

# Reconstruction of human mammary tissues in a mouse model

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**Establishing a model system that more accurately recapitulates both normal and neoplastic breast epithelial development in rodents is central to studying human breast carcinogenesis. However, the inability of human breast epithelial cells to colonize mouse mammary fat pads is problematic. Considering that the human breast is a more fibrous tissue than is the adipose-rich stroma of the murine mammary gland, our group sought to bypass the effects of the rodent microenvironment through incorporation of human stromal fibroblasts. We have been successful in reproducibly recreating functionally normal breast tissues from reduction mammaplasty tissues, in what we term the human-in-mouse (HIM) model. Here we describe our relatively simple and inexpensive techniques for generating this orthotopic xenograft model. Whether the model is to be applied for understanding normal human breast development or tumorigenesis, investigators with minimal animal surgery skills, basic cell culture techniques and access to human breast tissue will be able to generate humanized mouse glands within 3 months. Clearing the mouse of its endogenous epithelium with subsequent stromal humanization takes 1 month. The subsequent implantation of co-mixed human epithelial cells and stromal cells occurs 2 weeks after humanization, so investigators should expect to observe the desired outgrowths 2 months afterward. As a whole, this model system has the potential to improve the understanding of crosstalk between tissue stroma and the epithelium as well as factors involved in breast stem cell biology tumor initiation and progression.**

## INTRODUCTION

For more than four decades the mouse mammary fat pad transplantation technique has served as a central means to study mouse mammary gland morphogenesis, development and carcinogenesis. This assay, pioneered by DeOme<sup>1–3</sup>, has been used to understand the various endocrine, genetic and heterotypic interactions mediating the events of mammary ductal elongation, branching and alveolar differentiation. In addition, this system has also been used to serially passage mouse preneoplastic lesions (hyperplastic alveolar nodules) and various chemical and genetically induced mammary tumors<sup>4</sup>.

This system owes much of its success to the ability of the mouse mammary stroma to provide the natural stromal microenvironment for mouse mammary epithelial growth, making it an excellent site for transplantation. However, attempts at introducing human mammary epithelial cells (HMECs) into mouse mammary fat pads devoid of murine epithelium to propagate normal human structures have not been as successful<sup>5,6</sup>. These failures are probably due to the significant differences between the adipose-rich mouse mammary fat pad and that of the fibrous human breast stroma. Indeed, experiments in which murine stromal cells and HMECs were mixed into collagen gels before implantation beneath the renal capsule did augment the proliferation and expansion of HMECs<sup>7</sup>. However, we sought to create an orthotopic model that would (i) exploit the mouse mammary fat pad as a source of important endocrine signaling events and (ii) incorporate human stroma to support the growth and differentiation of human mammary epithelium<sup>8</sup>.

In this protocol we describe the procedure by which one can grow normal human breast tissues in a mouse fat pad by first humanizing the mouse stroma with immortalized human reduc-

tion mammary fibroblasts (RMF/EGs) and subsequently implanting a mixture of primary human breast epithelial organoids (oHMECs) and primary reduction mammary fibroblasts (RMFs; **Fig. 1**). To support the development of transplanted human cells into the fat pads of mice, NOD/SCID immunocompromised mice such as those available from Jackson Laboratory (NOD/LtSz-Prkdc<sup>scid</sup>) or Taconic (NODSC, NOD/MrkBomTac-Prkdc<sup>scid</sup>) are necessary. The consideration in choosing this line of immunodeficient mice over nude mice is important for several reasons. Nude mice are only deficient in T cell-mediated immune functions. This means that this strain shows fully competent B cell and natural killer cell functions. As a consequence, these mice show profound immune rejection in many xenograft models, including this one; this greatly complicates the experimental design (e.g., irradiation of the mice before implantation) and confounds the results.

That nude mice are extremely poor breeders also makes it difficult to generate the 3-week-old mice routinely required for these experiments. This breeding deficiency is due in part to their defects in hormonal profiles<sup>9</sup>. This raises another important issue regarding the use of NOD/SCID mice instead of nude mice. The hormonal defect in nude mice leads to poor development of the mammary gland. Furthermore, one must supplement these animals with hormones to overcome this defect and even to generate breast tissues in mice<sup>7</sup>. Whether NOD/SCID mice are superior hosts compared to Beige-SCID or Rag-1 null mice is uncertain. However, in a side-by-side comparison of nude, Rag-1 deficient and NOD/SCID mice in typical tumor xenograft assays, human cancer cells formed tumors with 100% efficiency in NOD/SCID mice compared with 50% and 0% in Rag-1 null and nude mice (unpublished observations), respectively.



A colony of NOD/SCID mice is maintained on the premises under sterile conditions. This includes autoclaved shavings, food and water, in filtered cages. After weaning, 3-week-old (10 g) female mice are used for this model system. This age and size combination is critical, because the mammary gland is not fully developed at this point, making it possible to completely remove the endogenous rudimentary mouse mammary epithelium<sup>10</sup>. Surgeries are performed under sterile conditions. To minimize infection, 70% ethanol (prepackaged wipes) and betadine are used to clean the animal. Sterile cotton balls and swabs are used as applicators. Instruments are sterilized in a bead sterilizer for 10–15 s at a temperature of 250 °C. After surgery animals are given 5 ml of Sulfatrim antibiotics (Alpharma) per 500 ml of drinking water for 2 weeks.

