroblast growth medium through the access holes of the stamp, ~4 ml into the bottom of the wells and ~1 ml into the insert.
i. Leave the plate in the tissue culture incubator (37 °C, 5% CO_2) for one day until the matrix is manually dislodged from the inner wall of the insert.

4. Preparation of 3D mammary acini

Matrigel Coating and Matrigel Suspension

a. Thaw Matrigel at 4 °C overnight and keep it on ice.
b. Coat a 12-well culture plate with ~500 µl Matrigel per well. Incubate for 15-30 min in a 37 °C tissue culture incubator to allow polymerization of Matrigel.
c. In a 15 ml centrifuge tube, prepare 4% Matrigel suspension in the mammary epithelial cell growth medium.

Preparation of Mammary Acini

Note: Normal or non-malignant mammary epithelial cells form mammary acinar-like structures)

a. Trypsinize, wash and resuspend mammary epithelial cells (primary mammary epithelial cells, MCF10A cells or HMT-3522 S1 cells) in 1 ml mammary epithelial cell growth medium.
b. Count the cell density and transfer the volume of the cell suspension to have 1 x 10^5 cells/sample in each well of a 12 well plate (0.26 x 10^5 cells/cm^2).
c. Centrifuge to collect the cell pellet, and resuspend cells in 1 ml 4% Matrigel suspension per sample.
d. Place the cell suspension onto Matrigel coat.
e. Leave the plate in the tissue culture incubator (37 °C, 5% CO_2) for six days until spheroid structures are formed.

Day 2

5. Dislodging collagen 1 (Acellular and Cellular) matrix (Figure 4)

a. Carefully lift up and remove the stamp from the co-culture.
b. Remove the growth medium.
c. Using a sterile glass Pasteur pipette, outline the circumference of the matrix for 2-3 times

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to dislodge the matrix from the inner wall of the insert.

Note: Do not pierce the transwell membrane.

d. Place the stamp back to fit into the grooves.

e. Add 1 ml fibroblast growth medium into the insert.

f. Leave the plate in the tissue culture incubator (37 °C, 5% CO_2) for five days. The matrix will become contracted over the next few days. The medium in the bottom of the well is replaced daily (no change of the medium in the insert).

