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PREPARATION OF EPITHELIAL AND MESENCHYMAL STEM CELLS FROM MURINE MAMMARY GLAND

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Abstract

The mammary gland is a complex organ consisting of multiple cell types that undergo extensive remodeling during pregnancy and involution, cyclical changes that suggest the existence of a resident stem cell population that is responsible for this remarkable tissue regeneration. The basic functional unit of the mammary gland is the terminal duct lobular unit, which invades the stromal tissue (fat, connective tissue, blood vessels, etc.). Luminal epithelial cells line the ducts while outer myoepithelial cells secrete the basal lamina that separates the mammary gland parenchyma from the mesenchymal cells of the stroma. Within the epithelial cell population of the ducts resides the mammary gland stem cells and it is believed that this population is the origin of the mammary gland cancer stem cells as well. In the mouse, epithelial stem cells can be separated from mesenchymal cells on the basis of CD24, CD44 and CD49f expression. This allows for the determination of both normal and cancer stem cell potential of these two populations and permits investigation into their interaction in tumour development.

Keywords

Mammary gland (tumour); cancer stem cell; epithelial; mesenchymal; polyoma middle T antigen transgenic mouse

Unit Title: The separation of epithelial and mesenchymal stem cells from mammary gland tumours in order to examine their potential stem and accessory cell roles in cancer development.

Unit Introduction

The interest in mammary gland epithelial cells (indeed epithelial cells in many cancers) lies with accumulating evidence that it is these cells that play a significant if not predominant role in tumour development. One of the current theories on the origin of cancer in both the

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haematopoietic system and solid tumours implicates tissue-specific stem cells as the tumour initiating cells (Hermann *et al*, 2010). Breast, prostate and colon cancers are among those solid cancers in which the epithelial cell fraction has been shown to contain a cancer stem cell (population) that has self-renewing and tumour-initiating potential. This hierarchical model of cancer, as opposed to the stochastic model, supposes that only a small fraction of the tumour cells has the potential for tumour induction (Johnsen *et al*, 2009). The evidence is now very convincing that most cancers are clonal in origin and although the stem cells from which these clones derive may not be completely characterized, it is widely accepted that stem cells are the responsible cells (Sell, 2010).

The field of mammary gland physiology has been particularly fruitful in stem cell biology due to the historical interest in the remarkable cyclical changes of proliferation, lactation and involution that occurs in the breast tissue throughout life and pregnancy. Research in the 1950's and 1960's laid the groundwork for the exploration of epithelial stem cell function in the mammary gland (Deome *et al*, 1959; Daniel *et al*, 1968) and two seminal papers, indicating the ability of a single cell to regenerate the entire functional mammary gland of a mouse (Shackleton *et al*, 2006) and the presence of a highly enriched population of cancer-initiating cells in the CD44⁺CD24⁻ fraction of human breast cancer (Al-Hajj *et al*, 2003), have stimulated investigations into tissue-specific (cancer) stem cells. For recent reviews on cancer stem cells, see Sell, 2010, Shackleton, 2010 and Bohl *et al*, 2011 (Sell, 2010; Shackleton, 2010; Bohl *et al*, 2011).

BASIC PROTOCOL 1

Isolation and flow cytometric sorting of epithelial and mesenchymal stem cells from mammary gland tumours of PyVT mice

This descriptive approach to isolate epithelial and mesenchymal stem cells from mammary gland tumours is applicable to several different transgenic models of mammary gland cancer in mice, including the FVB/N-Tg(MMTV-PyVT)634Mul/J (PyVT; Jackson Labs stock number 002374) and the FVB/N-Tg(MMTVneu)202Mul/J (*neu*; Jackson Labs stock number 002376) models but each model has distinct characteristics. For example, female PyVT mice present mammary gland tumours at 6–8 weeks of age, while males will be 20–24 weeks of age before tumours of adequate size are manifest. Thus, in the PyVT mouse, the age of tumours has a high degree of correlation with sex. In the *neu* mouse, only females get mammary gland tumours and these do not occur until 32–36 weeks of age. As tumours increase in size, necrosis and haemolysis will occur within the tumours. IACUC guidelines will dictate how large tumours will be allowed to grow (typically 1.5–2 cm³) and although necrotic areas do not prevent isolation of usable cells, the younger and typically smaller the tumour, the greater the proportion of the tissue will be made up of solid tissue and viable cells, and so the better the yield.

Female PyVT mice do not lactate, so male hemizygous PyVT mice are bred with wildtype FVB females in order to maintain the line and because of this, the offspring must be genotyped. The primer sequences and the PCR protocol provided by Jackson Labs (from where the mice are obtained) work well. FVB mice are also used for clearance of fat pads (see Support Protocol 3) in preparation for transplant of potential mammary gland (tumour) stem cells and must be between 3 and 3 and 1/2 weeks of age at the time of surgery. Do not use older mice, as complete removal of all host epithelial cells is unlikely to be achieved. Both male and female *neu* mice will breed successfully, but breeding should be confined to mice of 2–4 months of age for best results, as both males and females show increasing rates of aggression with ageing and this will interfere in timely pregnancies.