Two weeks before humanization and organoid implantation, 3-week-old NOD/SCID mice are cleared of their endogenous rudimentary epithelium from the fourth inguinal mammary gland. Those not familiar with this technique are strongly encouraged to watch the exceptional video and protocol available from <http://mammary.nih.gov/index.html> or from Young<sup>10</sup>. Subsequently, immortalized human breast fibroblasts are injected into the cleared fat pad (termed ‘humanizing’). This can be done at the time of clearing (although this is much more technically challenging) or approximately 14 d later. Depending on the type of experiments desired, several different types of fibroblasts can be used to humanize, such as immortalized, or immortalized versions expressing growth factors or other proteins or peptides of interest. According to our unpublished observations and others<sup>11–13</sup>, subjecting fibroblasts to irradiation results in an activated state, thus facilitating the engraftment of the stromal cells.

Two weeks after humanization (and until about 4 weeks, although this is not recommended) the mice are subjected to the last surgery, wherein oHMECs and RMFs are injected into the humanized area (**Figure 1**). The oHMECs can be normal, virus-infected or tumor organoids derived from cancer samples that are co-mixed with additional human breast fibroblasts in a collagen-extracellular matrix (ECM) mixture. These fibroblasts can differ in types depending on the desired conditions. For example, to maximize normal morphogenesis and outgrowth, normal primary RMFs are co-mixed, whereas to maximize hyperplasia and tumor growth, fibroblasts overexpressing hepatocyte growth factor (HGF) or cancer-associated fibroblasts (CAFs) can be used. In addition, mixtures of activated fibroblasts (e.g., irradiated, senescent or bleomycin-treated) can also be co-injected along with the epithelial cells to promote premalignant and malignant growth. Regardless of the type of stroma that is co-mixed and injected along with the epithelial cells, the same procedure is performed.

In combination, this procedure and model system allow for the investigation of normal human breast morphogenesis, as well as the creation and propagation of premalignant and malignant human breast lesions *in vivo*. We believe that this technique parallels the widely used and successful mouse mammary fat pad transplantation technique, but with the advantage that both the stromal and epithelial compartments can be easily modified. In accordance with experimental design ethical reviews as required for research on vertebrate animals, an accredited animal husbandry facility (e.g., American Association for Laboratory Animal Science (AALAS)) in addition to an Institutional Animal and Use Committee–approved protocol must be obtained before conducting any animal research or performing any of the surgical techniques described.

## MATERIALS

### REAGENTS

- DMSO (Sigma Aldrich)
- Epidermal growth factor (EGF; Sigma Aldrich; cat. no. E-9644) Insulin (Sigma Aldrich; cat. no. I-5500)
- Hyaluronidase (Sigma Aldrich; cat. no. H-3506)
- Hydrocortisone (Sigma Aldrich; cat. no. H0888)
- Tribromoethanol (Sigma Aldrich; cat. no. T48402)
- Collagenase (Roche; cat. no. 1088793)
- Fugene (Roche; cat. no. 11814443001)
- Matrigel Basement Membrane Matrix (BD Biosciences; cat. no. 354234)
- Rat tail type I collagen (Collagen I; Upstate; cat. no. 08-115)
- Polybrene (Fluka Biochemicals; cat. no. 52495)
- Trypsin (Cellgro; cat. no. 25-052-CI)
- DME–F12 medium (HyClone; cat. no. SH30023.01)
- Ham’s F12 medium (HyClone; cat. no. SH30026.01)
- DMEM (HyClone; cat. no. SH30243.01)
- Calf serum (HyClone; cat. no. SH30072.03)
- Pen/Strep/Fung (HyClone; cat. no. SV30079.01)
- Mammary epithelial growth medium (MEGM) (Cambrex; cat. no. CC3051)
- 293T cells (American Type Culture Collection)
- Betadine
- Ethanol (100%)
- Estrogen (1.7-mg 60-d slow-release pellet; Innovative Research of America)
- pLuc-hTERT-GFP retroviral construct (see ref. 8) (available from Weinberg laboratory)

### EQUIPMENT

- Razor blades
- Bunsen burner
- Polypropylene tubes (15 ml, conical)
- Tube rotator
- Tissue culture plates
- Cryovials
- Dissecting microscope
- Microscope lamps
- Electric or battery-powered cautery
- Styrofoam boards
- Clear tape
- Straight pins
- Shaver
- Bead sterilizer
- Operating scissors
- Dressing forceps
- DeWecker scissors (Roboz; cat. no. RS5802)
- Micro Dissecting scissors (Roboz; cat. no. RS5918, RS 5912, and RS6301)
- Autoclips, 9 mm (BD Biosciences; cat. no. 427631)
- Autoclip applicator (BD Biosciences; cat. no. 427630)
- Autoclip remover (BD Biosciences; cat. no. 427637)
- Syringe (1 ml)
- Syringe needles (20-gauge and 27-gauge)
- Ear tag applicator
- Ear tags

