

Modeling Breast Tumor Development with a Humanized Mouse Model

Lisa M. Arendt

Abstract

The tumor microenvironment plays a critical role in breast cancer growth and progression to metastasis. Here, we describe a method to examine stromal–epithelial interactions during tumor formation and progression utilizing human-derived mammary epithelial cells and breast stromal cells. This method outlines the isolation of each cell type from reduction mammoplasty tissue, the culture and genetic modification of both epithelial and stromal cells using lentiviral technology, and the method of humanizing and implantation of transformed epithelial cells into the cleared mammary fat pads of immunocompromised mice. This model system may be a useful tool to dissect signaling interactions that contribute to invasive tumor behavior and therapeutic resistance.

Key words Human-in-Mouse model, Human mammary epithelial cells, Breast cancer, Stroma, Mammary gland, Stromal–epithelial interactions

1 Introduction

Breast cancer is a complex disease, resulting from pathogenic genetic changes that occur in epithelial cells leading to unregulated epithelial cell proliferation. Tumor growth is supported through the recruitment of a reactive stroma, comprised of cancer associated fibroblasts and adipocytes, recruited immune cells, endothelial and lymphatic networks, and extracellular matrix. Functionally, this stromal compartment can promote tumor growth through increased angiogenesis, secretion of immunomodulating cytokines, and creation of niches for cancer stem-like cells [1–3]. An increasing body of evidence suggests that formation of the tumor stroma is a major regulator of tumor progression [4–7]. However, since many xenograft models recapitulate advanced disease, understanding the signaling interactions between epithelial and stromal cells during early tumor development is challenging. Here, we describe a xenograft model to study tumor progression. Both the breast epithelial cells and

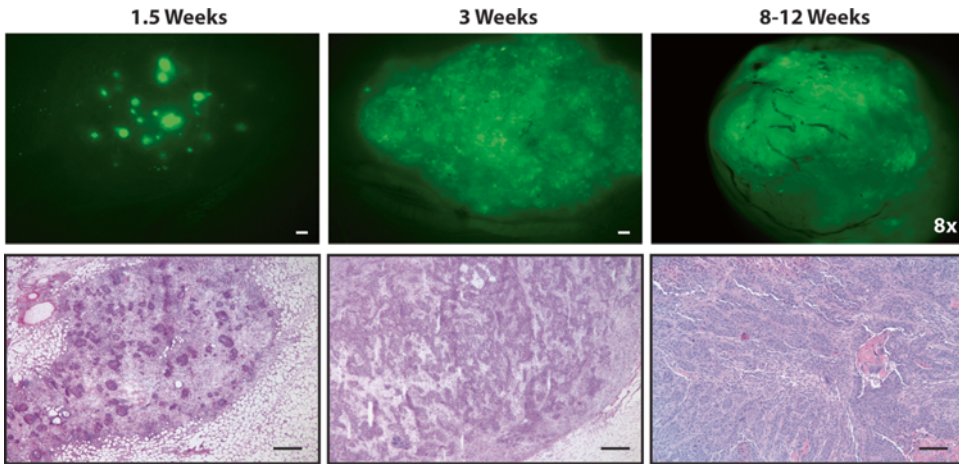


Fig. 1 Tumor progression utilizing the Human-in-Mouse (HIM) model. Following transplant of genetically modified mammary epithelial cells into humanized mammary fat pads, epithelial cells form hyperplasias which can be detected using green fluorescent protein (GFP) within 1.5 weeks. The tumors grow from approximately 1–2 mm in diameter at 3 weeks to large masses (1 cm in diameter) within 8–12 weeks. This timeline for growth may be altered due to the oncogenes used to induce tumor formation as well as genetic variation in the epithelial cells utilized. Scale bar = 100 μ m

stromal cells are of human origin, and these human-derived cells can be genetically modified in order to model genetic changes observed in human breast tumors then transplanted into the mammary fat pads of immunocompromised mice. This method allows for the stepwise progression of breast tumors from circumscribed hyperplasias to disorganized tumors (Fig. 1).