If age or genealogy are not critical factors, the grossly dissected glands from 2–3 animals can be pooled. This may be required since in any one mouse, tumours at each gland can vary enormously in size at any particular time and not all tumours will yield useful tissue. The mouse mammary tumour virus (MMTV) promoter essentially confines tumour expression to the mammary gland, although metastasis to lungs does occur in 80–90% of females. Another FVB model, the *Wnt1* transgenic mouse (Jackson Labs stock number 002934), has also been used successfully to derive mammary gland cancer stem cells (Cho *et al*, 2008). Although other models with different promoters do exist, these are unlikely to yield mammary gland tumours with such high penetrance, and tumours at other sites may dictate sacrifice or death before mammary gland tumours are sufficiently large enough for cell recovery. The use of isolated epithelial and mesenchymal cells can be applied in various fields, including biochemical, developmental and genetic studies and is not limited to cancer, as the methods described below can be used in the isolation of cells from normal mammary glands. This report will describe the separation of mammary gland epithelial and mesenchymal cells, allowing for assessment of their individual actions and interactions.

Basic Protocol Materials List

Mice—FVB/N-Tg(MMTV-PyVT)634Mul/J (PyVT) (Jackson Labs stock number 002374). All mice should be ordered at 3 weeks of age in the first instance (see discussion below). *PyVT mice are the first choice for mammary gland tumours, due to their rapid development. Plan to harvest tumours when female PyVT mice reach 6 weeks of age, although this time frame can vary by a week. Only male PyVT mice are used to maintain the line; their breeding performance is most successful between 2–5 months of age. The wildtype FVB mouse will be the source of control tissue for pathological studies and FVB females are used in breeding PyVT mice. The two transgenic mice, PyVT and neu, are recommended until one becomes adept at cell recovery, manipulation and animal husbandry. Experienced investigators may apply these techniques to other cancer or animal models. Investigators should use the same distributor consistently, as colonies founded by different companies may have diverged to the point of histoincompatibility, which would complicate interpretation of transplant experiments.*

Surgical scissors, tweezers and scalpels

Betadine solution

70% (v/v) ethanol

Disposable 35 and 100 mm petri dishes

Glass bead sterilizer. *A glass bead sterilizer is very convenient and is highly recommended (even if not required by IACUC).*

Dulbecco's Modified Eagle's Medium (DMEM - Gibco BRL)

Foetal bovine serum (FBS - Gibco BRL)

Hank's Balanced Salt Solution with and without 2% heat-inactivated FBS

0.25% (w/v) trypsin (see recipe)

Sterile 1, 5, 10 and 25 ml sterile serological pipettes

Sterile 0.22 µm syringe membrane filters (Millipore)

Sterile 70 µm cell strainers. *These are designed to fit inside a 50 ml tube.*

15 and 50 ml capped sterile centrifuge tubes

5 and 10 ml syringes

Temperature-controlled desk-top centrifuge

Trypan blue solution (see recipe)

Haemocytometer

Antibodies (all from Biolegend Inc., San Diego, CA): anti-CD24-fluorescein isothiocyanate (catalogue number 101805), anti-CD44-Pacific Blue (catalogue number 103019), anti-CD49f-AlexaFluor 647 (catalogue number 313609), anti-CD45-phycoerythrin (catalogue number 103105), anti-CD31- phycoerythrin (catalogue number 102407), anti-Ter119-allophycocyanin (catalogue number 116211), anti-Sca1- phycoerythrin (catalogue number 108109). *Fluorescent labels can be altered to suit investigator preferences or flow cytometer capabilities.*

Flow cytometer with sorting capacity (e.g. Becton Dickinson FACSVantage)

CO₂ regulated tissue culture incubator

Water bath

Ice buckets and crushed ice

Basic Protocol Steps and Annotations

Harvest tumours—

- 1 Euthanize mice by CO₂ exposure and cervical dislocation. *Choose mice with obvious mammary gland tumours that have not reached maximum size permitted by the IACUC, as this will yield the best chance of useful tissue. Mammary gland tumours are heterogeneous and rarely achieve similar size in more than two glands at a time so pooling several glands is standard practice.*
- 2 Lay mouse down in supine position and secure legs to prevent movement.
- 3 Swab all of the abdomen with 70% ethanol, followed by a betadine solution swab then another 70% ethanol wash.
- 4 Make an incision from pubis to just under the chin through the skin and fascia but do not penetrate the muscle layer. *There are 5 pairs of laterally symmetrical mammary glands in the mouse; thoracic glands (pairs 1–3) and inguinal glands (pairs 4 and 5). Pair 1 occurs between the shoulder and the head and pair 2 occurs just distal to the front legs and in a normal mouse these pairs are too small for routine collection of tissue, although tumours readily occur here. Pairs 3 (proximal to axillae) and 4 (just above the hip) are the glands frequently used for normal cell collection. Pair 4 are the glands traditionally removed when one prepares a cleared fat pad, due to their convenient location and size and therefore they are used most commonly for receipt of injections. Pair 5 glands occur just distally and proximal to pair 4, on either side of the genitalia.*
- 5 Make 4 lateral incisions through the skin from sternum right and left to just under axillae and from pubis right and left to just above each hip joint.
- 6 Grasp the skin layer with tweezers or forceps and gently but firmly pull each lateral skin flap fully to the right or left. Use the blunt edge of scissors to aid in separating the skin from the muscle.
- 7 With blunt dissection, remove tumour from fascia, gently pulling towards edge of opened skin flap while cutting away connective tissue between the tumour and the fascia. *Which glands will have large enough tumours is unpredictable. Tumours will vary in size and some may contain obvious necrotic or*

