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Rat Mammary Epithelial Cell Transplantation into the Interscapular White Fat Pad

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Cover Letter

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To: Vineeta Bajaj, Ph.D.
Review Editor
JoVE

08/04/2019

Dear Dr. Bajaj,

On behalf of all co-authors, please find enclosed the resubmission of our manuscript entitled "**Rat mammary epithelial cell transplantation into the interscapular white fat pad.**" to consider for publication in JoVE.

We have carefully considered all editorial and reviewers' critiques. Our responses are enclosed in the rebuttal document. The revised manuscript with track changes, figures and supplementary files are submitted for your review.

The reviews resulted in a major revision of the manuscript and we feel it greatly enhanced the clarity. We hope that you find our revised manuscript now suitable for publication in JoVE. Thank you for your consideration.

Sincerely,

Bart M. G. Smits, PhD

Affiliate Assistant Professor

Department of Pathology & Laboratory Medicine
Medical University of South Carolina

TITLE:

Rat Mammary Epithelial Cell Transplantation into the Interscapular White Fat Pad

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KEYWORDS:

rat mammary gland; epithelial transplantation; monodispersed cells; quantification; mammary gland development; breast cancer; carcinoma; graft

SUMMARY:

This article describes a transplantation method to graft donor rat mammary epithelial cells into the interscapular white fat pad of recipient animals. This method can be used to examine host and/or donor effects on mammary epithelium development and eliminates the need for pre-clearing, thereby extending the usefulness of this technique.

ABSTRACT:

As early as the 1970s, researchers have successfully transplanted mammary epithelial cells into the interscapular white fat pad of rats. Grafting mammary epithelium using transplantation techniques takes advantage of the hormonal environment provided by the adolescent rodent host. These studies are ideally suited to explore the impact of various biological manipulations on mammary gland development and dissect many aspects of mammary gland biology. A common, but limiting, feature is that transplanted epithelial cells are strongly influenced by the surrounding stroma and outcompeted by endogenous epithelium; to utilize native mammary tissue, the abdominal-inguinal white fat pad must be cleared to remove host mammary epithelium prior to the transplantation. A major obstacle when using the rat model organism is that clearing the developing mammary tree in post-weaned rats is not efficient. When transplanted into gland-free fat pads, donor epithelial cells can repopulate the cleared host fat pad and form a functional mammary gland. The interscapular fat pad is an alternative location for these grafts. A major advantage is that it lacks ductal structures yet provides the normal stroma that is necessary to promote epithelial outgrowth and is easily accessible in the rat.

Another major advantage of this technique is that it is minimally invasive, because it eliminates the need to cauterize and remove the growing endogenous mammary tree. Additionally, the interscapular fat pad contains a medial blood vessel that can be used to separate sites for grafting. Because the endogenous glands remain intact, this technique can also be used for studies comparing the endogenous mammary gland to the transplanted gland. This paper describes the method of mammary epithelial cell transplantation into the interscapular white fat pad of rats.

INTRODUCTION:

Postnatal mammary gland development and ductal morphogenesis are processes largely influenced by hormonal signaling at the onset of puberty. In mice and rats, commonly used model organisms of mammary gland biology, this process begins around 3 weeks of age, where rapid proliferation and differentiation result in the formation of the mature parenchyma. The mature mammary gland can undergo numerous rounds of expansion and involution, a property that has been under investigation since the early 20th century. Within the context of hyperproliferation and cancer development, mammary gland transplantation techniques were developed in the 1950s[1], and enhanced by the quantitative methodology contributed by Gould et al. in 1977[2-4]. Refinement of the transplantation technique in rodents has contributed to major advances in understanding normal mammary gland biology that are still widely used to study the effect of various treatments and genetic manipulation on normal mammary gland development and disease states.