For this model, human mammary epithelial cells (HMEC) and stromal cells are isolated using collagenase digestion of breast tissue from reduction mammoplasty surgeries (Fig. 2). Stromal cells can be immortalized with lentiviruses encoding human telomerase (hTERT) and genetically modified to express growth factors or other proteins of interest [8]. These stromal cells are then transplanted into the cleared mammary fat pads of NOD/SCID mice in order to generate connective tissue to support the growth of HMEC. HMEC can be transduced with lentiviruses encoding oncogenes observed in breast cancer subtypes to generate breast tumors [9, 10]. As the tumors develop, the human-derived stromal cells are replaced by immune cells and myofibroblasts from the recipient mouse [11]. This protocol outlines the methods for the isolation of epithelial and stromal cells used in this model, the surgical procedures for clearing and humanizing the mouse mammary gland, and the transplant of transformed epithelial cells into humanized mammary glands in order to generate tumors.

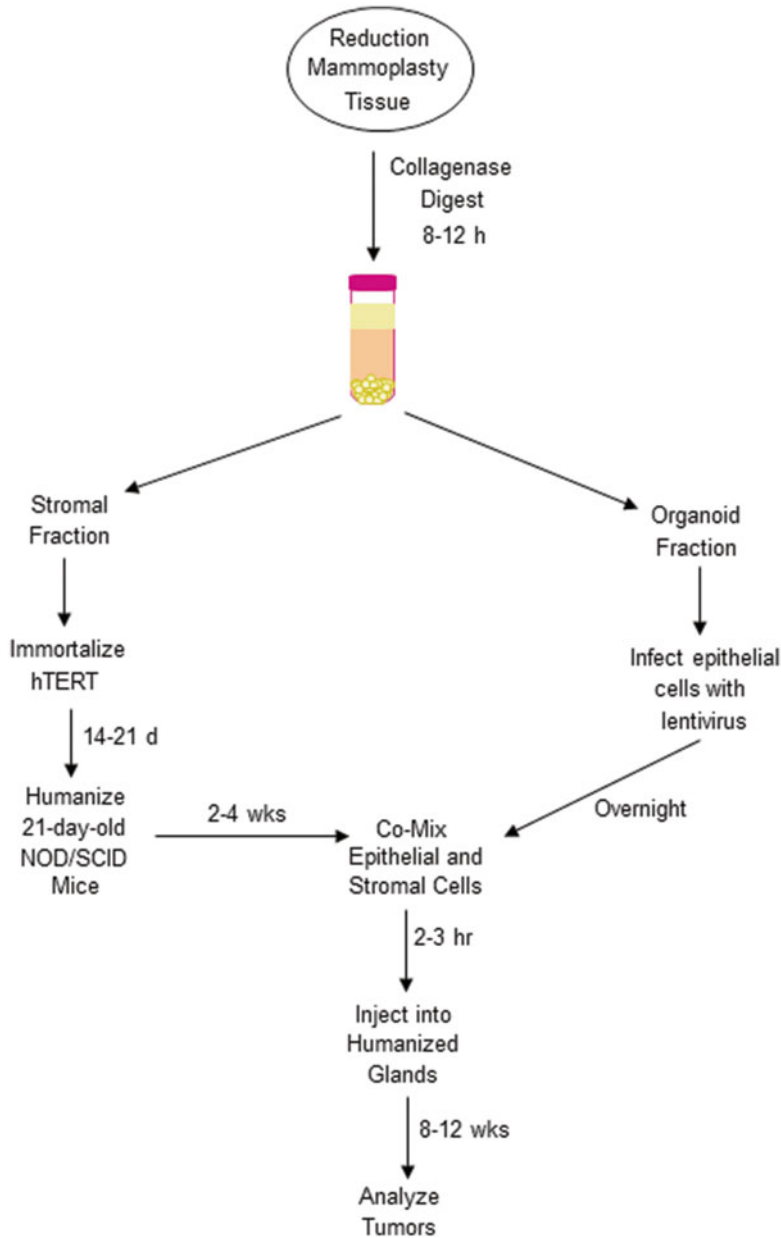


Fig. 2 Human-in-Mouse (HIM) model flowchart. Breast tissue from a reduction mammoplasty surgery is minced and digested overnight in collagen. The resulting suspension can be separated into a stromal fraction and organoids for use in culture or frozen in aliquots in liquid nitrogen for later use. The stromal fraction can be immortalized using lentivirus encoding human telomerase (hTERT) and then further genetically modified using additional lentiviral particles to express other genes of interest. The resulting stromal cell lines are used to humanize the cleared mammary fat pads of NOD/SCID mice. Two to three weeks later, epithelial cells from the organoid fraction can be dissociated into single epithelial cells, transduced with lentiviruses encoding oncogenes, co-mixed with stromal cells and implanted into the humanized glands for breast tumor formation

2 Materials

2.1 Equipment

1. Biosafety cabinet.
2. 37 °C incubator with tube rotator.
3. Tissue culture incubator held at 37 °C and 5% CO₂.