haemolysed areas, which should be avoided if possible. Blunt rather than fine excision of solid tumour tissue is adequate at this stage.

- 8 Immediately place the excised tumours into a 100 mm petri dish kept on a layer of crushed ice and containing 5–10 ml of DMEM. When all tumours have been collected, move petri dish to laminar flow hood. *Maintaining a sterile field is critical for any long-term culture of recovered cells. Although the mammary gland tumours of these mice contain mixed pathology including areas of sometimes extensive hemolysis, the recovered cells are usually uncontaminated. However, all isolation steps beyond the initial gross dissection of the tumours should be performed in a biological laminar flow cabinet.*

Prepare Cell Suspension—

- 9 Mince tissue in the petri dish into 1–2 mm-sized pieces, using scissors, scalpel or razor blade
- 10 Carefully remove most of the DMEM with a pipette, leaving the tissue pieces behind.
- 11 Add 5–10 ml of sterile 0.25% trypsin (see recipe), mix and incubate at 37 degrees C for 30 minutes
- 12 Inactivate the trypsin by adding an equal volume of DMEM/10% FBS to the petri dish and mix.
- 13 Pipette contents of dish through a 70 μ M filter screen into a new screw-capped test tube. *All future steps should be with cells on ice and solutions at 4 degrees Centigrade.*
- 14 Centrifuge for 5 minutes at $500 \times g$, 4 degrees Centigrade.
- 15 Resuspend cells in DMEM/10% FBS and place on ice. Remove a small aliquot of cells and count using a haemocytometer and trypan blue

Label cells with antibodies—

- 16 For staining, resuspend cells at 1×10^6 cells/100 μ l in cold HBSS/2% heat-inactivated FBS (staining medium).
- 17 Set aside a small aliquot of unlabeled cells and keep on ice. Add appropriate concentrations of antibodies as instructed by supplier. Incubate on ice in the dark for 30 minutes, gently shaking tubes every 10 minutes; keep light exposure (especially uv light) to a minimum.
- 18 Wash cells by centrifuging twice for 5 minutes at $500 \times g$ at 4 degrees Centigrade and resuspending in staining medium between washes and finally.

Perform sterile cell sorting—

- 19 Maintain cells on ice and proceed with flow cytometry and cell sorting. The initial gates should be set to exclude haematopoietic ($CD45^+$, $Ter119^+$) and endothelial cells ($CD31^+$) and sort based on CD24 expression. Mammary gland epithelial cells are $CD24^+CD49f^+CD44^{low}CD29^+Sca-1^{low}CD45^-CD31^-Ter119^-$. Mesenchymal cells are $CD24^-CD49f^-CD44^{high}CD29^+Sca-1^{high}CD45^-CD31^-Ter119^-$. *Flow cytometry and cell sorting is typically done by a core facility within an institution, since the equipment and expertise are major investments typically beyond the means of a standard laboratory. The flow cytometry facility will*

advise on appropriate controls e.g. aliquots of unlabeled cells will be required to set forward and side-scatter gates and propidium iodide-labeled cells will be needed to exclude dead cells.

At this point, cells are ready for culture, transplant or other manipulations, as the interest of the investigator dictates. For culture, see Support Protocol 1. For transplant studies, see Support Protocols 3 and 4.

ALTERNATE PROTOCOL 1

Alternate protocol for mesenchymal cell isolation from bone marrow

The mesenchymal cell population is made up of multiple cell types and their influence on tumour development is now widely recognized and is rapidly becoming a new discipline. Mesenchymal cell phenotypes are tailored to their organ of residence; probably the best known mesenchymal cell population is that of the bone marrow. Extensive in-depth analysis has explored the potential of these cells to differentiate into multiple cell types, including adipocytes, chondrocytes and myocytes (Friedenstein *et al*, 1968; Jiang *et al*, 2002). Given this potential, it may be of interest to examine if mesenchymal cells from other organs besides the breast might interact with or influence mammary gland epithelial cell behavior. Recent reports document bone marrow-derived mesenchymal cells playing a significant role in breast cancer metastasis to bone (Goldstein *et al*, 2010) and in prostate regrowth after cancer, in which the mesenchymal cells fuse with the prostate epithelial cells (Placencio *et al*, 2010). This alternative protocol describes an established, flow cytometric-based method to enrich and culture mesenchymal cells from mouse bone marrow. Ex vivo culture is necessary as these cells make up less than 1 in 10^6 bone marrow cells (Phinney *et al*, 1999), so initial yield will be low. These can then be coinjected with epithelial cells in any transplantation study or studied on their own. This description is based on the methods of Bonnet (Anjos-Afonso & Bonnet, 2008)

Additional Materials

- FVB mice (Jackson Labs stock number 001800)
- Bone scissors
- Red Blood Cell Lysing Buffer (Sigma)
- Tissue culture flasks (25, 75 and 175 ml filter-top flasks)
- Mesencult complete medium (see recipe).
- Anti-CD11b-FITC (Biolegend; catalogue number 101205).