Many hypotheses have been generated and subsequently tested using mammary gland transplantation, first described by DeOme et al. in 1959[1]. Experiments across several decades showed the propensity of ductal tissue excised from donor mammary glands to repopulate the entire fat pad[5-7] and indicated that a critical component of mammary gland development resides in these epithelial structures. Later studies in mice showed that a single mammary stem cell can repopulate a cleared fat pad and contributed to the discovery of a single, common progenitor of basal and luminal mammary epithelial cells[8-10]. In line with these conclusions, it has been suggested that transplantation increases the pool of cells with multilineage-repopulating potential as a result of plasticity, allowing the grafted cells to grow a functional mammary gland[7,10-13]. Importantly, the use of transplantation techniques in rodents overcomes the limitations of cell culture-induced abnormalities[14] and often provides results in just a matter of weeks.

While the procedure was originally described in the context of preneoplastic lesions in mice, it was soon expanded to rats and used in conjunction with the carcinogen treatment to establish multiplicity as a measure of cancer susceptibility[15], but the popularity of transplantation techniques has followed the development of genetic tools for each species. Although mouse studies incorporating transplantation have contributed many translational findings, the parenchyma of the rat mammary gland resembles the human more closely[16,17] and offers distinct advantages for studying estrogen receptor-positive (ER+) breast cancer. Mammary tumors are inducible in both species, but they differ in terms of hormone sensitivity and gene expression profiles. A primary difference is that rat mammary tumors express and depend on the function

of ovarian and pituitary hormone receptors, namely, estrogen and progesterone (PR), similar to the luminal-A subtype of human breast cancer. Indeed, mammary epithelial cell transplantation, as described in this protocol, has been used to study genetic variants involved in breast cancer and determine the cellular autonomy of effects on mammary epithelial cells[18].

In addition to the tumor biology, the ductal epithelium of the normal rat mammary gland exhibits a higher level of branching and is flanked by a thicker layer of stroma than the mouse. The importance of the stroma is well-documented in mammary epithelial transplantation studies. Mammary epithelium must interact with fatty stroma, and ideally its own mesenchyme, to undergo its characteristic morphogenesis[19,20]. Grafting tissue into a recipient mammary gland provides an optimal environment; however, the presence of endogenous epithelium can interfere with results. Preclearing the mammary gland of endogenous epithelium is commonly performed in mouse transplantation assays and requires surgical excision of endogenous mammary tissue and/or removal of the nipple[1,21,22]. Although possible, preclearing the mammary epithelium in post-weanling rats is not as widely-performed, mainly due to the ineffectiveness of clearing the growing mammary tree in post-weanling rats. Since it has been shown that regions of adipose tissue elsewhere in the body could support the growth of transplanted mammary epithelium[21,23,24], the process of preclearing can be easily avoided in rats by grafting tissue into the interscapular white fat pad.

The transplantation method described in this paper involves the injection of enzymatically dissociated mammary gland organoids (fragments of mammary ductal epithelium and other cells types capable of morphogenesis) or monodispersed cells into the interscapular fat pad in inbred, isogenic or congenic strains of laboratory rats[2]. Because the interscapular fat pad is normally devoid of mammary tissue, it provides a suitable environment for multiple transplantation sites without the need to pre-clear endogenous epithelium. As a result, the host animal's endogenous, abdominal-inguinal mammary glands are not subject to surgical manipulation, develop normally, and cannot interfere with interpretation of results. Additionally, the intact mammary glands can be used for comparison to evaluate host versus donor effects on the mammary epithelium development and tumorigenesis[18,25]. Although repopulation of the mammary gland from a single stem cell is available for mice, it has not yet been developed for rat, mainly due to the lack of availability of antibodies to select for rat mammary stem cells[25-27]. Despite this, transplantation of monodispersed mammary epithelial cells to quantify repopulating potential can be successfully performed, and those cells will develop normally when grafted into the appropriate framework[2-4]. While organoids are good for many purposes, monodispersed cells are required for quantitative applications, for example, to determine the number of mammary epithelial cells required for the cancer initiation following ionizing radiation treatment[28] or for comparing characteristics of flow cytometrically selected mammary epithelial cell populations[29].