4. Water bath set to 37 °C.
5. Centrifuge.
6. Isoflurane vaporizer, induction chamber, anesthesia circuits with nose cone, heating pad.
7. Hemocytometer.
8. Heat lamp.
9. Hair clipper.

2.2 Reagents

1. Reduction mamoplasty tissue (*see Notes 1 and 2*).
2. 21-day-old NOD/SCID (NOD.CB17-Prkdcscid/J) female mice (*see Notes 3 and 4*).
3. Mayo scissors or razor blades.
4. 15 and 50 mL conical tubes (*see Note 5*).
5. 100 mm × 20 mm tissue culture plates.
6. Cryovials.
7. Plastic aspirating pipettes (*see Note 6*).
8. Dulbecco's Modified Eagle's medium.
9. DMEM–F12 medium.
10. 0.05% trypsin–EDTA.
11. Calf serum.
12. Antibiotic–antimycotic.
13. Epidermal growth factor (EGF).
14. Insulin.
15. Hydrocortisone.
16. Collagenase A.
17. Hyaluronidase.
18. Red blood cell lysis buffer: (0.15 M NH₄Cl, 0.01 M KHCO₃, 0.03 mM EDTA).
19. 1× phosphate buffered saline (PBS).
20. Dimethyl sulfoxide (DMSO).
21. DNase I.
22. Lentiviral particles encoding human telomerase (hTERT; *see Notes 2 and 7*).
23. Lentiviral particle encoding oncogene of interest (*see Note 8*).

24. Protamine sulfate (*see Note 9*).
25. Bleomycin sulfate (2 mU/mL).
26. 0.1% bovine serum albumin (BSA) in PBS, filter sterilized.
27. Growth factor-reduced Matrigel.
28. Rat tail type I collagen.
29. 0.1 N NaOH.
30. 0.01 N glacial acetic acid.
31. 40 μm basket filter.
32. Low adherence 24-well plates.
33. 10 mL syringes and 18 g needles.
34. pH indicator paper.
35. Parafilm.

2.3 Media

1. Epithelial Cell Media: Mammary epithelial cell basal media supplemented with 52 $\mu\text{g}/\text{mL}$ bovine pituitary extract, 0.5 $\mu\text{g}/\text{mL}$ hydrocortisone, 10 ng/mL human EGF, 5 $\mu\text{g}/\text{mL}$ insulin.
2. Organoid Media: DMEM–F12 media supplemented with 5% calf serum, 10 $\mu\text{g}/\text{mL}$ insulin, 10 ng/mL EGF, 0.5 $\mu\text{g}/\text{mL}$ hydrocortisone, and 1% antibiotic–antimycotic.
3. Digestion Media: Organoid Media supplemented with 3 mg/mL collagenase and 600 $\mu\text{g}/\text{mL}$ hyaluronidase.
4. Fibroblast Media: DMEM supplemented with 10% calf serum and 1% antibiotic–antimycotic.
5. Wash Buffer: PBS + 5% calf serum.