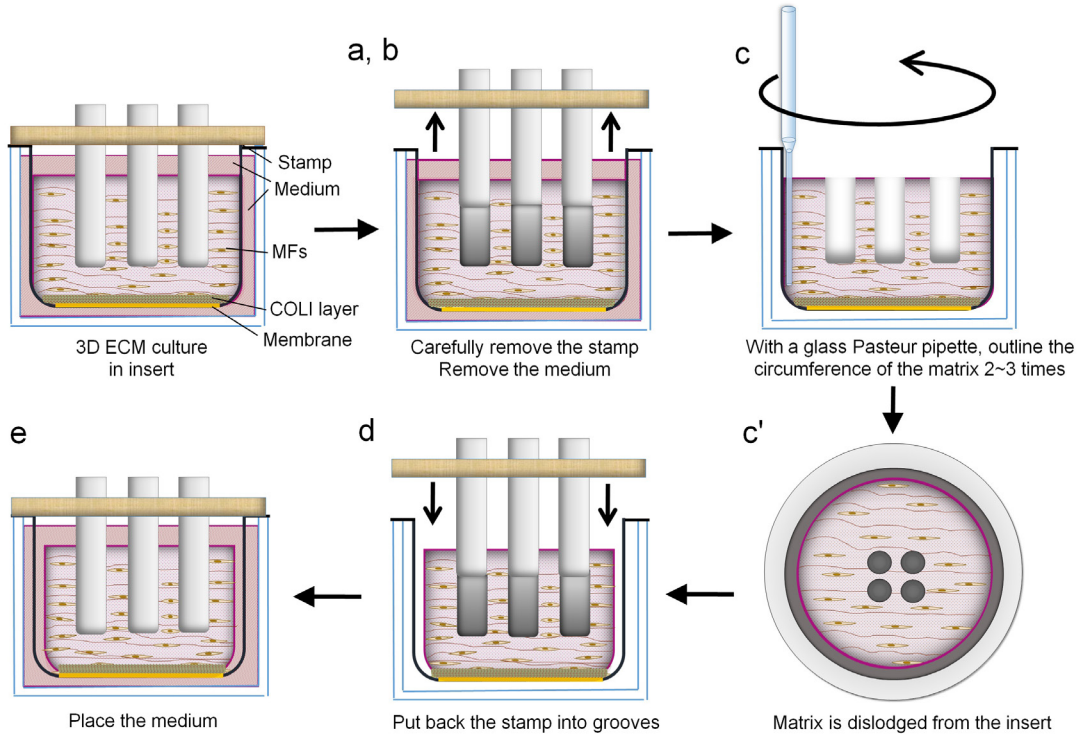

Figure 4. Dislodging collagen I (COL1, acellular and cellular) matrix

Day 7

6. Combining the basement membrane harboring mammary acini

Dissociation of Mammary Acini from Matrigel

a. Dissolve Dispase I (2 mg, 20 U) in 200 µl PBS to make the stock solution (10 mg/ml, 100 U/ml). Further dilute Dispase I solution to make the working solution (0.2 mg/ml, 2 U/ml).

b. Remove the Matrigel suspension from the mammary epithelial culture in the 3D matrix. Wash with PBS.

c. Add 1 ml working solution of Dispase I (2 U/ml). Incubate for 30 min in a 37 °C tissue culture incubator for enzymatic digestion of Matrigel.

d. Add 20 µl 0.5 M EDTA (pH 8.0, final concentration of 1 mM) to inactivate Dispase I.

e. Collect the digest in a 15 ml centrifuge tube. Centrifuge and wash the cell pellet in PBS 2~3 times.

f. Resuspend the pellet (~2 x 10^4 acini) in 500 µl Matrigel and keep it on ice

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Addition of Mammary Acini in Matrigel to Collagen Matrix (Figure 5)

a. Carefully remove the stamp.
b. Remove the growth medium from the insert.
c. Into four grooves formed after removing the stamp, transfer ~100 µl/groove Matrigel/mammary acini mixture. Place the remaining mixture (~100 µl) on the surface of the cellular layer. Incubate for 30 min in a 37 °C tissue culture incubator for polymerization of Matrigel (Figure 5).
d. Prepare fibroblast growth medium/mammary epithelial cell growth medium (50:50) mixture and add ~4 ml into the bottom of the wells and ~1 ml into the insert.
e. For the analysis of drug treatments, different drugs can be added to the growth medium.
f. Leave the plate in the tissue culture incubator (37 °C, 5% CO_2) for seven days. Change the medium daily.