To date, the procedure described here is the most robust method of performing mammary gland transplantation in the rat with an overall goal of studying mammary gland development and mechanisms underlying breast cancer development. Often, the donor and/or recipient animals are exposed to different variables before, during, or after the epithelial transplantation. Examples include single gene studies involving chemical carcinogenesis[30], radiation[28,31,32], genetic

manipulation of host/donor genome[18], and hormonal manipulation[12]. A major advantage of the enzymatic dissocation described in this protocol is the opportunity to isolate epithelial organoids or monodispersed cells for complementary experiments involving flow cytometry, 3-D culture, gene editing, and more. Future applications of this technique will include additional manipulation of donor and/or host tissue with genetic engineering. For example, donor cells can be genetically altered ex vivo at any chosen genomic locus using the CRISPR-Cas9 gene editing system. Similarly, recipient rats can also be genetically altered to study the interaction between donor and recipient engineered genetic factors.

PROTOCOL:

All animals were housed and maintained in an AAALAC-approved facility, and experiments described in this protocol were approved by the MUSC Institutional Animal Care & Use Committee (IACUC). Animals for use in reciprocal transplantation should be an inbred or isogenic strain, with congenic status preferred or backcrossed for at least 6 generations.

1. Harvesting donor rat mammary gland epithelium

1.1. Determine the number of donor rats needed for transplantation.

NOTE: Generally, 1 donor rat (4 weeks of age) can provide enough cells for transplantation into 4 recipient animals. Certain applications of this protocol will require additional numbers of cells, and there can be strain-specific differences in total yield.

1.2. Label all supplies and ensure accessible placement for the surgeon (**Table 1**).

1.3. Record the body weight of each female donor rat. Follow institutional guidelines to fully anesthetize or euthanize the donor rat. Check for the depth of anesthesia by the lack of response to the toe pinch.

1.4. Move the animal to the sterile surgical field. Place the animal on its back and spray the entire ventral surface with 70% ethanol.

1.5. Make a sagittal, X-shaped incision, allowing access to thoracic, abdominal and inguinal mammary glands. Dissect all the mammary gland tissue from the donor rat using scissors (**Figure 1**). Remove all visible lymph nodes.

1.6. Extract the mammary tissue using forceps and place in a labeled 60 mm dish on ice. Add 500 µL of serum-free DMEM/F12 media to the 60 mm dish. Adjust the placement of the mammary gland tissue so it stays completely wet.

1.7. Finely mince the mammary tissue using scissors. To do so, cut the tissue into pieces 1-2 mm^3 in size (**Figure 1C**). Keep the gland on ice until all the donor tissue has been harvested (do not exceed 60 min).

NOTE: Additional personnel can mince mammary glands and additionally prepare collagenase solution while the surgeon proceeds with tissue extractions.

2. Extract brain tissue from euthanized donors

2.1. Carefully turn the body of the donor animal over to place it in a prone position. Secure with pins. Spray the head and upper back with 70% ethanol.

2.2. Locate the base of the skull and make an incision beneath the occipital condyles. Insert sharp scissors beneath the skin and cut the skin away from the skull, including the sides of the head.

2.3. Use bone cutters or strong scissors to cut the skull along the midline, from the occipital to frontal bones. Keep the blade as superficial as possible and angle upward to prevent the destruction of the underlying brain tissue.

2.4. Peel the bone away using rongeurs or strong forceps. Insert the tool lateral to the cerebellum to break the bone on either side, exposing the bony auditory canal. Sever the connections to the meninges.

2.5. Gently lift the brain with curved, fine-tip forceps. Place the brain on a piece of foil and record the weight, and then immediately transfer to a 15 mL tube with an equal amount (w:v) of media, stored on ice.

2.5.1. Optionally, use fine-tip forceps to remove the pituitary gland (located beneath the brain) for additional use in the transplant procedure, if needed.

2.6. Use a mechanical homogenizer to disrupt the tissue. Homogenize the brain for 10-15 s on low speed. Let the mixture sit on ice for at least 1 min, and then homogenize again. Homogenization is sufficient when the final mixture is free of large pieces.

2.7. Filter the homogenate by passing it through a 100 µm filter. Keep the filtrate on ice until use (less than 4 h).

3. Digestion and processing of mammary gland extracts

3.1. Thaw or warm reagents as indicated (**Table 1**). Follow the appropriate steps for recovering organoids or monodispersed cells.