2.4 Surgical Supplies

1. Analgesic (*see Note 10*).
2. Ophthalmic lubricant (such as Dechra NDC).
3. Betadine solution (1% iodine).
4. Cotton tip applicators.
5. Alcohol wipes (70% ethanol).
6. 9 mm Autoclips.
7. Autoclip applicator.
8. Autoclip remover.
9. Hamilton syringe 100 μL size.
10. Hamilton syringe needles 22G, 2", point style 2.
11. 1 mL syringes and 27G needles.
12. Surgical scissors.
13. Surgical forceps.

3 Methods

3.1 Dissociation of Reduction Mammoplasty Tissue

1. Transfer tissue into a biosafety cabinet, and mince the sample with sterile razor blades or Mayo scissors until tissue pieces measure approximately 5 mm³ (*see* **Notes 11** and **12**).
2. Fill a 15 mL conical tube with 10 mL of Digestion Media. Add minced tissue to fill the tube to the 15 mL marker (approximately 2 g of tissue). Cover the tube and seal with Parafilm. Invert the tube to make sure that the tissue can mix with the media. If the tissue does not mix, remove tissue from the top of the tube to create more space (*see* **Note 12**).
3. Incubate filled 15 mL tubes at 37 °C with rotation for approximately 8–10 h.
4. Remove the tubes from the incubator, then centrifuge the tubes for 1 min at 9 × *g* to separate the Digestion Media into an oil/fat layer, middle aqueous layer enriched for stromal cells, and bottom organoid pellet.
5. Remove oil/fat layer (*see* **Note 13**).
6. Separate the stromal and organoid fractions into 50 mL conical tubes. Centrifuge the tubes for 5 min at 300 × *g*. Aspirate the supernatant.
7. Resuspend the organoid and stromal cell pellets in 2 mL of red blood cell lysis buffer. Incubate at room temperature for 2–5 min.
8. Add 10 mL of Wash Buffer and centrifuge for at 300 × *g* for 5 min.
9. Repeat **step 8** three times.
10. The fractions can be used for further experiments now or frozen for later use. Centrifuge cells at 300 × *g* for 5 min, and remove supernatant. Resuspend the stromal fraction in Fibroblast Media + 10% DMSO; resuspend the organoid fraction in Organoid Media supplemented with an additional 5% calf serum and 10% DMSO (*see* **Note 14**).

3.2 Generation of Immortalized Stromal Cells

1. Thaw or use the stromal fraction pellet as generated in Subheading 3.1 (*see* **Note 15**).
2. Plate cells onto 100 mm × 20 mm plates in Fibroblast Media and allow stromal cells to adhere to the plastic overnight. Cells should be grown in a tissue culture incubator.
3. Culture cells in Fibroblast Media, feeding every other day, until they approach 80% confluence.
4. To split the cells, aspirate the media from the plate. Add trypsin, and incubate the cells at 37 °C for 5 min. Gently tap the plate to dislodge remaining adherent cells. Quench the trypsin with Fibroblast Media, collect the cells into a conical tube, and centrifuge at 300 × *g* for 5 min. Aspirate the supernatant.