Prepare bone marrow cell suspension

- 1 Euthanize mouse and prepare for bone marrow extraction by dipping entire rear legs in a 70% ethanol solution, swab with betadine solution then redip legs in 70% ethanol.
- 2 Incise skin from ankle to hip, cut through tibia at ankle and femur at hip joints and remove legs, without skin, from body. Place legs in a 100 mm Petri dish containing 5–10 ml HBSS on ice. *Operation should now be moved to laminar flow hood.*
- 3 Remove all tissue from bones then separate at knee joint.
- 4 Fill a.3 ml syringe with HBSS and attach a 25 gauge needle.

- 5 Grasp tibia or femur with tweezers, insert needle into end of bone at knee joint and push syringe contents gently through bone, collecting extruded marrow/cells into a 50 ml capped centrifuge tube. *Removing patella and gently rotating needle while entering ends of bone will aid penetration.*
- 6 Flush bones several times with more HBSS and pool. *Two femurs and two tibias from one mouse will yield on average $30\text{--}50 \times 10^6$ bone marrow cells. The bone marrow from a minimum of 5 mice should be pooled for mesenchymal cell culture and purification.*
- 7 Centrifuge cells for 5 minutes at $500 \times g$, 4 degrees Centigrade
- 8 Discard supernatant, agitate tube to break up pellet of cells and add 1 ml red blood cell lysing buffer.
- 9 Incubate on ice 1 minute, add 10–20 ml HBSS and recentrifuge.
- 10 Resuspend cells in HBSS/2% HI-FBS and count on a haemocytometer.

Culture Cells

- 11 Spin cells down and resuspend in Mesencult complete medium at $5 \times 10^6/\text{ml}$.
- 12 Dispense into tissue culture flasks at a density of $1 \times 10^6/\text{cm}^2$.
- 13 Discard non-adherent cells on day 3; replenish medium every 4–5 days.
- 14 When cells have achieved approximately 70% confluence, remove media, rinse cells once with 5–10 ml HBSS then discard HBSS.

Passage Cells and Expand Cultures

- 15 Passage cells by exposing to 0.25% trypsin for 5 minutes at 37 degrees Centigrade. *Warm trypsin solution to 37 degrees Centigrade before infusion into flasks and use approximately 2 ml/25cm².*
- 16 Remove trypsin solution and detached cells by pipetting flask contents up and down and decanting all into a centrifuge tube containing 1 ml cold FBS for each 25 cm² flask equivalent in order to inactivate the trypsin.
- 17 Rinse flask with Mesencult (without supplements) and add to cells in centrifuge tube.
- 18 Spin cells for 5 minutes at $500 \times g$ at 4 degrees Centigrade
- 19 Resuspend cells in fresh Mesencult complete medium and split 1:3 into new flasks.

Label Cells for Flow Cytometry

- 20 At passage 3, trypsinize cells, resuspend in staining medium and label with anti CD11b and anti CD45 antibodies, according to manufacturer's directions.
- 21 Incubate on ice for 30 minutes, with regular agitation, as described above.
- 22 Proceed with cell sorting, collecting double negative CD11b[−]CD45[−] cells.
- 23 Reculture cells in Mesencult complete medium for expansion. *At least two aliquots of cells should be cryopreserved (see Support Protocol 2) at this stage. One must also confirm differentiation potential (e.g. adipocytes, myoblasts) of these mesenchymal cells by exposing them to specific inducing media. See Phinney et al, 1999 for details (Phinney et al, 1999).*

24 Cells are now ready for transplantation or other studies.

SUPPORT PROTOCOL 1

Culture of epithelial and mesenchymal cells

Additional Materials—

- Isolated and sorted cells (see above)
- CO₂-regulated tissue culture incubator
- Sterile pipettes (1, 5, 10 and 25 ml serological pipettes)
- Sterile 0.22 µm membrane filters (Millipore)

Culture medium for epithelial cells: Dulbecco's Modified Eagle's Medium (DMEM-Gibco BRL). Prepare for cell culture by adding 10% (v/v) HI-FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 ng/ml epidermal growth factor (Sigma), 5 µg/ml bovine insulin (Sigma). *Ideally, cultures should be maintained without antibiotics, but this requires constant vigilance that comes with experience, so initial cultures can contain penicillin and streptomycin and one can gradually stop supplementing the culture media with these antibiotics.*

Culture medium for mesenchymal cells: Mesencult complete medium (see recipe).