3.2. Prepare 10 mL of serum-free digestion media (without collagenase) for every donor animal (**Supplemental File 1**).

NOTE: The volume of digestion media used can be adjusted (scaled up or down) to accommodate the grouped tissue, if applicable.

3.2.1. For organoids skip to 3.3.

3.2.2. For monodispersed cells, prepare fresh Monodispersion Mixture and Inactivation Solution (**Supplemental File 2, Supplemental File 3**) in addition to the serum-free collagenase digestion media. Proceed to step 3.3.

3.3. When all the mammary tissue from donors has been extracted and minced, add the collagenase enzyme to the warm (or room-temperature) digestion media (**Supplemental File 1**). Mix by inverting.

3.4. Pass the collagenase digestion media through a 20 µm filter. Dispense 10 mL of filtered media in the labeled 50 mL tubes for digestion.

3.5. Use a new 1,000 µL pipette tip for each sample and transfer the minced donor tissue from each 60 mm dish to the collagenase digestion tube. Cut 1 cm off the end of a 1,000 µL tip and manually place it on the device before use. Gently mix the minced tissue by pipetting up and down 1-2 times.

NOTE: If the tissue is difficult to pipette during transfer, use a small amount of the collagenase digestion media from the 50 mL tube, and then transfer it back.

3.6. Place the samples in the horizontal position in a shaking incubator and allow the samples to digest 1.5-2 h at 37 °C, 200-220 rpm.

3.6.1. For organoids skip to 3.7.

3.6.2. For monodispersed cells add DNase I (0.2 µg/mL) to the mixture for the last 10 min of the digestion. Incubate as before, with vigorous shaking. Proceed to step 3.7.

3.7. When the tissue is fully digested, pellet the suspension using cold centrifugation (4 °C) for 10 min at 1,200 *x g* (**Figure 1D**).

NOTE: Keep tubes on ice between every step to increase viability of cells.

3.8. Ensure that a pellet has formed, and then carefully pour off the supernatant and fat layer. Gently resuspend the pellet in 10 mL of fresh DMEM/F12 media.

3.9. Briefly spin at 68 *x g* for approximately 10 s. The length of the spin (but not the speed) may be increased if there is no clear separation of cells. Visually inspect the pellet before proceeding (**Figure 1D**).

3.10. Carefully remove the supernatant, leaving behind a small volume. Proceed to the next step, based on whether organoids or monodispersed cells are needed.

NOTE**:** It is important to leave a small volume of media in the tube because the pellet will be very loose. The residual volume of media will be diluted through wash steps.

3.10.1. For organoids, add another 10 mL volume of DMEM/F12 media and repeat the wash/spin. After the second wash, resuspend the cells in a smaller volume (1-2 mL) of DMEM/F12 media for filtration. Proceed to step 3.11.

3.10.2. For monodispersed cells, dissolve in 2 mL of pre-warmed HBSS with 0.025% (w/v) Trypsin and 6.8 mM EDTA. Digest for 3-5 min at 37 °C. Inactivate immediately.

3.10.2.1. Add 4 mL of DMEM/F12 with 10% FBS to stop inactivate the trypsin.

3.10.2.2. Spin the cells at 270 *x g* for 5 min, and then resuspend in DMEM/F12 with 10% FBS again. Proceed to step 3.11 to filter the cells.

3.11. Filter the cells using a 40 µm cell strainer placed in a new 50 mL tube. Pre-wet the strainer by pipetting 1 mL of the same base medium used to suspend the cells, and then pass the cell suspension through the filter using a pipette to collect ductal fragments/organoids.

NOTE: The approximate yield of filtered epithelium from the mammary gland tissue of a single, 4-week-old donor rat is 1 x 10^6 cells.

3.11.1. For organoids, discard the filtrate. Mammary organoids will remain inside the basket of the cell strainer and smaller, unwanted cells will be eliminated. Invert the cell strainer over a new 50 mL tube, and rinse with any volume necessary to collect the cells. Proceed to step 3.12.

3.11.2. For monodispersed cells, discard the cell strainer and keep the filtrate because the mammary epithelial cells will pass through the filter, along with smaller stromal and immune cells. Rinse the tube that was used for the digestion/centrifugation with another volume of DMEM/F12 with 10% FBS and pass through the same cell strainer. Pellet the monodispersed cells by centrifugation at 1,200 *x g* for 5 min. Proceed to step 3.12.

