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Running head: Organotypic 3D assay for primary human mammary epithelial cells.

Title: An organotypic 3D assay for primary human mammary epithelial cells that recapitulates branching morphogenesis.

Summary

We have developed a three-dimensional organotypic culture system for primary human mammary epithelial cells (HMECs) in which the cells are cultured in free floating collagen type I gels. In this assay, luminal cells predominantly form multicellular spheres, while basal/myoepithelial cells form complex branched structures resembling terminal ductal lobular units (TDLUs), the functional units of the human mammary gland *in situ*. The TDLU-like organoids can be cultured for at least three weeks and can then be passaged multiple times. Subsequently, collagen gels can be stained with carmine or by immunofluorescence to allow for the analysis of morphology, protein expression and polarization and to facilitate quantification of structures. In addition, structures can be isolated for gene expression analysis. In summary, this technique is suitable for studying branching morphogenesis, regeneration and differentiation of human mammary epithelial cells as well as their dependence of mechanical environment.

Key words

Primary human mammary epithelial cells, branching morphogenesis, organoid, 3D assay, regenerative potential, collagen gel, mechanotransduction, Forskolin, Rho-associated protein kinase

1. Introduction

The mammary gland (MG) is a unique characteristic of mammals. Its function is to produce milk following pregnancy for the nursing of offspring. The mature human MG consists of a network of branched ducts that terminate in alveoli-like clusters, the terminal-ductal lobular units (TDLUs) (1, 2). In the human MG, the TDLUs are surrounded by collagen-rich stroma that contains fibroblasts and immune cells. The epithelial ducts and alveoli contain two major cell lineages, a basal layer of myoepithelial cells that contract and thereby excrete the milk, and a second layer of cells surrounding the lumen of the ducts, the luminal cells, which produce milk. A unique characteristic of the MG is that most of its development takes place postnatally: under the influence of steroid hormones during puberty, a rudimentary ductal tree grows out into the concomitantly developing fat pad, undergoing branching morphogenesis through duct elongation and side-branching (3). Taken together, the function of the MG derives directly from its branched morphology: differentiation of both myoepithelial and luminal cells depend on their respective *in situ* localization. Moreover, an increasing loss of glandular, branched morphology is a hallmark of tumor progression. Therefore, we reasoned that organotypic assays to model pubertal MG development need to recapitulate branching morphogenesis.

Consequently, it is apparent that culture of human mammary epithelial cells (HMECs) in standard two-dimensional conditions does not allow for the analysis of morphological aspects and limits assessment of functional features. However, in contrast to mouse mammary epithelial cells, HMEC do not recapitulate branching morphogenesis in the commonly used three-dimensional Matrigel *in vitro* assay.

We have developed an organotypic assay for HMEC that recapitulates branching morphogenesis of the breast (Figure 1, 2). This assay is based on a protocol for free-floating collagen gels described to enable breast cancer cell lines to generate tube-like structures (4).

In our protocol, the addition of Forskolin is critical to enable the generation of TDLU-like organoids (5). Forskolin is a direct agonist of adenylyl cyclase, thereby increasing intracellular cAMP levels (6). Moreover, we add the Rho-associated protein kinase (ROCK) inhibitor Y-27632, for the initial plating of cells, which promotes survival.

Taken together, our protocol enables single primary HMEC to generate both spheres and complex branched structures. Specifically, by using published cell surface markers for fluorescent-activated cells sorting (FACS), we determined that luminal cells predominantly generate spheres while basal/myoepithelial cells generate complex branched structures (Figure 3). This is in line with the finding that in transplantation assays using mouse mammary epithelial cells, regenerative capacity is enriched in myoepithelial cells (7-9).