5. Resuspend the cells in Fibroblast Media and count the number of cells using a hemocytometer.
6. Plate and infect the cells using lentivirus encoding hTERT, according to the manufacturer's instructions, and subsequently select the cells using a mammalian selection marker or by fluorescence activated cell sorting (FACS) depending on the viral construct backbone (*see Note 7*).
7. Expand the number of fibroblasts in culture prior to humanizing. Each mammary gland that is humanized will require 2.5×10^5 untreated and 2.5×10^5 bleomycin-treated fibroblasts. The fibroblasts should not reach confluence during growth as this may have a negative effect on the engraftment during humanization (*see Note 16*).
8. 24 h prior to surgery, add 2 mU/mL bleomycin sulfate to the media of half of the fibroblasts to be used for humanizing. Incubate the cells for 30 min at 37 °C, then aspirate off the media, wash the cells with PBS, and add fresh Fibroblast Media.
9. On the day of the surgery, wash the cells with PBS, trypsinize and count cells (*see Subheading 3.2 step 4*). Prepare enough cells to inject at least four extra glands for both bleomycin-treated and untreated fibroblasts. Pool the correct number of treated and untreated cells, centrifuge at $300 \times g$ and resuspend in 25 μ L Fibroblast Media per gland prepared. Keep the cells on ice until the mice are ready for humanizing.

3.3 Humanizing Mammary Fat Pads

1. Clean surgical area with disinfectant and sterilize surgical instruments. Inject mice with analgesic prior to the start of surgery (*see Note 10*).
2. Place a mouse in the induction chamber of the anesthesia machine, and induce anesthesia using 1–3% isoflurane with an O₂ flow rate of 1–2 L/min. When the mouse no longer withdraws its foot in response to pinching its toes, the mouse is in a surgical plane of anesthesia. Apply ophthalmic ointment to its eyes and place the mouse in lateral recumbency with a nose cone to supply anesthesia.
3. Shave a 2–3 cm area extending from the rib cage to the leg. Disinfect the shaved area completely with betadine applied with a cotton tip applicator, and then wash the skin with 70% ethanol wipes. The lymph node should be visible though the skin as a small, dark oval at the level of the kneecap.
4. Make a 1 cm incision in the skin over the surface of the mammary gland at the level of the lymph node. Expose the lymph node, and with forceps, lift the lymph node and cut the mammary gland dorsal to the lymph node. Retract the mammary gland gently from the skin with the forceps, and excise the mammary gland ventrally toward the nipple. This will remove the lymph node and the endogenous mammary epithelium.

5. Using forceps gently retract the remaining mammary fat pad. Using a Hamilton syringe and needle, slowly inject 25 μL of immortalized fibroblasts into the remaining mammary fat pad. Insert the needle deeply enough into the fat pad to prevent leakage around the needle. Slowly withdraw the needle, and twist the syringe upon removal to prevent leakage.
6. Pull the skin together and tent over the injection site to avoid puncturing the injected mammary gland. Apply wound clips to close skin incision.
7. Turn mouse over and repeat **steps 3–6** on the contralateral mammary gland.
8. Place mice in clean cage with an area of the cage under a heat lamp, and monitor the mice until they are able to walk on their own. Continue to monitor mice daily post-surgically. Surgical clips can be removed after 10–14 days (*see Note 17*).

3.4 Preparation of Human Mammary Epithelial Cells

1. Injections of epithelial cells for both tumor studies and normal outgrowths should be made 2–4 weeks following humanization for the best results.
2. Expand fibroblasts for co-injection with epithelial cells. For this injection 2.5×10^5 fibroblasts are co-mixed with epithelial cells at the time of the injection.
3. 24 h before injection, thaw a vial of collagenase pellet generated in Subheading 3.1 in a 37 °C water bath. Resuspend the pellet in 10 mL of Fibroblast Media and plate on a 100 mm \times 20 mm plate. Incubate in a tissue culture incubator for 1–2 h (*see Note 18*).
4. Decant the non-adherent cells into a 50 mL conical tube with a pipette, and wash the plate with 10 mL PBS to remove any remaining organoids (*see Note 19*). Centrifuge cells at $300 \times g$ for 5 min. Aspirate and discard the supernatant.
5. Add 10 mL of 0.1% BSA in PBS, and resuspend the pellet by it passing through an 18 g needle attached to a 10 mL syringe 8–10 times. Centrifuge at $300 \times g$ for 5 min. Aspirate and discard the supernatant.
6. Resuspend the pellet in 2 mL trypsin. Triturate the solution with a 1 mL pipette for 1 min to break up the organoids. Incubate the tube in a 37 °C water bath for 5 min. Triturate the solution again, and then continue the incubation in the water bath for an additional 5 min.
7. Add 10 mL of Fibroblast Media to quench the trypsin and 100 μL of DNase to remove cellular clumping due to cell death. Mix thorough with a 10 mL pipette until the cell clumps disperse, then filter through a 40 μm basket filter into a 50 mL conical tube. Wash the filter with an additional 10 mL of Fibroblast Media.

