Breast cancer Now cell bank- Epithelial cells

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The Breast Cancer Now tissue bank takes tissue from patients with varying disease status, we can provide epithelial cells from:

Normal reduction mammoplasty

Prophylactic mastectomy (removal of breast tissue due to family history and/or BRCA 1/2 genes)

Contralateral- prophylactic (following tumour in 1 breast worried about spread or new tumour in other), mastectomy (following removal of tumour in 1 breast even up for appearance).

Ductal Carcinoma In-Situ- pre-invasive cancer (very limited numbers, possibly mix of normal and DCIS cells)

The tissue, with informed consent from patients, is assessed by a pathologist who will ascertain what is required for diagnostics and supply us with the excess material.

Before the cells reach you:

Isolation

All tissue on arrival is washed once with ethanol (if big enough) and 3 times with RPMI-1640 media plus 25mM Hepes, supplemented with 5% foetal bovine serum (FBS), penicillin (100U/ml), streptomycin (0.1mg/ml), amphotericin-B (5µg/ml) and Gentamicin (20 µg/ml). After washing the tissue is measured.

Tissue is chopped in to small pieces and is digested for 12 to 16 hours at 37˚C in RPMI-1640 medium plus 25mM Hepes, supplemented with 5% foetal bovine serum (FBS), penicillin (100U/ml), streptomycin (0.1mg/ml) and amphotericin-B (5µg/ml) containing 1mg/ml collagenase 1A and hyaluronidase on a rotary shaker. The digested tissue was centrifuged at 380g for 20 minutes and washed in medium three times to remove enzymes. The tissue isolates were then sedimented three times at 1g for 30 mins to collect the denser organoids (ductal tree containing TDLUs and ducts). The organoids were centrifuged (380g x 3minutes), at this point organoids can either be frozen or specific cell isolation can be done.

The organoids, either fresh from tissue digestion or frozen are digested using trypsin, and DNAse is added to ensure the cells are single. The cells are counted before adding fluorescently conjugated antibodies to EPCAM for epithelial cells and CD10 for myoepithelial cells. A FACS machine is then used to separate the EPCAM+ cells (epithelial cells), CD10+ cells (myoepithelial cells) and double negatives (microenvironment cells). The fraction containing epithelial cells is then centrifuged (380g x 3minutes) and the cell pellet re-suspended and cultured in DMEM:F12 supplemented with 10% FBS, hydrocortisone, transferrin, insulin, EGF, penicillin/streptomycin and amphotericin-B (BCM), on collagen coated tissue culture plates.

Epithelial cells are grown for 1 passage and frozen down at 2 different densities to allow flexibility of cell number for requests. Cells are stored in liquid nitrogen before being sent to you on dry ice. If you are not culturing straight away, on arrival cells should be kept in liquid nitrogen.

Each cell line is cultured separately and a laboratory management system is in place to ensure there is no cross contamination. Previously we have both stained via immunocytochemistry a number of

our cultures, and probed via western blotting for various epithelial markers including EMA and CK8 and CK14 (Gomm et al, Anal Biochem. 1995 Mar 20;226(1):91-9. Gomm, et al J Cell Physiol. 1997 Apr;171(1):11-9). Due to the consistency seen across these markers and in the cells grown we are confident of the cell type. The cells are also checked for mycoplasma by PCR on the cell culture media.

Media Recipes

BCM

Make up 500ml of BCM by adding penicillin (100U/ml), streptomycin (0.1mg/ml) (pen/strep Sigma P4333), 2.5µg/ml amphotericin-B (Fungizone Sigma A2942), 50ml FBS, 0.5µg/ml hydrocortisone (H0888 Sigma), 10µg/ml apo-transferrin (T1147 Sigma), 10ng/ml EGF (E9644 Sigma), 5µg/ml insulin (I9278 Sigma) to 500ml DMEM:F12 (D8437 Sigma, already contains glutamine).

To buy for media:

Base medium: DMEM:F12 with 15mM Hepes Sigma D8437

Plus the following additives:

Hydrocortisone (H0888 Sigma)

1g stored at room. temp.

Weigh out approx. 10mg into an eppendorf tube and dissolve in 1ml of absolute ethanol. Transfer to 50ml tube.
Wash out eppendorf with DMEM:F12 and top up to 20ml with same to give a conc. of 0.5mg/ml.
Filter through 0.22 filter and aliquot in 500µl aliquots to add to 500ml to give working strength of 0.5µg/ml.
Store aliquots at -20°C.

Apo-transferrin (T1147 Sigma)

100mg stored at 4°C.

Dissolve whole amount in 10ml DMEM:F12 by adding medium directly to bottle.
Filter sterilise. This gives a stock concentration of 10mg/ml. Aliquot in 500µl aliquots to add to 500ml to give working conc. of 10µg/ml.
Store aliquots at -20°C.

Human Insulin (I9278 Sigma)

5ml solution at 10mg/ml stored at 4°C.
Add 250µl to 500ml medium to give working conc of 5µg/ml.

Human EGF (E9644 Sigma)

200µg human recombinant EGF. Lyophilised from PBS. Stored at -20°C.
Need to make up 2 solutions before reconstituting EGF:

1. 10mM acetic acid by adding 6µl glacial acetic acid to10ml sterile water.
Add 100µl FBS (1%) and filter through 0.22 filter.

2. 20ml DMEM:F12 + 200µl FBS (1%).
Filter through 0.22 filter.

