

**Breast cancer
Now cell bank-
Myoepithelial
cells**

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The Breast Cancer Now tissue bank takes tissue from patients with varying disease status. 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. We can provide myoepithelial 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)

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.

Organoids either fresh from tissue digestion or thawed from 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 myoepithelial cells were centrifuged (380g x 3minutes) and the cell pellets re-suspended and cultured in MEGM (Mammary Epithelial Growth Medium) consisting of a serum-free basal medium (HUMEC) supplemented with 5 µg/ml human insulin, 0.5 µg/ml hydrocortisone, 10ng/ml human Epidermal Growth Factor (EGF), and 52 µg/ml bovine pituitary extract, on collagen coated tissue culture plates.

Myoepithelial cells were grown for either 2 or 3 passages depending on growth requirements and frozen down at 2 different densities to allow flexibility of cell number for requests. Cells are stored in liquid nitrogen before being transferred to you on dry ice. If you are not culturing straight away, on arrival cells should be kept in liquid nitrogen until required.

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 myoepithelial markers including fibronectin, integrin β 4 and β 6, 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 in the cell type. The cells are also checked for mycoplasma by PCR on the cell culture media.

Media Recipes

MEGM

Make up 50ml of MEGM at a time because it is only used for myos and the base medium is expensive. To 50ml of HuMEC (Invitrogen, 12753-018) add 50 μ l hydrocortisone and EGF, 25 μ l insulin, 200 μ l BPE 100 μ l Fungizone and 10 μ l Gentamicin.

To buy for media:

Base medium: HuMEC (Invitrogen, 12753-018)

Plus the following additives:

Gentamicin (G1397 Sigma)

10ml solution stored at 4°C.

Amphotericin-B (Fungizone Sigma A2942)

Aliquot in 5ml aliquots. Add 100 μ l to 50ml MEGM.

Bovine pituitary extract (13028-014 Invitrogen)

25/26 mg in 2ml = 12.5/13mg/ml.

Thaw and aliquot into 100 μ l and 200 μ l aliquots.

Store at -20°C.

Add 200 μ l to 50ml HuMEC medium.

Final concentration = 50/52 μ g/ml.

Human Insulin (I9278 Sigma)

5ml solution at 10mg/ml stored at 4°C.

Add 250 μ l to 500ml medium to give working conc of 5 μ g/ml.

Human EGF (E9644 Sigma)

200µg human recombinant EGF. Lyophilised from PBS. Stored at -20°C.

[NOTE: We previously used to dissolve the EGF in DMEM:F12 containing 10% FBS (Sigma suggested 0.1 to 1% HSA or BSA or 1-10% FBS). But this was when it was lyophilised in acetic acid . Now it is in PBS so need to add acetic acid. However, decided to still include FBS at 1%.]

So need to make up 2 solutions before reconstituting EGF:

1. 10mM acetic acid by adding 6µl glacial acetic acid to 10ml 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.
(Probably should use a syringe!)

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.

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.

(Eg. 02/09/11. Weighed out 11.8 mg in 2ml eppendorf tube on microbalance. Dissolved in 1ml absolute ETOH. Transferred to 50ml tube. Washed out eppendorf and topped up to 23.6ml.)

Quick guide for collagen coating of tissue culture plasticware.

For myoepithelial 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/cm² is the collagen density recommended.

So first you need to know the cm² of your plasticware.

For a T25 flask it is 25cm².

For a 24 well plate it is 2cm²/well. So that is 48cm²/plate in total.

For a 6 well plate it is 9.5cm²/well. So that is 57cm²/plate in total.

Then decided on 10µg/cm².

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 myoepithelial 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 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 MEGM 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% CO₂.

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 myoepithelial 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 MEGM.

Grow until almost confluent. These cells will compact as they become more confluent and areas of compressed cells will appear. The cells should not detach unless they overgrow.

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

Usually only required for P2 cells, P3 cells should be used directly in experiments due to limited onward growth.

Volumes are per well of a 6 well plate, alter as required.

Wash the cells with 2mls PBS (without calcium and magnesium), remove and add 1.5ml 1 x trypsin* Incubate at 37°C until the cells lift off. Trypsinising can vary so check every couple of minutes. Myoepithelial cells round up quickly but do not detach until 5-7'. If very dense may take 10-12'. Myoepithelial cells should not be left in trypsin more than 15 minutes as they are very sensitive to enzyme treatment. Once the majority of the cells have detached the rest can be scraped. Wash the wells twice with room temperature MEGM. 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 myoepithelial cells

Freezing medium

Cell Freezing Medium-DMSO 1x (Sigma C6164). Aliquoted and frozen at -20°C in 5ml 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 130,000 cells and for 300,000 we freeze down 330,000 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 LN₂. When freezing the cell samples, it is best to try and minimise the time they sit at room temp in the DMSO.