## PROTOCOL

- Heat lamp
- Hemacytometer
- $^{137}\text{Cs}$  Irradiator
- Hamilton syringe (50  $\mu\text{l}$  with 22-gauge needle)
- 18-gauge needles
- Ultracentrifuge tubes
- 0.45- $\mu\text{m}$  filters (Millipore; cat. no. SLVH033RS)
- Syringes
- Packaging plasmid mix (Invitrogen; cat. no. K4975-00)

### REAGENT SETUP

**Epithelial cell medium** DMEM–Ham's F12 (1:1 ratio) medium supplemented with 5% calf serum (CS), insulin (10 ng ml<sup>-1</sup>), EGF (10  $\mu\text{g}$  ml<sup>-1</sup>), hydrocortisone (10  $\mu\text{g}$  ml<sup>-1</sup>) and 1 $\times$  Pen/Strep/Fung, stored at 4 °C. Preparation time: 10 min.

**Organoid plating medium** Ham's F12 medium supplemented with insulin (10 ng ml<sup>-1</sup>), EGF (10  $\mu\text{g}$  ml<sup>-1</sup>), hydrocortisone (10  $\mu\text{g}$  ml<sup>-1</sup>) and 1 $\times$  Pen/Strep/Fung, stored at 4 °C. Preparation time: 10 min.

**Primary cell medium** MEGM supplemented with bovine pituitary extract (included with purchase), stored at 4 °C. Preparation time: 5 min.

**Fibroblast medium** DMEM supplemented with 10% CS and 1 $\times$  Pen/Strep/Fung, stored at 4 °C. Preparation time: 10 min.

**10 $\times$  Collagenase solution** 15 mg ml<sup>-1</sup> collagenase, 1250 U ml<sup>-1</sup> hyaluronidase and 1 $\times$  Pen/Strep/Fung in epithelial cell medium, stored at -20 °C. For each 1–2 g of tissue, use 10 ml of a 1:5 diluted solution for a final working solution of 3 mg ml<sup>-1</sup> collagenase, 250 U ml<sup>-1</sup> hyaluronidase and 1 $\times$  Pen/Strep/Fung in epithelial cell medium. The longer you wish to digest the tissues, the more dilute the solution can be; thus, a 1 $\times$  solution will work but requires longer dissociation time. Preparation time: 10 min. 100 $\times$  and 10 $\times$  stock solutions can be stored at -20 °C for several months.

### PROCEDURE

Organoid separation

- 1| Sterilize instruments using a bead sterilizer or bunsen burner.
- 2| Bring the tissue into a clean, sterile tissue culture hood and remove yellow adipose tissue with operating scissors.  
**▲ CRITICAL STEP** When breast tissue is received it is kept on ice until dissociation. The tissue can be held on ice for several hours after harvesting.  
**! CAUTION** In accordance with institutional review board guidelines and regulations, human breast tissue is obtained from freshly discarded material of patients undergoing elective reduction mammoplasty surgery. Therefore, this tissue should be handled using the appropriate biosafety precautions, because it should be considered to be blood-borne pathogen/biohazardous.
- 3| Mince the sample with sterile razor blades until the tissue pieces are ~3–5 mm<sup>3</sup>.
- 4| Transfer 1–2 g of tissue to a 15-ml conical polypropylene tube filled with 10 ml of working collagenase solution.
- 5| Incubate the tubes on a rotator at 37 °C until the large tissue fragments are dissociated.  
**■ PAUSE POINT** This step typically requires 4 h but can be extended to overnight.
- 6| Remove the tubes from the incubator and let them stand for 2–5 min to allow organoids to settle. These large clusters of tissue represent the epithelial portion and are thus composed of luminal epithelial and myoepithelial cells.
- 7| Decant the supernatant to a fresh tube.  
**■ PAUSE POINT** This supernatant contains single stromal cells and can therefore be grown in fibroblast medium to expand, can be frozen back or can be simply discarded.
- 8| Wash the organoid-containing tube with PBS + 5% CS and centrifuge (300g on a tabletop centrifuge at 4 °C) for 5 min.
- 9| Repeat Step 8 three more times with 10 ml of PBS + 5% CS. (If stromal cells are to be plated they should be washed in a similar fashion.)

## BOX 1 | GENERATION OF PRIMARY EPITHELIAL CELLS

1. Resuspend cells in 10% of the volume of your desired culture plate in organoid plating medium, plated dropwise, and wait 1–2 min to allow the organoids to settle.
2. Slowly add the remaining 90% of the medium. Incubate cells in a humidified 37 °C tissue culture incubator with 5% CO<sub>2</sub>, feeding with the appropriate medium (pre-warmed) every other day after aspirating off the old medium.
3. Leave cells in the plating medium until the fibroblasts are gone; however, if the cells approach confluence, their volume should be split (this will accelerate fibroblast loss).
4. One will notice long, spindle-type cells contrasting with the smaller 'cobblestone' epithelial cells. Eventually (in ~1–2 weeks) these long fibroblasts will senesce, enlarge and die. From then on the epithelial cells should be cultured in MEGM. For more detailed information on culturing primary HMECs see Martha Stampfer's excellent review of the HMEC culture system at <http://www.lbl.gov/~mrgs/>.