These basal/myoepithelial cell derived structures resemble the TDLUs of the MG: they contain p63-positive cells (p63, a p53 homologue, is a selective nuclear marker of myoepithelial cells of the human breast) at a basal position (10) that secrete laminin-1 to generate a basement membrane-like extracellular structure. On top of these p63-positive cells are one or more layers of p63-negative cells, many of which express the luminal markers Gata-3 and ZO-1 (11) (Figure 4). The degree of lumen-formation varies between donors and also depends on time in culture. 10-12 days after initial plating of single cells, the p63-positive, myoepithelial cells within the TDLU-like structures spontaneously contract the collagen gel in which they are embedded. This can be observed by shrinking of the gels circumference. Thereby, the contractile functional features of basal/myoepithelial cell *in vivo* are recapitulated. Importantly, free-floating collagen

gels represent a compliant matrix that closely resembles the mechanical properties of the human MG extracellular matrix (12). These mechanical properties are crucial to obtain TDLU-like organoids. If the collagen gels are left attached to the bottom and sides of the well, the myoepithelial cells within the branched structures are unable to contract the gels. As a consequence, both luminal differentiation, as gauged by the appearance of Gata-3 positive cells at a luminal position, as well as the formation of alveoli-like structures at the tip of the ducts are inhibited.

The organoids obtained in this assay can be cultured for at least three weeks and passaged multiple times. The structures within the gel can be visualized by carmine staining for morphological analysis and quantification. Immunofluorescence stainings allow for the analysis of protein expression and polarization. In addition, cells can be isolated for gene expression analysis.

In summary, the assay allows for the analysis of branching morphogenesis, the quantification and characterization of regenerative cells that give rise to branched TDLU-like structures and the influence of the mechanical environment on cell function and differentiation.

2. Materials

2.1 Coating components

1. Silicone coating reagent: 2.5% siloxane in n-Heptane. Add 2.5 mL of 1,7-Dichloro-1,1,3,3,5,5,7,7-octamethyltetrasiloxane to 1L of n-Heptane. Swirl to mix. Close bottle tightly and store in a dry, well-ventilated space at room temperature.
2. Phosphate buffered saline (PBS).
3. Ultrapure water (Milli-Q = MQ).

4. 24-well polysterene tissue culture plate.

2.2 Collagen gel preparation, culture and passaging components

1. Cells: Cryopreserved primary human mammary epithelial cells. The cells are commercially available from different providers or can be isolated from reduction mammoplasties as described in (5, 13).
2. Neutralizing solution: 550 mM HEPES in 11x PBS. Weigh 5.25 g of PBS powder and 6.55 g of HEPES into a beaker. Dissolve in approximately 40 mL of MQ. Adjust pH to 7.4 using NaOH. Add MQ to 50 mL. Filter sterilize, then aliquot into Eppendorf tubes and store at -20°C. Aliquots in use can be stored at 4°C.
3. MECGM basic medium: Mammary Epithelial Cell Growth Medium (MECGM, PromoCell), 1% penicillin/streptomycin (10,000 units/mL penicillin and 10 mg/mL streptomycin stock).
4. 10x initial plating medium: MECGM basic medium, 5% FCS, 100 µM Forskolin, 30 µM Y-27632 (see Note 1, 2).
5. 10x passaging medium: MECGM basic medium, 100 µM Forskolin, 30 µM Y-27632 (see Note 3).
6. Maintenance medium: MECGM basic medium, 10 µM Forskolin.
7. Collagen type I from rat tail (BD Biosciences)
8. Digestion medium: MECGM basic medium, 300 U/mL collagenase (see Note 4)
9. 40 µm cell strainer
10. 0.15% Trypsin, pre-warm in waterbath at 37°C before use.