8. Centrifuge cells at $300\times g$ for 5 min and resuspend the pellet in Epithelial Cell Media. Count cells with a hemocytometer. Centrifuge cells at $300\times g$ for 5 min and resuspend the cells in Epithelial Cell Media at a concentration of 1×10^6 cells/mL.
9. Aliquot 500 μ L of the resuspended epithelial cells into each well of a 24-well non-adherent plate. Add lentivirus encoding oncogenes to the cells in the presence of 5 μ g/mL protamine sulfate (see **Note 20**). Incubate the epithelial cells with the lentivirus in a tissue culture incubator overnight.
10. Using a 200 μ L pipette tip, triturate the transduced mammary epithelial cells to break up clumps that formed overnight. Collect the epithelial cells into a conical tube. Wash each well with 1 mL Wash Buffer, and collect the wash fraction with the infected cells in the conical tube. Centrifuge at $300\times g$ for 5 min.
11. Aspirate off the supernatant and wash cell pellet with 10 mL of Wash Buffer. Centrifuge at $300\times g$ for 5 min. Aspirate the supernatant and repeat the wash step two additional times.
12. Resuspend cells in 5 mL Epithelial Cell Media and count the cells. The target number of cells for injection to make tumors is between 1 and 2×10^5 cells/gland. Remove the number of cells necessary to complete the injections, including enough cells for at least four additional injections, and put the cells in a conical tube on ice until the fibroblasts and matrix components have been prepared.
13. Aspirate off media from fibroblasts, wash with PBS, and trypsinize as described in Subheading 3.2 **step 4**. Count fibroblasts using a hemocytometer and remove the number of cells necessary for the injections. The total number for each gland is 2.5×10^5 , and enough fibroblasts should be prepared to inject four additional glands. Place the correct number of fibroblasts in a 15 mL conical tube on ice until the matrix has been prepared.
14. The fibroblasts and epithelial cells are mixed in a mixture of collagen and Matrigel for injection. Matrigel should be thawed and maintained on ice, because it will gel at 37 °C. For tumor growth, use a 1:1 mixture of collagen and Matrigel. Dilute stock collagen to a concentration of 2 mg/mL with 0.01 N glacial acetic acid, then add 0.1 N NaOH dropwise until the pH reaches approximately 5.0 when measured with pH paper.
15. Combine collagen and Matrigel together. The mixture will turn yellow/clear due to the low pH of the collagen. Keep on ice. Check the pH of the mixture using pH paper. Add 0.01 N NaOH to the mixture dropwise, mixing well, and check the pH frequently until it reaches approximately 7–7.5. The color of the matrix mix should be pale rose pink.
16. Combine fibroblast and epithelial cells into one conical tube and centrifuge at $300\times g$ for 5 min. Aspirate the supernatant

and resuspend the cell pellet in collagen–Matrigel. The amount of matrix used for each injection is 30 μL . Keep the cells in matrix on ice until surgery.