Epithelial cell culture—

1. Infuse single cell suspensions of mammary gland epithelial cells into tissue culture flasks (seed at between 5×10^4 and 5×10^5 cells/cm²) and place in a 37 degrees centigrade incubator with 5% CO₂.
2. Change medium every 2–3 days thereafter.
3. When cells have achieved approximately 70% confluence, remove media and rinse cells once with 5–10 ml HBSS and discard HBSS.
4. Passage cells by exposing to 0.25% trypsin, as described above.
5. Resuspend cells in fresh culture medium and split 1:3 into new flasks.

Frozen stocks of early passage (2–5) should be cryopreserved (see Support Protocol Number 2).

Mesenchymal cell culture—Proceed exactly as for epithelial cell culture, using the mesenchymal cell sorted fraction and replacing DMEM with Mesencult Complete Medium.

SUPPORT PROTOCOL 2

Cryopreservation of Cells

Additional Materials—

- Cryopreservation Solution (see recipe).
 - 1 ml cryotubes
 - Freezing container (e.g. Nalgene Mr Frosty)
1. Trypsinize cells in flasks when 70% confluent, as described above.

2. Resuspend cells in multiples of $5\text{--}10 \times 10^6$ (depending upon availability) in culture media i.e. DMEM-based for epithelial cells, Mesencult complete medium for mesenchymal cells.
3. Centrifuge cells for 5 minutes, $500 \times g$ at 4 degrees Centigrade.
4. Decant culture medium and add 1 ml (dropwise, with constant tube agitation) of cryopreservation medium per $5\text{--}10 \times 10^6$ cells.
5. Place cells on ice for 30 minutes.
6. Dispense 1 ml aliquot into cryotubes.
7. Place cryotubes in freezing container and place in a -80 degree Centigrade freezer overnight.
8. Move cryotubes to a liquid nitrogen storage tank within 24–48 hours.

SUPPORT PROTOCOL 3

Clearance of Mammary Gland Fat Pad and Transplantation of Cells

Additional Materials—

FVB mice (must be no older than 3 and 1/2 weeks)

Ketamine/Xylazine mix (see recipe)

PBS

1 ml syringes and 25 gauge needles

Hamilton 25–50 μ l microsyringe (Hamilton, Reno, NV.) and 27 gauge needles

Prepared cells ready for transplant

Cauterizer (e.g. Model RS-320; Roboz Surgical Instrument Co., Inc., Gaithersburg, MD)

Wound closing clips and applicator (Clay Adams Autoclips; Parsippany, NJ)

This procedure is based on the pioneering work of Deome, which is still widely used today (Deome *et al.*, 1959). It was originally designed to detect the repopulating ability of epithelial cells but is now most commonly employed to detect cancer stem cells. The mammary gland fat pad (typically number 4) is cleared of host epithelial cells. The fat pad remains as a receptacle for transplanted cells, allowing for demonstration of any mammary gland repopulation potential or interaction with host cells.

1. Anaesthetize the mouse with an intraperitoneal injection of ketamine/xylazine mixture.
2. Lay mouse down in supine position and secure legs.
3. Swab all of the abdomen with 70% ethanol, followed by a betadine solution swab then another 70% ethanol wash.
4. Make a vertical incision from mid-abdomen (at a point between the number 4 nipples) to the sternum and then 2 lateral incisions from the bottom of the mid-abdominal incision, ending between the number 4 and number 5 mammary glands on either side. The resultant incision will have an inverted Y shape.
5. Retract skin and fascia from the abdominal muscle wall to clear the surgical field. *The mammary glands appear as greyish or pinkish bodies with a slightly denser appearance and darker colour than the surrounding fat or fascia and will be attached*

to the underside of the dermis. Careful inspection will distinguish glandular material from the surrounding fat, which can be identified by the presence of shiny, tightly packed spherical lipid globules.

6. Cauterize the nipple, the blood vessel proximal to the lymph node and at the point where the number 4 and number 5 fat pads touch. The mammary gland anlage at this age should not have grown beyond the lymph node. Excise the mammary gland between the cautery points from the nipple to the associated lymph node using fine scissors and precise incisions. *One can use a single mouse as its own control by removing the number 4 gland from one side only. This depleted side will receive cells prepared for transplant. Alternatively, one can perform complete removal of both glands and use sham-operated mice as controls.*
7. Transplant cells into the fat pad in a volume of 10–15 μ l of PBS or HBSS using a Hamilton microsyringe and a 27 gauge needle. *Ideally, cells to be transplanted are injected at this time, while the mammary gland fat pad is exposed. This avoids a second operation.*
8. Close incisions with wound clips.
9. Remove clips 7 days later. *If cells were not transplanted at the time of clearance, the above surgical procedure is repeated when cells are ready for transplantation into the residual fat pad.*

SUPPORT PROTOCOL 4

Ectopic transplantation of isolated cells

If the tumorigenicity of cells is to be tested and not epithelial mammary gland function per se, a second surgery to expose the cleared fat pad can be avoided and cells can be injected subcutaneously in the rear legs of a host. This procedure is best performed with two people to avoid anaesthesia.