2.3 Fixation, immunofluorescence and carmine staining components

1. Permeabilization solution: 0.2% Triton X-100 in PBS. Dilute 100 µL Triton X-100 in 50 mL PBS (see Note 5).
2. PFA fixation solution: 4% PFA in PBS
3. 0.15 M glycine in PBS
4. Antibody buffer solution: 0.1% BSA in PBS. For a 1% BSA stock solution, dissolve 0.5 g BSA fraction V in 50 mL of PBS, sterile filter, and store at -20°C. For a 0.1% BSA solution, dilute the stock 1:10 in PBS.
5. Blocking solution: 10% goat or donkey serum and 0.1% BSA in PBS. Add 1 mL of goat or donkey serum and 1 mL of 1% BSA stock to 8 mL of PBS (see Note 6).
6. Primary antibody solution: Prepare 250 µL of primary antibody solution per gel by adding the desired volume of primary antibody to antibody buffer solution (see Note 7).
7. Secondary antibody solution: Prepare 250 µL of secondary antibody solution per gel by adding the desired volume of secondary antibody to antibody buffer solution (see Note 8).
8. DAPI staining solution: 167 ng/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS (see Note 9).
9. Mounting medium suitable for immunofluorescence.
10. Clear nail polish.
11. Carmine Alum staining solution: Weigh 1 g of Carmine and 2.5 g of Aluminum Potassium Sulfate and place in a beaker. Add 500 mL of distilled water. Boil for 20 min while mixing with a magnetic stirrer. Adjust final volume to 500 mL with distilled water. Filter sterilize, add a crystal of thymol for preservation. Store at 4°C.

12. Tweezers.

3. Methods

The methods described below are optimized for 24-well cell culture plates. We have also successfully used other formats like 48-well and 6-well cell culture plates by simply adjusting the used volumes. All centrifugation steps are performed at 500 x g at 4°C for 5 min. Cells are incubated at 37°C in the presence of 5% CO_2 and 3% O_2.

3.1 Silicone coating of cell culture plates

1. In a sterile flow cabinet, transfer 1 mL of fresh coating solution to the first well of a 24-well cell culture plate using a Pasteur pipette. Wait for 10 seconds, then transfer coating solution to the second well, wait for 10 seconds and proceed in this manner until the whole plate is coated (see Note 10, 11).
2. Remove the remaining coating solution with the Pasteur pipet, and transfer to designated waste container (see Note 11).
3. Rinse each well with 1 mL of PBS.
4. Rinse each well with 1 mL of MQ.
5. Open the lid of the plate and allow the wells to dry. Plates can be used immediately or sealed with parafilm, wrapped in cling wrap and stored at 4°C until usage (see Note 12).

3.2. Determination of final volumes of collagen solution, neutralizing solution and cell suspension

1. Based on the concentration of the collagen I stock solution, determine the volume of collagen I stock solution needed to make a 400 µL collagen gel with a final collagen concentration of 1.3 mg/mL.
2. Calculate the amount of neutralizing solution which is $1/10^{th}$ of the volume of collagen stock solution.
3. The remaining volume consists of cell suspension with the desired amount of cells in MECGM basic medium (see Table 1).
4. Multiply determined volumes with the number of gels you wish to produce, add volume for one extra gel to account for fluid loss due to the viscosity of collagen.

3.3 Plating cells into floating collagen gels

Until polymerization of collagen, all steps are carried out at 4°C or on wet ice unless otherwise described.

1. Place collagen stock solution on ice and bring neutralizing solution to room temperature to allow for complete dissolution of salts. If necessary vortex until all crystals have resolved.
2. Quickly thaw primary human mammary epithelial cells in a water bath at 37°C.
3. Take up cell suspension in MECGM basic medium.
4. Filter cell suspension using a 40 µm strainer, to remove residual fragments and cell aggregates.

   Spin down cell suspension, remove supernatant and resuspend the cells in the desired volume of MECGM basic medium (determined in Section 3.2). For bulk HMEC, we

recommend cell densities of 2000-3000 cells per 400 µL of collagen gel to obtain 2-6 TDLU-like organoids (see Note 13)