■ **PAUSE POINT** At this point each conical tube of organoids can be frozen back as single aliquots in cryovials by resuspending organoids in 900  $\mu$ l of epithelial medium and 100  $\mu$ l of DMSO, and storing at  $-80^{\circ}\text{C}$ .

10| At this point cells can be plated to generate primary epithelial cells (**Box 1**) or used for generating normal structures in the mammary fat pad as discussed below.

11| To generate immortal fibroblasts from primary fibroblast cultures, infect a population with a retroviral construct generated to encode for the catalytic subunit of human telomerase (hTERT)<sup>8</sup>, and subsequently select the cells using a mammalian selection marker or by cell sorting depending on the viral construct backbone.

**Clearing**

12| Anesthetize a 3-week-old female mouse with tribromoethanol, administered i.p. at a dose of 250 mg kg<sup>-1</sup>. Place mouse ventral side up on a sterile Styrofoam board, and secure the limbs with clear tape.

13| Completely shave the area from the vagina to just below the third nipple, or an area approximately 150% greater than the area of surgery.

14| Liberally clean the shaved area from the center outward with betadine, and then 70% ethanol.

15| Locate the fourth and fifth sets of nipples and make an inverted Y incision from the midline point between the fourth set of nipples and ending at a point between the fourth and fifth fat pads on one side as previously shown<sup>11</sup>. Use dressing forceps to separate the skin from the body cavity.

16| Cauterize the nipple connection, the junction of blood vessels near the lymph node and the blood vessels and fat joining the fourth and fifth set of fat pads, forming a triangular area.

17| Use DeWecker scissors to remove the tissue in the cauterized triangle, and then repeat Steps 15 and 16 on the opposite side.

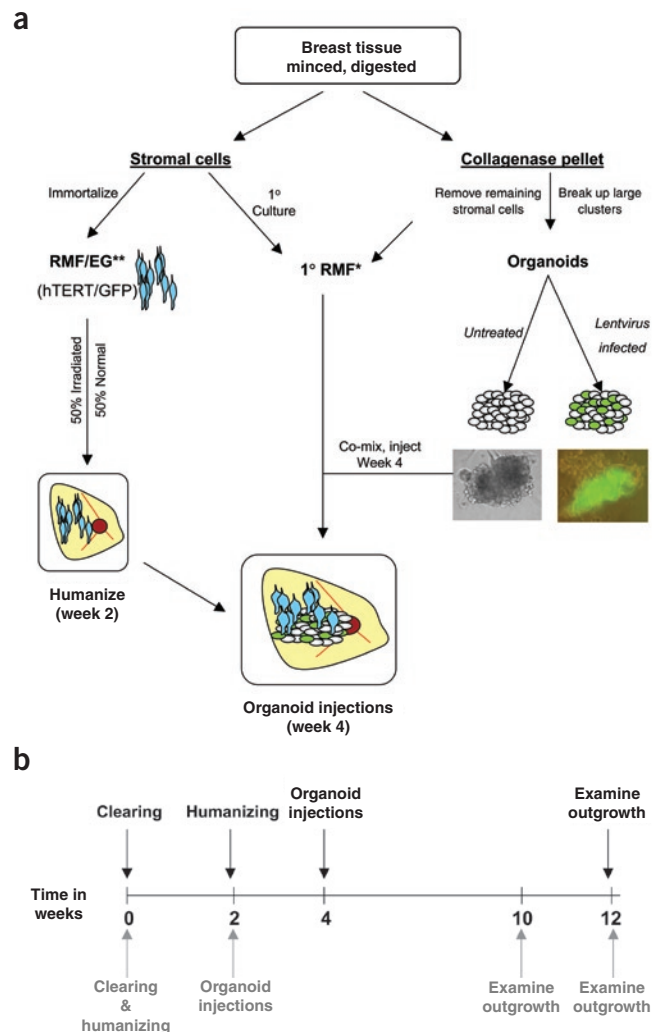
▲ **CRITICAL STEP** Insufficient clearing will lead to growth of mouse mammary epithelium and may affect your results; therefore, be sure to remove all of the tissue.

■ **PAUSE POINT** At this stage the animal can either be sutured and humanized as long as 2 weeks later, or can be humanized directly after clearing. If suturing, suture the animal with 9-mm autoclips as previously shown<sup>11</sup> and provide Sulfatrim in the water for as long as 2 weeks, changing at 7 d.

18| Apply ear tags to mice, place them in a clean cage and monitor them until they awake, keeping them warm with a heat lamp .

**Humanizing**

19| Culture and expand the RMF/EGs (**Box 2**).