Resuspend EGF by adding 1ml of 10mM acetic acid directly to tube and transfer to 50ml tube.
Rinse out original tube several times with DMEM:F12/1%FBS.
Top up to 20ml with same. This is 10µg/ml stock.
Do not filter EGF solution.
Aliquot in 500µl aliquots to add to 500ml medium to give working conc. of 10ng/ml.
Store aliquots at -20°C.

Amphotericin-B (Fungizone Sigma A2942)
Aliquot in 5ml aliquots. Add 5ml to 500ml BCM.

Quick guide for collagen coating of tissue culture plasticware.

For epithelial cells we collagen coat all plasticware prior to adding cells as it helps with attachment of cells.

Firstly make up 0.02M acetic acid by adding 60µl glacial acetic acid(A6283 Sigma) to 50ml of sterile PBS.
Filter sterilise through 0.22 filter. This is what you will dilute the collagen in.

The stock collagen I solution is 3.6mg/ml or 3.6 µg/µl.
(Rat tail. BD Biosciences 354236. 100mg)
6-10µg/cm^2 is the collagen density recommended.

So first you need to know the cm^2 of your plasticware.
For a T25 flask it is 25cm^2.
For a 24 well plate it is 2cm^2/well. So that is 48cm^2/plate in total.
For a 6 well plate it is 9.5cm^2/well. So that is 57cm^2/plate in total.

Then decided on 10µg/cm^2.
So for T25 = 250µg
For 24 well plate = 480µg
For 6 well plate = 570µg

Then divide by 3.6 to give you the total number of µl required.
For T25 – 250/3.6 = 69.444
For 24 well plate – 480/3.6 = 133.333
For 6 well plate 570/3.6 = 158.333

Round up for ease of remembering:

T25	70µl
24 well plate	140µl
6 well plate	160µl

Volume for even coating:

T25	5ml	
24 well plate	1ml/well	24ml/plate
6 well plate	2ml/well	12ml/plate

Plasticware is incubated for at least 1 hour in incubator at 37°C.

After incubating plate, remove collagen and wash wells twice with sterile PBS and leave wells in same. Place plate back in incubator.

When cells are ready remove PBS and replace with cells.

Reviving epithelial cells

Keep the sample on dry ice until ready to thaw. Thaw as quickly as possible using hands to warm the sample. When just starting to melt, slowly add room temp medium dropwise to the sample. Decant by pouring into 15ml tube containing 5mls of fresh medium. Wash out the cryotube with medium to collect any cells left behind. Spin the cells at 380g for 5mins. Remove supernatant and re-suspend the cells in a small volume of BCM medium, using a 200µl Gilson pipette set at 180µl. Viability and cell numbers can be checked at this point (Number of cells before freezing is on the vial and the information sheet attached when cells sent. Viability can vary). Make cell volume up to required level with more medium and seed wells or flask as required. Our incubators are set at 5% CO_2.

Seeding density will depend on how soon you require the cells. We routinely use:

70,000-100,000/well for 6 well plate.
~15,000-20,000/well for 24 well plate.
3,000-5,000/well for 96 well plate.

Culturing epithelial cells

Medium is changed twice a week. Leave a small amount of conditioned medium (0.5ml) in the well or flask and make the volume back up with warmed BCM.

Grow until almost confluent. These cells will compact as they become more confluent and ridges may form between colonies. Small numbers of cells may then detach.
If you see anything that doesn't look right e-mail the cell team and they'll attempt to advise, adding a picture to this is particularly useful.

To passage

We recommend using the passage 1 epithelial cells directly in your experimental scheme.
However, If passaging is required, then proceed as follows (volumes are per well of a 6 well plate, alter as required).
Wash the cells with 2mls PBS, remove and add 1.5ml 1 x trypsin*(see below). Incubate at 37°C until the cells lift off. Trypsinising can vary so check every couple of minutes. Some cells will come off quickly (5-7 minutes), others take longer (10-15 minutes). Epithelial cells should not be left in trypsin more than 20 minutes. Wash the wells twice with room temperature BCM. We suggest scraping the well to retrieve as many cells as possible. Spin down the cells at 380g for 5mins. Use a 200µl pipette set at 180µl to triturate the pellet before counting with trypan blue to check viability.

Suggested seeding density as in initial reviving (above).

*Trypsin/EDTA (Hyclone SV30031.01 Fisher Scientific HYC 020 010K)
0.25% trypsin plus 0.1% EDTA in PBS without calcium and magnesium.
Thaw and aliquot in 5ml aliquots.
Store at -20°C. Dilute this 1/5 in PBS w/o ca/mg (D8537 Sigma) to give a final concentration of 0.05% trypsin and 0.02% EDTA (1 x).

Freezing epithelial cells

Freezing medium
DMEM:F12 with 10% FBS, Pen/Strep, Fungizone (final conc 2.5 µg/ml)
Add FBS to a final concentration of 40% and 6% DMSO (Sigma D2650).
Kept in fridge in 50ml aliquots.

Passage the cells as normal and count with trypan blue. Spin down again and re-suspend in freezing medium. We freeze the myoepithelial cells down in a slightly higher cell number than required. So for 100,000 cells for an experiment we would freeze 120,000 cells and for 300,000 we freeze down 320,000 to 350,000 cells in 0.5ml aliquots. This allows for loss in freezing and thawing.

Place cryovials in a freezing jar as soon as possible and place in a -80°C freezer overnight before storing in LN_2. When freezing the cell samples, it is best to try and minimise the time they sit at room temp in the DMSO.