3.5 Injection into Humanized Glands

1. Prepare the surgical area and induce anesthesia as in Subheading 3.3 steps 1 and 2.
2. Re-shave the area from the humanization surgery, scrub with Betadine, and wash with 70% ethanol wipes.
3. Make a small incision dorsal to the scar formed from the previous humanization incision. Gently retract the skin from the mammary gland with a forceps. The area of humanization will be evident as a whitish, striated area in the gland.
4. Redistribute the cells in the collagen–Matrigel mixture by flicking the tube, and draw 30 μL into a Hamilton syringe.
5. Inject the 30 μL bolus slowly in the same area that was used to humanize. Turn the needle while withdrawing to prevent leakage from the gland.
6. Close the wound as described in Subheading 3.3 step 6. Repeat Subheading 3.3 steps 2–5 on contralateral gland. Recover the mice and monitor as described in Subheading 3.3 step 8.
7. Monitor mice weekly for tumor formation. Tumors may be palpable (approximately 2 mm in diameter) within 3 weeks following epithelial cell injection.

4 Notes

1. Human breast tissue for these studies is obtained from patients undergoing elective breast reduction mammoplasty surgeries. All studies should be performed in accordance with guidelines for human research subjects and institutional policies.
2. Working with cells from human breast tissue and lentiviruses requires the utilization of biosafety containment procedures. Human breast tissue may be a source of blood-borne pathogens. Risk assessments should be carried out in conjunction with institutional guidelines for biosafety prior to starting any procedures.
3. All animal procedures must be conducted in accordance with institutional animal care and use committees and ethical guidelines. All surgeries should be conducted under sterile conditions with aseptic technique.
4. We typically use 21-day-old NOD/SCID females that weigh between 8 and 10 g of body weight. If a pup weighs less than 7 g, it is advisable to wait until the pup gains weight in order to improve viability following surgery. Mice greater than 12 g of body weight should be avoided, because they might be older than suspected. The mammary ductal epithelium

continues to grow allometrically, and increasing age may reduce the ability to remove the endogenous epithelium by clearing the fat pad.

5. We recommend using polypropylene centrifuge tubes with a plug cap. This type of tube is less likely to leak or crack during rotation, reducing the potential for laboratory contamination from the human breast tissue.
6. The use of plastic aspirating pipettes greatly reduces the possibility of sharp injuries from glass potentially contaminated with blood-borne pathogens.
7. Lentiviral particles are available from a number of commercial sources. Lentivirus is particularly good for generating stable lines from primary stromal cells, because the virus infects both proliferating and non-proliferating cells. Alternatively, vectors encoding hTERT for generation of lentivirus or retrovirus as well as packaging and envelope plasmids are available from Addgene. For generation of viruses in the laboratory, 293T packaging cells can be obtained from ATCC (CRL-3216). After selection, the immortalized stromal cells can be further modified to overexpress other genes, growth factors and proteases, such as transforming growth factor beta, hepatocyte growth factor, or vascular endothelial growth factor. Lentiviruses can also be purchased to create stable cell lines encoding these genes. When selecting a second lentiviral construct, it is necessary to choose lentiviruses that encode a second selectable target, such as resistance to a different antibiotic or expression of a separate fluorescent protein.
8. In order to generate tumors, our laboratory routinely uses lentivirus for SV40 T antigen and mutant KRas G12V, which are both commercially available (abm and GenTarget, Inc, respectively). Other oncogenes may be utilized and are available from other commercial vendors. Alternatively, constructs for oncogenes in lentivirus plasmids as well as packaging and envelope plasmids can be obtained from Addgene and 293T cells from ATCC (CRL-3216) for generation of lentivirus in the laboratory.
9. We have found that protamine sulfate is less toxic to primary epithelial cells than polybrene (hexadimethrine bromide). Polybrene can be used in place of protamine sulfate at a concentration of 8 $\mu\text{g}/\text{mL}$.
10. For analgesia, we treat mice with ketoprofen at a dose of 2–5 mg/kg subcutaneously every 12–24 h. Alternatively, buprenorphine may be administered subcutaneously or intraperitoneally at a dose of 0.05–0.1 mg/kg. However, buprenorphine is a controlled substance and has strict regulations for its usage. The institutional laboratory animal veterinarian can provide guidance for analgesia as necessary.