5. The following steps should be performed quickly in order to prevent premature polymerization of collagen: Add the desired volume of neutralizing solution (determined in Section 3.2) to the cell suspension and mix well by pipetting.
6. Add according amount of collagen stock solution (determined in Section 3.2) and mix well by pipetting without introducing any bubbles.
7. Immediately transfer 400 µL of the mixture to one well of the silicone-coated 24-well cell culture plate (prepared in Section 3.1). Repeat to plate desired number of gels.
8. Carefully tilt the plate to ensure even coverage of the wells. Place the plate in an incubator at 37°C for 1 h to allow for polymerization of collagen.
9. Add 500 µL of MECGM basic medium and 100 µL of 10x initial plating medium to each well (total volume per well including 400 µL of collagen gel volume: 1 mL).
10. Encircle the gels using a small pipet tip and carefully shake the plate to detach the gels from the bottom of the culture dish. Place the plate back in incubator.
11. Structures start to form at about 7 days of culture. Structures grow and expand in size until about 14 days of culture. After this, the size of structures does not increase but organoids continue to differentiate, form alveoli like buds and contract the gels. Feeding and passaging of cells is described in Section 3.4 and 3.5. For analysis of organoids see Section 3.6 – 3.8.

3.4 Feeding of cells in floating collagen gels

After 5 days of culture the initial plating medium is replaced by maintenance medium. After this, fresh maintenance medium is added every 2-4 days, depending on the growth rate of primary mammary epithelial cells and on the number of cells seeded per well.

1. To remove medium, carefully hold back the floating collagen gel to one side of the well using a small pipet tip.
2. Take a 1000 µL pipet in the other hand and remove the medium from the opposite side of the well.
3. Carefully add 600 µL of fresh maintenance medium to the well. Make sure not to pipet the medium on the gel directly, to avoid damaging it.

3.5 Passaging of cells in floating collagen gels

1. Preheat water bath to 37°C. Prepare a tube containing digestion medium (1 mL digestion medium per collagen gel).
2. Use sterile tweezers to transfer the collagen gels from the cell culture plate into the digestion medium.
3. Put the tube into the preheated water bath for around 30 min up to 1 h. Occasionally check dissolution of collagen gels and vortex briefly to ensure even digestion of collagen gels.
4. Spin down cells and remove supernatant.
5. Resuspend the pellet in 0.15% pre-warmed trypsin (use 1.5 mL trypsin for a pellet of five digested collagen gels) and mix well by pipetting for about 3 min.
6. Add trypsin-neutralizing solution (for the required volume of trypsin neutralizing solution refer to your manufacturer's manual).

7. Filter cell suspension through a 40 µm mesh to get rid of any residual clumped cells. If desired, cells can now be counted.
8. Spin down cell suspension, remove supernatant and resuspend the cell pellet in desired volume of maintenance medium.
9. Cells can now be plated into floating collagen gels as described in Section 3.3. Use 10x passaging medium instead of 10x initial plating medium and switch to maintenance medium after 5 days of culture.

3.6 Immunofluorescent staining of breast epithelial cells cultured in floating collagen gels

All steps are carried out at room temperature unless otherwise described. Between all incubation and washing steps, carefully remove the solutions with a 1000 µL pipet while holding back the gel with a 200 µL pipet as described above. Perform all fixation, permeabilization, blocking, washing and staining steps on an orbital shaker (see Note 14).

1. To pre-digest collagen, pre-warm collagen digestion solution at 37°C in water bath and prepare a container with wet ice.
2. Remove the medium from the collagen gels, add 1 mL of digestion medium to each collagen gel and place the plate back into the incubator for 5 min.
3. Put the plate on ice to stop collagenase digestion immediately.
4. Carefully remove the digestion medium and wash with 1 mL of PBS (see Note 15).
5. To fix collagen gels, add 1 mL of PFA fixation solution to each well and incubate for 15 min.
6. Remove PFA fixation solution and wash the gels in 1 mL of PBS for 10 min.