**Figure 1** | Flowchart and timeline of the HIM protocol. (a) Breast reduction tissue is minced and digested, yielding a stromal and epithelial cell fraction. The epithelial cell clusters are additionally fragmented and frozen back in aliquots. The reduction mammary fibroblasts (RMFs) can be expanded as primary (1°) cultures or immortalized. Primary RMFs can also include CAFs isolated from dissociated tumor tissues or tumor organoids harvested from mastectomy samples. Such immortalized RMF/EGs can be modified further to overexpress any gene, growth factor, protease, etc. (e.g., HGF, transforming growth factor- $\beta$  (TGF- $\beta$ ), matrix metalloproteinase 9), and utilized for humanization. RMF/EGs are prepared for humanization by subjecting half of the needed cells to irradiation and mixing with an equal part of untreated cells. The resulting cells are implanted into the fourth inguinal gland of cleared 3-week-old immunocompromised mice. Two weeks later, organoids (infected with a gene(s) of choice or not) are co-mixed with normal, immortalized or other fibroblasts as described in the PROCEDURES and xenografted into the area of previous humanization. (b) Timeline for the protocol.

## BOX 2 CULTURE AND EXPANSION OF THE RMF/EG FIBROBLASTS

1. Culture cells in fibroblast medium (feeding every other day) at 37 °C 5% CO<sub>2</sub> until they approach 80–90% confluence.
2. Wash cells with PBS and trypsinize for ~2 min.
3. Use a cell lifter if not all cells are floating, and pipette the cells several times to break up cell clumps.
4. Wash off the plate with fibroblast medium (approximately four times the volume of trypsin used). Plates can be washed again to be sure all of the cells are harvested.
5. Spin the harvested cells at 300g for 5 min at 4 °C. Wash with PBS and repeat spin. Resuspend the pellet in fresh medium, and generate three to four plates from the single harvested plate.

These cells grow fairly quickly and thus will generate similarly confluent plates in a few days. If the cells are plated too sparsely, or were allowed to reach confluence previously, they will respond by growing extremely slowly and should be replaced with frozen back stocks. An 80–90% confluent 15-cm plate should yield  $\sim 6 \times 10^6$  to  $8 \times 10^6$  cells. If this number is not reached, either the serum or tissue culture methods are not optimal and the cells or media should be replaced.

**20|** One day before humanization but when the cells are 80–90% confluent, irradiate the necessary number of plates with 4 Gy, and leave the same number of plates untreated. (Each gland will require 250,000 untreated and 250,000 treated fibroblasts; one can generally retrieve 4–6 million cells from a 15-cm<sup>2</sup> plate at this confluence.)

**▲ CRITICAL STEP** Do not allow the fibroblasts to reach confluence at any point during their growth. This has profound negative effects on subsequent proliferation/expansion and engraftment.

**21|** On the day of humanization, harvest the fibroblasts by first washing the cells with PBS, and then trypsinizing them from the plates.

**22|** Count cells with a hemacytometer, and pool an equal number of irradiated and unirradiated cells in a 15-ml conical polypropylene tube. Spin cells at 300g at 4 °C and resuspend pellet in a volume of fibroblast medium corresponding to the number of cells (e.g.,  $5 \times 10^5$  cells in 35  $\mu$ l, thus  $1 \times 10^6$  cells in 70  $\mu$ l, 5 million in 350  $\mu$ l). Transfer resuspended cells into a microcentrifuge tube, and place on ice until injection. (As a general rule, always count and harvest at least 20% more cells than will be injected.)

**23|** As long as 2 weeks after clearing (**Fig. 3g**, stage i), reopen the animal using the same incision lines and expose the cleared gland. Inject slowly 35  $\mu$ l of the fibroblast cell suspension into the middle of the cleared fat pad using a 50- $\mu$ l Hamilton syringe and a 22-gauge needle. A bolus will be observed; take care to insert the needle deeply enough to prevent leaking. Twist the syringe upon removal to help prevent leaking.

**24|** Repeat on the contralateral side.

**25|** Follow Steps 17–18 for animal closing.

### Organoid injections

**26|** Prepare fibroblasts. If using primary fibroblasts (whether normal reduction or CAFs), culture as described in **Box 2** but make sure they have not been cultured for more than 14 d before injection. If using engineered or modified fibroblasts, use procedure as described in Steps 19–22 of the humanization protocol to expand and treat. For this injection, 500,000 fibroblasts are co-mixed with epithelial cells in each gland, so expand appropriately. Spin cells at 300g at 4 °C for 5 min to pellet and leave on ice while preparing the organoids.

**27|** Whether using a fresh organoid preparation or an aliquot of previously frozen back stock, add 1 ml of organoid plating medium to the 1- to 2-g preparation and pass through an 18-gauge syringe 5–10 times to break up any large clusters of cells. We prefer to first plate the organoids on a 15-cm<sup>2</sup> plate in fibroblast medium to allow the stromal cells to settle and help purify the epithelial fraction. The organoids will not settle; after 1–2 h at 37 °C, remove the supernatant containing the organoids, spin down at 300g at 4 °C for 5 min and continue with Step 28. To maintain the primary fibroblast culture, feed the plate with fibroblast medium, and in a short time any remaining epithelial components will slough off, leaving a pure population of fibroblasts.