1. Gently restrain mouse and extend rear leg.
2. Swab injection site with 70% ethanol
3. Inject cells in a volume of 25–50 μ l using a 1 ml syringe and a 26 gauge, 5/8" needle. *Needle should be inserted at a 10–20 degree angle relative to the femur; as cells are injected, a bleb should become visible under skin. Use the full length of the needle and wait 5–10 seconds after injection before withdrawal, to avoid leakage of cells.*
4. Monitor mice daily by palpation and note time to first evidence of tumour growth.

Reagents and Solutions

All solutions should be made using purified water systems, such as Millipore or Barnstedt.

Cryopreservation Solution. Prepare a solution of 70 ml HI-FBS, 20 ml DMEM (or Mesencult if mesenchymal cells are being frozen) and 5 ml DMSO. Aliquot into 10 ml fractions and store at –20 degrees Centigrade. Storage life 24 months.

Dulbecco's Modified Eagle's medium (DMEM). Prepare in 500 ml bottles. Leave one litre untreated. Prepare another litre for cell culture by adding 10% heat-inactivated foetal bovine serum (HI-FBS;), 50 U/ml penicillin, 50 μ g/ml streptomycin. Store in dark at 4 degrees Centigrade. This volume is a compromise between having sufficient amount for 1–2 weeks of culture maintenance but not so much in one bottle that the loss due to contamination is a serious setback. Ordering the DMEM as powder (Gibco BRL) and making it up oneself is

much more economical. Sterile reusable glass bottles, a pH meter and a vacuum filtration system are required. Ideally, cultures should be maintained without antibiotics, but this requires constant vigilance that comes with experience, so initial cultures can contain penicillin and streptomycin and one can gradually stop supplementing the culture media with these antibiotics. Storage life 6 months.

Epidermal Growth Factor (suitable for cell culture). Make a stock solution at 10 µg/ml in HBSS by adding 100 µg to 10 ml. Filter sterilize and store at –80 degrees Centigrade in cryovials in aliquots of 500 µl. Add one vial per 500 ml bottle of DMEM. Storage life 24 months.

Ethanol solution. Make up 70% (v/v) ethanol in purified water. Storage life 12 months in closed container.

Foetal bovine serum (FBS) Heat-inactivate serum by placing sealed bottles in a water bath at 56 degrees Centigrade for 60 minutes. There is no need for further sterilization. Store at –20 degrees Centigrade in 100 ml aliquots.. Storage life 24 months.

Hank's Balanced Salt Solution (HBSS). Prepare in 1 litre bottles from powder (Gibco BRL), filter sterilize and store at 4 degrees Centigrade. Storage life 12 months.

Insulin (suitable for cell culture). Make a stock solution at 5 mg/ml in HBSS by adding 50 mg insulin to 10 ml HBSS. Filter sterilize and store at –80 degrees Centigrade in cryovials in aliquots of 500 µl. Add one vial per 500 ml bottle of DMEM. Storage life 24 months.

Ketamine/Xylazine. Make a working solution by mixing 1 ml of ketamine stock solution (100 mg/ml), 0.5 ml of xylazine stock solution (20 mg/ml) and 8.5 ml of PBS. Inject 100 µl per 10 grammes body weight by intraperitoneal injection using a 1 ml syringe and a 25 or 26 gauge 1/2 inch needle. Anaesthesia will last about 20–30 minutes and sedation about 60–90 minutes. If more anaesthesia is required, use only a ketamine solution (make up as above, omitting xylazine). Storage life of stock solutions 18–24 months (expiry dated by manufacturer. Discard working solution every month.

Mesencult Complete Medium. This consists of Mesencult MSC Basal Medium (catalogue number 05501) plus Mesenchymal Stem Cell Stimulatory Supplements (catalogue number 05502), obtainable from Stem Cell Technologies, Vancouver, BC. This has a limited shelf-life based on date of receipt; mix only the amount needed for 1–2 weeks. Store supplements at –20 degrees Centigrade.

Penicillin-Streptomycin. 100 × concentrated solutions are widely available and inexpensive. Store at –20 degrees Centigrade. Storage life 24 months.

Phosphate buffered saline (PBS). For a 10× solution, prepare the following, per litre:

- 80 grammes NaCl
- 2 grammes KCl
- 11.5 grammes Na₂HPO₄·7H₂O
- 2 grammes KH₂PO₄
- 1 litre of purified H₂O

Adjust pH to 7.4 then autoclave. Dilute 1:10 before use. Shelf-life 12 months in sealed bottle.

Red Blood Cell Lysing Buffer (Sigma). Store at room temperature. Shelf life 24 months.

Staining Medium. Prepare HBSS with 2% (v/v) HI-FBS, Store at 4 degrees Centigrade. Storage life 6 months.

Trypan blue. Make a 0.25% (w/v) solution in PBS. This does not have to be sterile; keeps indefinitely.