7. Add 1 mL of 0.15 M glycine to each gel and incubate for 10 min to quench the paraformaldehyde.
8. Wash again with 1 mL of PBS for 10 min. Collagen gels can now be stained directly or stored in PBS at 4°C for several months. (see Note 16).
9. To permeabilize the gels, add 1 mL of permeabilization solution to each well and incubate for 10 min.
10. Wash the gels in 1 mL of PBS for 10 min.
11. Next, add 1 mL of blocking solution and incubate at 4°C overnight.
12. Remove blocking solution from collagen gels and wash with 1 mL of PBS for 10 min (see Note 17).
13. Add 250 µL of primary antibody solution (see Note 7, 18) to each collagen gel and incubate at 4°C overnight (see Note 19).
14. Wash collagen gels three times with 1 mL PBS for 10 min to remove unbound primary antibodies.
15. Add 250 µL of secondary antibody solution (see Note 8, 20) to each collagen gel and incubate in the dark for around 3 hours. From this point on, exposure to light should be avoided (e.g by wrapping the plate in aluminum foil).
16. Wash collagen gels two times with 1 mL of PBS for 10 min.
17. Add 250 µL of DAPI staining solution to each collagen gel and incubate for 2 min.
18. Wash three times with 1 mL of PBS for 10 min and two times with MQ for 5 min.
19. For mounting, transfer the gel onto a microscope glass slide using tweezers, spread the gel out with a pipet tip, and carefully soak up excess water with a tissue.

20. Add approximately 2 drops of mounting medium on top of the gel (see Note 21) and lay a coverslip on top without introducing any bubbles (see Note 22).
21. Let the mounting medium dry at room temperature overnight (see Note 23).
22. Seal the rims of the coverslip with colorless nail polish as soon as the mounting medium has dried.

3.7 Carmine staining of breast epithelial cells cultured in floating collagen gels

Carmine Alum solution stains cellular structures red and thereby allows better visualization.

All steps are carried out at room temperature unless otherwise described. Between all incubation and washing steps, carefully remove the solutions with a 1000 µL pipet while holding back the gel with a 200 µL pipet. Perform all fixation, permeabilization, blocking, washing and staining steps on a shaker (see Note 14).

1. Fix structures as described in step 3.6 (see Note 24).
2. Add 1 mL Carmine Alum staining solution to each gel and incubate plate on a shaker overnight at room temperature
3. The next day, use tweezers to transfer the gels to a glass microscopy slide (see Note 25). Using the tweezers, make sure that the gels lay flat and in the middle of the microscopy slide. Remove excess fluid with a tissue.
4. Mount as described in 3.6

3.8 Cell lysis for RNA extraction

1. Digest collagen gels as described in section 3.5.
2. After the gels have dissolved, fill up the tube with PBS and centrifuge.

3. Wash the pellet with PBS and centrifuge again.
4. Use the pellet for your regular RNA isolation protocol (see Note 26).

4. Notes

1. 10x initial plating medium is prepared for easier handling. The resulting concentration in cell culture (1x initial plating medium) is 0.5% FCS, 10 µM Forskolin and 3 µM Y-27632 in MECGM basic medium.
2. We recommend making 10 mM Forskolin and 10 mM Y-27632 stock solutions in DMSO. These can be stored in small aliquots at -20°C.
3. 10x passaging medium is prepared for easier handling. The resulting concentration in cell culture (1x passaging medium) is 10 µM Forskolin and 3 µM Y-27632 in MECGM basic medium.
4. Preparation of collagenase stock: We recommend preparing a 100x collagenase stock. For this, get the collagen digestion unit (CDU) of your collagenase from the data specification sheet and dilute the collagenase in PBS to a final stock concentration of 30.000 CDU/mL.
5. For pipetting viscous Triton X-100, cut off the end of pipet tip.
6. Use serum from the same species as the secondary antibody.
7. Use antibody concentrations recommended by the manufacturer or determine the optimal concentration empirically; 1:100 is often a good starting point.
8. Use antibody concentrations recommended by manufacturer or determine the optimal concentration empirically; 1:250 often is a good starting point.