**28|** Transfer the fragmented material to a 15-ml conical polypropylene tube and let the cells settle for ~1 min. At this point the large clusters of cells will fall to the bottom, while the single cells and organoid clusters (~100–1,000 cells) will still be in suspension.

**29** | Determine the number of organoids via a hemacytometer or by plating a known volume onto a tissue culture plate. Again, organoids are defined as clusters of cells several times the diameter of single cells but not overtly large. We typically yield about 5–10 organoids  $\mu\text{l}^{-1}$ .

**30** | Add the appropriate number of organoids to the pelleted fibroblasts such that each injection site per gland results in 30–50 organoids and 500,000 fibroblasts. For example, if one will inject five mice (and therefore need enough material for 10 glands), then prepare enough for 11 glands (e.g., 330–550 organoids mixed with  $5.5 \times 10^6$  fibroblasts). Pellet the epithelial-fibroblast mixture for 5 min at 300g on a tabletop centrifuge at 4 °C.

**31** | Resuspend the mixture pellet with 35  $\mu\text{l}$  of an ECM mixture. For normal outgrowth use a ratio of 1:3 (Matrigel–Collagen I). For preneoplastic and tumor conditions resuspend cells in 1:1 (Matrigel–Collagen I) mixture. (Again, if following numbers in Step 29, for 10 glands, resuspend the cell mixture in 385  $\mu\text{l}$  matrix.) Keep on ice until injection.

**▲ CRITICAL STEP** Matrigel must be kept cold at all times; thus it should be thawed on ice and then placed in prechilled tubes using chilled pipette tips. Moreover, because of the acidity of Collagen I, the Collagen I solution must be neutralized on ice and the Matrigel–Collagen I mixture should be allowed to neutralize for an additional 10–30 min after mixing, on ice, before adding cells.

**32** | Prepare mice for injections by reopening the animal using the same incision lines, and expose the cleared gland. Inject 35  $\mu\text{l}$  of the co-mixed cell suspension into the area of humanization with a Hamilton syringe. A bolus will be observed; take care to insert the needle deeply enough to prevent leaking. Twist the syringe upon removal to help prevent leaking.

**33** | Repeat on the contralateral side.

**34** | Close mice with autoclips.

**35** | If desired, hormone pellets can be implanted during this time as well. If using estrogen, a 1.7-mg 60-d slow-release pellet (Innovative Research of America) is desirable.

**36** | Normal outgrowths are evident 8 weeks after organoid injection. At that time the mice are killed ( $\text{CO}_2$  and subsequent cervical dislocation) and the glands are subjected to whole-mount analysis<sup>14</sup>, histological analysis or endpoint assays (e.g., serial passaging).

### Preparing virus

**37** | Plate  $\sim 1.3 \times 10^6$  293T cells in 10-cm<sup>2</sup> dishes using fibroblast medium.

The next day transfect the cells with a lentiviral vector encoding the gene of interest (pLenti6/V5, for example, from Invitrogen) and the packaging plasmid mix, following manufacturer's directions, via Fugene.

**! CAUTION** It is critical to follow institution rules and guidelines for these biohazardous agents.

**38** | The next day aspirate the medium from the plates and replace with fresh, pre-warmed fibroblast medium.

**39** | Collect the supernatant from the cells (filter through a low protein-binding 0.45- $\mu\text{m}$  filter), and spin in an ultracentrifuge at 85,000g for 1.5 h at 4 °C.

**40** | Immediately after centrifugation, carefully decant and discard the supernatant and add a small volume of plain DMEM to the pellet. Viral titers will depend on many things such as transfection efficiency and size of gene insert, so we typically add between 150 and 300  $\mu\text{l}$  of DMEM.

**41** | Let the pellet remain in the DMEM for 2–4 h at 4 °C. Gently pipette to resuspend, taking care not to introduce bubbles.

**■ PAUSE POINT** This step can be extended to overnight at 4 °C.

**42** | At this point the virus can be frozen back in aliquots at  $-80$  °C or used immediately for infections. Because our viral vectors contain GFP, we are able to determine our viral titers by fluorescence-activated cell sorting; thus, before infecting organoids we determine the viral titer.

### Infecting organoids

**43** | Following Steps 27–29, add 30–50  $\mu\text{l}$  of organoids to one well of a six-well plate (this can be scaled up for more than one gland) in a total of 2 ml of epithelial cell medium.

## PROTOCOL

**44** | Add  $10^5$ – $10^7$  colony-forming units of lentiviral particles plus  $8 \mu\text{g ml}^{-1}$  of polybrene, and spin the plate at  $32$ – $37^\circ\text{C}$  at  $300g$  for 1.5 h.