11. After clearance with Surgical Pathology, the tissue should remain in a closed specimen cup on ice until mincing and collagenase digestion. Recovery of viable cells is maximal if the tissue is processed on the same day as the surgery.
12. Following incubation, if the digestion media contains tissue pieces $>2 \text{ mm}^3$, the tissue may not have been minced enough in Subheading 3.1 **step 1** or the tubes were filled with too much tissue which inhibited collagenase digestion (Subheading 3.1 **step 2**).
13. The oil/fat layer is enriched for mature adipocytes, although the cells that grow in culture will not be a pure population of adipocytes. This layer can be plated in Fibroblast Media on tissue culture plates, and adipocytes without lipid droplets will grow within 3–7 days.
14. To aliquot cells for freezing, we usually freeze one vial for each 15 mL digestion tube. Although this method usually results in $2\text{--}3 \times 10^6$ epithelial cells in each vial, the number of epithelial cells isolated from organoids can be variable depending on the patient tissue sample. To enhance viability after thawing, each vial of cells should be cooled to $-80 \text{ }^\circ\text{C}$ in an isopropanol-based cooling chamber, then transferred to liquid nitrogen for long-term storage.
15. Thaw vials of cells quickly in $37 \text{ }^\circ\text{C}$ water bath. Immediately after the media in the cryovial is thawed, dilute the cells in Fibroblast Media and plate on tissue culture $100 \text{ mm} \times 20 \text{ mm}$ plates.
16. Immortalized fibroblasts grow quickly and may become confluent in several days. We recommend expanding the fibroblasts following selection and freezing back stocks for storage in liquid nitrogen. Immortalized fibroblasts may grow poorly and need to be replaced by a different stock if the cells are grown too sparsely or confluent. During passaging, the cells will optimally be split at a 1:3 or 1:4 dilution. To minimize genetic variations between experiments, we maintain a culture of fibroblasts for ten passages, and then replace them with frozen stocks. Generally, an 80–90% confluent $150 \text{ mm} \times 25 \text{ mm}$ plate will yield $2\text{--}4 \times 10^6$ cells.
17. Surgical clips should be removed at least 1–2 days prior to the next surgery for implanting mammary epithelial cells. This allows any remaining swelling in the tissue to reduce prior to the next surgical procedure.
18. The organoid pellet is enriched for mammary epithelial cells. However, there are still fibroblasts present in the pellet. Plating the cells in Fibroblast Media for 2 h depletes the fibroblasts in our experience by approximately 50%, although this may be patient sample-dependent or dependent upon the collagenase digestion efficiency.

19. The fibroblasts depleted from the organoid fraction can be maintained by feeding the cells that adhered to the plate with Fibroblast Media. The fibroblasts grow faster than any remaining epithelial cells, leading to a pure culture of stromal cells within a couple of passages. Fibroblasts cannot be easily differentiated from adipocytes growing in this culture, since adipocytes grown in culture lose their lipid droplets.
20. We generally add 1×10^6 colony forming units of lentiviral particles to 5×10^5 epithelial cells for a multiplicity of infection of 3. However, depending on the size of the target gene, it may be necessary to titer the appropriate amount of the virus needed to obtain acceptable infections. In planning the number of epithelial cells to infect for each experiment, we plate and infect 2–3 times more cells than the number that we will need for the injections the following day. Dissociation of cryopreserved organoids and overnight infection with lentiviruses decreases epithelial cell viability. By plating at least twice the number of cells that we need for our experiment, we will have an acceptable yield of viable cells the following day.

Acknowledgements

The author would like to thank Victoria Thompson for helpful discussions. This work is supported by the Susan G. Komen Foundation.

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