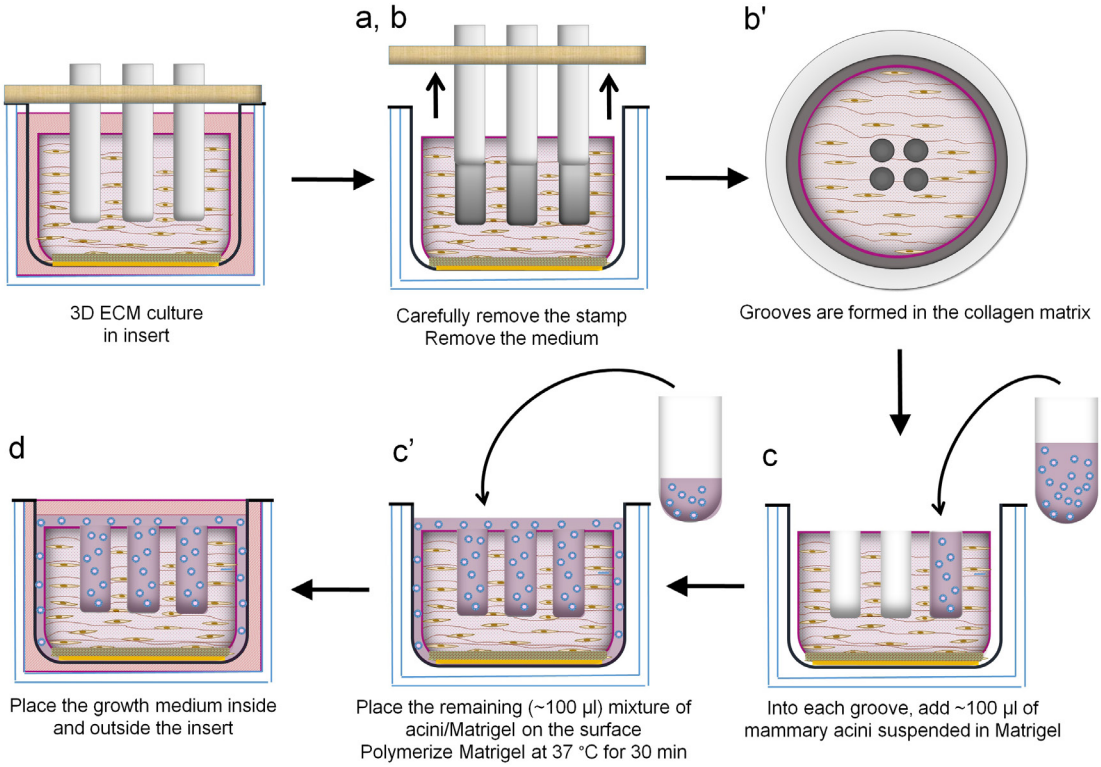

Figure 5. Adding mammary acini/Matrigel mixture to the collagen matrix

Day 14

7. Harvesting the co-culture for paraffin-embedding
   a. Fix the co-cultures with 4% paraformaldehyde at 4 °C for 2~3 h. Wash the cultures in PBS at RT for 10 min.
      Note: Do not fix for too long. Over fixation will destroy certain antigens.
   b. Flip the insert and cut out the membrane. Gently push out the co-culture from the insert into the well plate. Add PBS to cover the culture.

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c. Place a Bio-Wrap inside a tissue cassette. Position the co-culture face-down on the Bio-Wrap. Fold the Bio-Wrap to wrap the co-culture and close the cassette.
d. Put the cassettes in 70% EtOH and keep at 4 °C until processing for paraffin-embedding.
e. Section paraffin-blocks at 10 µm and analyze the sections by immunohistochemistry (Figure 3, bottom right).

Recipes

1. MCF10A additives

Reagent	Final Concentration
Horse serum	5%
Penicillin/streptomycin	1%
Insulin	10 µg/ml
EGF	20 ng/ml
Hydrocortisone	0.5 µg/ml
Cholera toxin	100 ng/ml

2. HMT-3522-S1 additives

Reagent	Final Concentration
Prolactin	5 µg/ml
Insulin	250 ng/ml
EGF	5 ng/ml
Hydrocortisone	1.4 µM
β-estradiol	0.1 nM
Sodium selenite	2.6 ng/ml
Transferrin	10 µg/ml

3. Phosphate buffered saline (PBS)

Reagent	Final Concentration
NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	1.8 mM

Adjust pH to 7.4 and autoclave to sterilize (121 °C, 15 min)
Store PBS at 4 °C

4. Acellular layer matrix (Final concentration of 1 mg/ml collagen, total 1.8 ml for 6 samples)

Reagent	Volume
10x DMEM/F12	180 µl
FBS	18 µl
L-Glutamine	18 µl
Na-bicarbonate	18 µl

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Type I collagen (2 mg/ml, pH 7.4)	900 µl
Sterile water	Up to 1.8 ml

Add in this order. Keep the mixture on ice until use. Mix gently and readjust the pH to 7.4 with sterile 1 M NaOH

5. Cellular layer matrix (Final concentration of ~1 mg/ml collagen + ~3 mg/ml Matrigel, total 10 ml for 6 samples)

Reagent	**Volume**
10x DMEM/F12	1.0 ml
FBS	100 µl
L-Glutamine	100 µl
Na-bicarbonate	100 µl
Type I collagen (2 mg/ml, pH 7.4)	5.0 ml
Readjust the pH to 7.4 with sterile 1 M NaOH.	
Matrigel	2.5 ml
Fibroblasts (1 x 10^8/ml)	1.0 ml
Sterile Water	Up to 10 ml

Add in this order. Keep the mixture on ice until use

6. Paraformaldehyde (4%, 1 L)
   a. Add 800 ml of 1x PBS to a glass beaker on a stir plate and heat to ~60 °C while stirring
   b. Add 40 g of paraformaldehyde powder and stir
   c. Add 1 N NaOH dropwise until the solution becomes clear
   d. Once the paraformaldehyde is dissolved, cool down the solution to the room temperature
   e. Recheck the pH, and adjust it with 1 N HCl or NaOH to ~pH 6.9
   f. Adjust the volume of the solution to 1 L with 1x PBS
   g. Store the solution at 2-8 °C for up to one month

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Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics

All animal experiments conformed to The Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, D.C., 2010) and were performed with the approval of the Institutional *Animal* Care and Use Committee of the University of Toledo, Toledo, OH (Protocol No: 108658).

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