9. Prepare 5 mg/mL DAPI stock solution in sterile H_2O, keep at 4°C and protect from light. To get DAPI staining solution, add 0.33 µL of stock solution to 10 mL PBS, keep at 4°C and protect from light.
10. We suggest transferring 1mL of coating solution from well to well, as this saves a lot of coating solution. Theoretically, more than one 24-well plate can be coated with the described volume. To save time, it is also possible to coat more than one well at a time. For instance, add 1mL of coating solution to four wells each, wait for 10 seconds and then transfer the coating solution to the next four wells. As there is loss of coating solution with every well coated, one might have to add fresh coating solution for any additional plates.
11. The siloxane coating solution is flammable and causes burns. Therefore, wear protective clothing when handling. Refer to safety data sheet for correct disposal.
12. The siloxane coating solution reacts with plastic and forms a hydrophobic surface. With this coating, collagen hydrogels are much easier to detach and are less likely to get damaged by detachment.
13. Importantly, this approach does not guarantee clonality. However, at densities of 100-500 cells, most organoids are clonal. The enrichment of myoepithelial cells with the capacity to generate TDLU-like organoids and limiting dilution assays to determine the frequency of these cells are described in Linnemann et al.
14. Do not use a vacuum pump or an electric pipet aid to remove fluids as the gels might be aspirated.
15. After the collagenase-predigest and before fixation the gels are very fragile and slippery and tear easily! Be very careful when removing collagenase solution and

PBS. Also, in order to better visualize the pre-digested gels, this step can be done on the bench where one can look into the well from a closer distance. Visibility of the gel is also promoted by placing the wells on a dark surface.

16. For storage, plates should be sealed with parafilm and cling wrap to avoid dehydration.
17. If desired, the collagen gels can now be cut into smaller pieces using a scalpel. This allows for more antibody combinations for one collagen gel.
18. Not all antibodies recommended for immunofluorescence work in 3D staining. The suitability of each antibody has to be determined by the experimenter. For a list of suitable antibodies, refer to Linnemann et al. (5).
19. If primary antibodies are directly conjugated to a fluorophore, the plate should be wrapped with aluminum foil to avoid bleaching and to ensure high quality of staining.
20. Phalloidin for visualization of F-actin can be added at this point
21. When mounting multiple gels on one glass slide, we usually place an additional drop of mounting medium between the gels.
22. We do this by carefully putting one side of the coverslip on the microscope slide and then slowly lowering the coverslip. This prevents the formation of bubbles.
23. Alternatively, mounting medium can be dried in the fridge, which takes about 2-3 days. We have the impression that this longer process of drying reduces the formation of bubbles.
24. For the carmine alum staining gels do not need to be pre-digested.
25. Be careful not to break the gels. We use tweezers like a shovel in order not to squish the gels.

26. We usually take up pellet in RLT lysis buffer (RNeasy mini kit, Qiagen) containing beta-mercaptoethanol, mix by pipetting ten times and vortex for 30 sec. Next, the lysates are frozen at -80°C for at least 1 h or until further usage. After thawing, the lysate is given on QIAshredder and centrifuged at max speed for 2 min. The flow through is used for isolation of RNA using the RNeasy mini kit (Qiagen).

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Figure legends:

Figure 1: Workflow for the generation of floating collagen type I gels. First, cell suspension, neutralizing solution and collagen type I are added to a 15 mL conical on ice. Mind the correct order and mix by thoroughly pipetting up and down after every step. Next, the mix is transferred to a silicon-coated culture dish and incubated at 37°C. After one hour, medium is added and gels are detached by encircling the gel with a pipet tip and shaking the plate carefully.

Figure 2: Development of a TDLU-like branched structure. Bright-field images were taken starting at day 9 of culture and then every 24 hours. Scale bar: 200 µm.

Figure 3: Typical luminal cell (left) and basal cell (right) derived structures stained with carmine. Scale bar: 200 µm.

Figure 4: Confocal microscopy of branched structures stained by immunofluorescent antibodies. (a) The presence of the basement membrane component laminin and expression of its receptor integrin-α6 indicate deposition of a basement membrane. (b) Branched structures show expression of luminal cell marker ZO-1 and basal cell marker p63 at correct positions. Scale bars: 50 µm.

Table legends:

Table 1: Example for volumes used to make one or five collagen gels, assuming a collagen type I stock concentration of 4.04 mg/mL. Figures in µL.

Tables:

Table 1

	1 collagen gel	**5 collagen gels**
Collagen I stock solution	128.71	643.55
Neutralizing solution	12.87	64.36
Cell suspension	258.42	1292.10
Total volume	400	2000