0.25% Trypsin. Make up 0.25% (w/v) trypsin solution in sterile PBS or HBSS, sterilize using a 0.22 μ m syringe filter and store at -20 degrees Centigrade in 10–25 ml aliquots. Keep on ice when not in use; unused portions can be refrozen multiple times, but will gradually lose activity. Replace after 1 year.

COMMENTARY

Background Information

The mammary gland derives from the embryonic ectoderm, from which a small population of cells invades surrounding stroma to form branching ducts which terminate in lobules (Daniel & Silberstein, 1987; Stingl *et al*, 1998). This terminal duct lobular unit (TDLU) is the basic functional unit of the mammary gland and it is within this unit that the mammary gland stem cells reside (Woodward *et al*, 2005; Villadsen *et al*, 2007). The mammary gland has two epithelial layers; an outer myoepithelial/basal layer and a luminal layer lining the ducts. In addition, there are a variety of non-epithelial cells, including mesenchymal cells, endothelial cells, lymphocytes, adipocytes, neurons and myocytes (Sleeman *et al*, 2006). In 2006 it was shown that a single cell lacking haematopoietic (CD45 and TER119) and endothelial (CD31) antigens, positive for CD24 and expressing high levels of CD29 (i.e. Lin⁻CD24⁺CD29^{hi}) could generate the entire mammary gland (Shackleton *et al*, 2006). Mammary gland stem cells have subsequently been shown to play key roles in both regeneration of the mammary gland and in the development of mammary gland tumours.

In the normal human breast, there are three populations within the epithelial progenitors: luminally restricted, basal myoepithelial restricted and bipotential cells (LaBarge *et al*, 2007). The luminally restricted cells are sialomucin (MUC)1⁺, epithelial cell adhesion molecule (EpCAM)⁺ (also known as epithelial specific antigen – ESA) and these cells subsequently develop cytokeratin (CK)8, CK18 and CK19 antigens. The myoepithelial cells demonstrate CK5, CK14 and alpha smooth muscle actin (α -SMA) antigens; this is the population enriched for stem cells (Stingl *et al*, 1998; Clarke, 2005). The bipotential progenitors can be found as a core of cells expressing CK19 surrounded by myoepithelial cells expressing CK14 (Stingl *et al*, 2001). This stem cell enriched pool of myoepithelial cells correlates highly with cells expressing low levels of CD24; it is these cells that demonstrate robust repopulation of cleared mammary gland fat pads while CD24^{high} cells show limited repopulation (Sleeman *et al*, 2006; Fillmore & Kuperwasser, 2008).

The CD44/CD24 antigens are the most commonly cited antigens identifying mammary gland cancer stem cells. CD44 is a transmembrane hyaluronan receptor with a role in cell migration, chemotaxis and adhesion (Sleeman & Cremers, 2007; Vigetti *et al*, 2008). CD44 has been used as a marker of cancer initiating cells in various cancers, including prostate, pancreas and colon (Collins & Gibson, 1999; Dalerba *et al*, 2007; Li *et al*, 2007). CD44⁺CD24⁻ESA⁺ cells have been found to be highly enriched for human breast cancer-initiating ability; this report was one of the first demonstrations of a cancer-initiating stem cell in solid organ tumours (Al-Hajj *et al*, 2003).

The CD24 antigen, a glycosylphosphatidylinositol of heterogeneous molecular weight, was established as a mammary gland tumour marker in 1999 (Fogel *et al*, 1999). The expression of CD24 correlates with tumour stage and metastasis (Baumann *et al*, 2005; Bircan *et al*, 2006) and it has also been recently identified as being required for self-renewal regulation in transit-amplifying cells (the stage between stem cells and differentiated cells) (Nieoullon *et al*, 2007). Recent investigation of human breast cancer cell lines has revealed the intriguing finding that invasive CD44⁺CD24⁻ mesenchymal cells can be derived from a single non-invasive epithelial CD44⁺CD24⁺ cell (Meyer *et al*, 2009). For a recent review of mammary gland stem cells, see Stingl, 2009 (Stingl, 2009).

The mouse is an excellent model in which to investigate mammary gland physiology and pharmacology. An extensive body of literature exists on surgical manipulation and transplant of cells to the mammary gland in wildtype and transgenic mice. Investigation of potential mammary gland stem cell function is typically performed by injecting or transplanting cells into a cleared mammary gland fat pad, for which innate epithelial cells from the mammary gland have been surgically removed from the host at 3 weeks of age. There are several transgenic strains, such as the Polyoma Middle T antigen mouse and the Her2/*neu* mouse that have been engineered to spontaneously develop mammary gland cancers that closely reproduces the cellular pathology that is seen in human breast cancer (Cardiff & Wellings, 1999; Lim *et al*, 2010). These and other mice are available commercially and allow for a relatively simple isolation of (cancer) stem cells as well as non-epithelial cells such as mesenchymal cells. When these donor cells are labeled with antibodies and subjected to cell sorting, the population can be broken down into specific cell types and when transplanted into a host animal, the influence of the non-epithelial cell population on tumour growth can be studied. Epithelial and mesenchymal cells can be injected together in defined ratios into the cleared mammary gland fat pad or the contribution of the host mesenchymal cells can be studied by transplanting only epithelial donor cells (Guest *et al*, 2010).