**45** | Place at  $37^\circ\text{C}$  for 1 h.

**■ PAUSE POINT** Repeat Steps 44–45 for optimal infection, then proceed with injections, or stop, add an additional 1 ml of pre-warmed epithelial cell medium and let incubate overnight at  $37^\circ\text{C}$  (**Fig. 2**).

**46** | Proceed with Step 30 of the organoid injection protocol, pipetting the infected organoid suspension into a 15-ml conical polypropylene tube containing the harvested fibroblasts (**Fig. 3**).

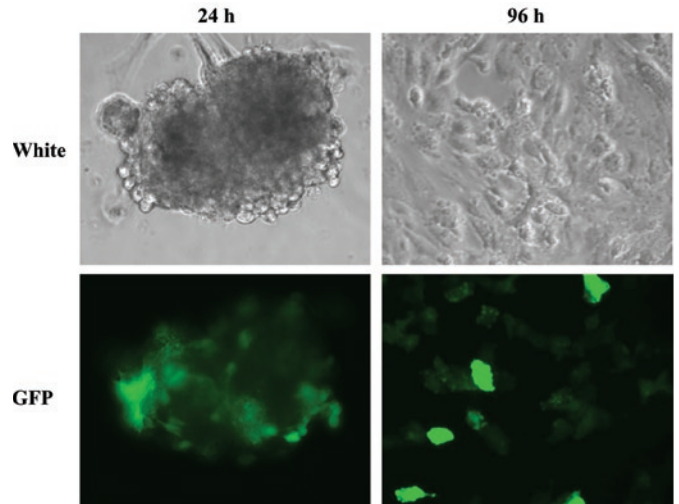
### ? TROUBLESHOOTING

#### Organoid separation

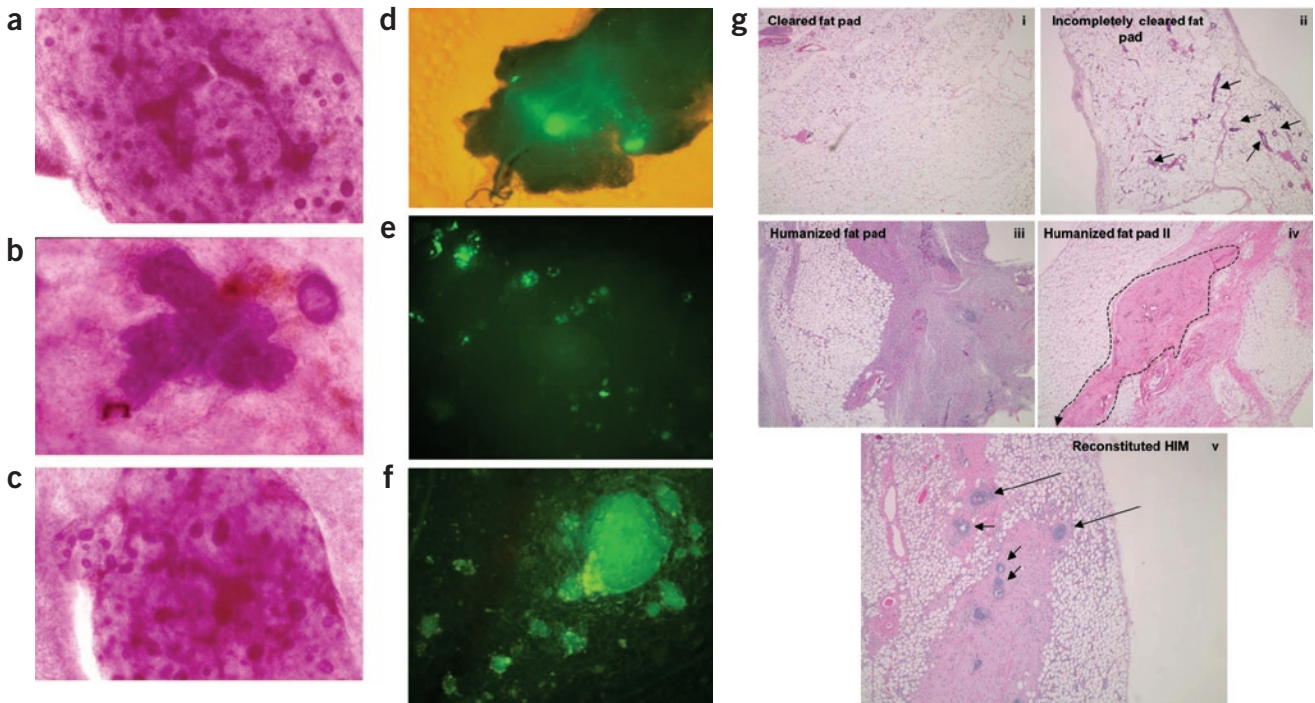
This procedure is straightforward and is unlikely to cause problems for the user; however, contamination is certainly possible. Therefore, it is important to make fresh solutions with Pen/Strep/Fung and maintain a high degree of sterility.

#### Clearing

Upon whole-mount analysis or immunohistochemistry, if mouse ducts are visible it is probable that the glands were not sufficiently cleared of their epithelium or that the epithelium of the fifth gland grew into the fourth fat pad. Be sure to



**Figure 2** | Lentiviral-mediated transduction of GFP into human breast organoids. Human breast organoids were isolated after digestion of reduction mammoplasty tissue and infected with a GFP lentivirus. Organoids were plated and examined for GFP expression. Phase contrast and fluorescence images of an organoid 24 h after infection and 96 h after infection.



**Figure 3** | Formation of ductal, lobular and acinar structures in HIM model. Carmine-stained whole mount of typical outgrowths seen from normal organoids humanized with (a) RMF/EG-TGF- $\beta$ , (b) RMF/EG or (c) RMF/EG-HGF. (d–f) GFP whole mount under a fluorescence dissecting microscope shows outgrowths of organoids infected with GFP lentiviral constructs 8 weeks after injection. (d,e) GFP whole mount of organoids infected with GFP vector at  $2.5\times$  and  $4\times$ , respectively. (f) Organoids infected with p53si and Her2/neu-GFP. (g) H&E-stained sections of tissues at various stages of the HIM protocol. All sections are taken at  $4\times$  magnification to illustrate the outgrowth proportion. (Stage i) A section of a cleared NOD/SCID mammary fat pad. (Stage ii) A section of an improperly cleared NOD/SCID gland. Note the outgrowth of endogenous mouse mammary epithelium (arrows). (Stage iii) A section of a cleared and humanized NOD/SCID mammary fat pad. (Stage iv) A section of a cleared and humanized NOD/SCID mammary fat pad that was injected with Matrigel–Collagen I. Note the injection site of the matrix (dashed line). (Stage v) Fully reconstituted HIM gland with human breast ductal structures evident (arrows).

cauterize the points highlighted, and remove all the tissue from the junction of the fourth and fifth gland to the lymph node in the fourth gland.

Although we typically use 21-day-old mice at ~8–10 g body weight, there are variations between manufacturers in the quality of mice. Therefore, if a 21-day-old mouse weighs less than ~7 g, wait until they have developed to this weight and avoid using mice heavier than ~12 g, because the epithelium may have overgrown at this point.

### Humanizing

Keys to success at this stage are proper culturing of the fibroblasts and a well-cleared fat pad. Primary fibroblasts should not be maintained in culture for >2 weeks, and none of the fibroblast lines should be allowed to reach confluence before harvesting.

If too much tissue is removed during clearing, it will be very difficult to humanize the sparse tissue remaining; therefore, remove only the triangle area of tissue defined by the cauterization. Additionally, if leakage occurs during injecting, attempt to inject the same area at a different location or humanize a different area. Injections should be done slowly to avoid leakage as well as to keep the cells localized in one area.

### Organoid injection

If organoid yield is a problem after plating to remove fibroblasts, ascertain that the medium contains serum, because the organoids will rest in serum-free medium. The plate can be gently washed with PBS to rinse off loosely attached organoids.

If no large structures form, it is possible that the organoids used were either too large or too small. Therefore, it is necessary to fragment the large organoids through a syringe, and after allowing the large clusters of cells to settle, take the supernatant for use in injecting. At this point the new supernatant can be allowed to rest, yielding organoids of the appropriate size, and the remaining supernatant containing single cells can be discarded.

At the time of injection, the area of humanization is not often easily recognizable. It is helpful to humanize approximately the same general area every time. We often see a white area at the site of humanization, which serves as a landmark for organoid injection. Stunted and deformed structures may be the result of not implanting into the humanized zone. We have also noticed such structures when organoids are injected into humanized fat pads in the absence of co-mixed fibroblasts.

If clearing was not sufficient, fat from the fifth gland may invade the fourth gland, so it is important not to inject in this area.

As with humanizing, inject slowly to avoid leakage and to keep the organoids concentrated in one area, not diffuse.

### Virus preparation and infection

Poor virus efficiency can have several causes; the most frequent cause is over- or underconfluence of 293T cells during transfection, not feeding the cells 24 h after transfection, and absence of polybrene. Certainly, the size of the target gene will have an impact on titer yield.

Because of the size of viruses, it is important to perform double-spin infections. Once the virus has been titered, it would be to the user's benefit to determine the appropriate amount of virus needed to obtain acceptable infections. Again, polybrene is an important component here, and if the organoids are too large, infection rates will be low.

Attachment of the organoids to the plates when cultured overnight can cause problems, so be sure to use the epithelial cell medium, and rinse the plates to dislodge those organoids that may settle out.

### ANTICIPATED RESULTS

Shown in **Figure 3** and ref. 8, normal organoids xenografted into humanized fat pads yield acinar structures with hollow lumina, as well as ductal outgrowths with limited side branching. Immunohistochemistry shows these structures to be functionally similar to normal human structures. Luminal epithelial cells stain for expected markers such as cytokeratin 19, E-cadherin and the estrogen receptor. On the basal side of the ducts, cells positive for smooth muscle are visible, indicating an intervening layer of myoepithelial cells.

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