There is a great deal of current interest in the role that mesenchymal and other stromal cells play in cancer maintenance and progression. It has been known for some time that the mammary gland stroma plays an important role in mediating breast tissue response to hormones (Woodward *et al*, 1998) but more recently the function of stromal/mesenchymal cells in mammary gland tumour development has been recognized. For example, mesenchymal stem cells not only promote migration, invasiveness and metastasis but also play roles in their hormone independence and regulation by cytokine pathways (Goldstein *et al*, 2010; Rhodes *et al*, 2010; Halpern *et al*, 2011; Liu *et al*, 2011). In some cases, stromal cells have fused with and transformed mammary gland cancer epithelium (Jacobsen *et al*, 2006). Stromal fibroblasts have also been found to promote other cancers, including pancreatic cancer (Hwang *et al*, 2008). For reviews of tumour-associated fibroblasts, see Xouri and Christian and Franco *et al*, 2010 (Franco *et al*, 2010; Xouri & Christian, 2010).

Critical Parameters

Transplantation of cells into the cleared mammary gland fat pad is best done immediately after removal of the gland while the mouse remains anaesthetized. This avoids a second surgery, which would otherwise be required because precise delivery of cells into the fat pad by injection through the skin is not possible. The age of the recipient dictates the surgery schedule, not the availability of cells because after 3 and ½ week to 4 weeks of age, epithelial growth within the mammary gland fat pad is too extensive to permit complete removal. Proliferation of residual host epithelial cells would then complicate any interpretation of transplanted cell growth.

Mesenchymal cells require specific media; do not substitute traditional culture media (e.g. DMEM), as this will permit growth of other cell types. It must be cautioned that there is as yet no universally accepted distinct antigen profile for mesenchymal cells, so any purification procedures may include cells or populations with other lineage fates. It would behoove the investigator to monitor the literature for reports that may suggest a new and distinct antigen profile for mesenchymal cells in the context of mammary gland biology. Several groups have established procedures to isolate mesenchymal cells from different sources and these populations differ in their antigenic profiles.

Troubleshooting

The establishment of cancer stem cell clones (immortal cell lines) from PyVT mammary gland tumours may take multiple attempts, due to the rarity of epithelial stem cells in any given tissue sample. Initial cell fractions from these tumours should be very conservatively sorted, such that there is no reasonable chance of including ungated cells. Sorting with high stringency allows for the greatest confidence in data generated by the cells in later experiments. One must confirm differentiation of mesenchymal stem cells early on in passaging, to prevent expansion and investigation of stem cell or other potential of cell cultures which may not be enriched enough.

When collection of bone marrow cells from multiple femurs and tibias is desired, first form holes in all bone ends with one 25 gauge needle mounted on a syringe. Penetrating the bone ends with a needle frequently plugs the needle orifice with bone fragments or damages the bevel, which blocks dispensing of syringe contents (but does not prevent one from drilling the holes). This will avoid switching to a new needle with each bone.

Anticipated Results

The collection of only 1 or 2 mammary gland tumours from a PyVT mouse can be enough to yield a stem cell line. On the other hand, several mice may be sampled with no long-term cultures surviving. In general, sampling of 10 mice with tumours should result in the successful establishment of at least one immortal cell line from which epithelial cells can be isolated. Mesenchymal cells from mammary gland typically grow more slowly than epithelial cells but the chances of establishing a line from e.g. 10 mice is similar. Transplanted cells in high numbers (e.g. $> 10^5$) usually yield tumours within 6–8 weeks. Lower numbers can take weeks to months, depending upon stem cell activity and dose. Bone marrow derived mesenchymal cells are typically slow to expand and obtaining numbers sufficient for sorting and purifying (i.e. passage 3) can take 2–3 months.

Time considerations and Limitations

If a colony of PyVT mice is to be established by an investigator, it will take a minimum of 12–14 weeks between receipt of mice to the point of obtaining useful mammary gland tumours. Dissection of mammary gland tumours, dissociation into single cell suspensions, labeling and flow cytometry/sorting can be achieved in one day. If yield of sorted cells is sufficient, cells could be transplanted immediately. However, if large numbers of cells are required, culture for 4–8 weeks may be necessary. This is particularly true for mesenchymal cells, which grow more slowly. The development of transplanted cells into tumours is strictly dependent upon the tumorigenic potential and cell number. Injecting cells in moderate to large numbers (on the order of 1×10^4 to 1×10^6) can yield tumours in a matter of weeks, while injecting single cells could conceivably take months to develop into tumours. The surgical preparation of cleared mammary gland fat pads in 10–20 mice and the transplant of cells into the cleared pad can be accomplished in one day.

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
FBS	Foetal bovine serum
HI-FBS	Heat-inactivated foetal bovine serum
HBSS	Hank's balanced salt solution
neu	FVB/N-Tg(MMTVneu)202Mul/J mouse
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PyVT	FVB/N-Tg(MMTV-PyVT)634Mul/J mouse

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