NOTE: The resuspended cells are ready for subsequent applications.

3.12. Pulse spin the solution and ensure the formation of a cell pellet before proceeding to the next step. Pulse spin again, if necessary.

3.13. Carefully remove the supernatant. Resuspend the pellet in a small volume (1,000-2,000 µL of DMEM/F12) to concentrate the cells for counting.

3.14. Count cells and dilute if needed. Resuspend the desired number of cells for transplantation in 20 µL of DMEM/F12 media for each animal. Always keep cells on ice.

NOTE: Donor cell counts in the range of 1 x 10^5 – 1 x 10^6 cells will be required for each graft site based on the endpoint of the study. For example, carcinogenesis experiments often require a greater number of transplanted cells, relative to other applications. The number of cells needed must be experimentally determined for each strain. The procedure described in this protocol utilized 250,000 donor cells in 20 µL media per graft site, prior to mixing with brain homogenate, as described in step 3.16.

3.15. Prepare any aliquot(s) of cells needed for other experiments (e.g., FACS isolation[3,26,27,30,33]).

3.15.1. For organoids, proceed to step 3.16.

3.15.2. For monodispersed cells, quantify the viable cells using Trypan or methylene blue staining, and then proceed.

3.16. Prepare single batches of donor material for all the transplant recipients. Combine equal volumes of the cell suspension (20 µL) with 50% brain homogenate (20 µL) for every site of transplantation.

NOTE: A total of 40 µL per site will be injected at each site, but it is recommended to include a minimum of 25% extra volume for waste.

3.17. Immediately proceed to transplantation or freeze cells for the transplantation later (potential stopping point).

NOTE: Frozen cells have not been tested with this protocol and will require optimization in advance of generating a cohort of animals for transplantation experiments. It is strongly recommended to transplant fresh cells.

4. Tranplantation procedure (recipient rats 4-5 weeks of age)

4.1. Weigh each recipient rat and calculate the correct dose of approved analgesic that will be used in the procedure.

NOTE: Body weights can be measured up to 24 h in advance of the procedure. Follow institutional guidelines to restrain or briefly anesthetize each animal for the duration of shaving.

4.2. Shave the surgical area on each animal using electric clippers. Identify the base of the skull and start of the vertebral column. Approximately one-third of the way down the spine, shave a 3 cm x 2 cm area on the upper thoracic portion of the back.

NOTE: Shaving can also be performed under anesthesia on the day of transplantation but must be performed outside the sterile field. Return the rat to its home cage until it is needed.

4.3. Locate all of the supplies needed for transplantation (**Table 1**).

4.4. Evaluate the Laboratory Animal Anesthesia System before use. Top off any fluids and replace any tanks or parts that are needed. Ensure the gas line to the anesthesia chamber is open and all peripheral lines are closed so isoflurane anesthesia and oxygen may freely flow to the animal once it is placed in the chamber.

4.5. Warm heating pads to support the body temperature of recipient animals.

4.6. Generate the sterile field that will be used for surgery. Arrange the supplies as described in **Table 1.**

4.7. Administer preoperative analgesic to recipient rats as indicated by institutionally-approved animal care protocol.

4.8. Flush the Hamilton syringes with sterile DMEM/F12 media to prevent loss of cells. Ensure the needle is secured to the body of the syringe, insert the needle into the liquid, and draw back the plunger. Fill to the maximum volume. Press the plunger down and expel the contents into a waste collection tube. Repeat 3-5 times.

4.9. Load the entire volume of donor material (prepared in step 3.16) into a separate syringe for each condition (e.g., control, treated, wildtype, knockout, etc.). Insert the tip of the needle into the liquid, draw back the plunger and keep the needle beneath the surface of the mixture as the volume in the tube decreases.

NOTE: Include at least 10% extra volume in each syringe. Do not dispose of the remaining mixture close the tube and keep it on ice in case more is needed.

4.10. Invert the syringe after it is fully loaded and press the plunger slightly to remove air bubbles at the tip of the needle. Proceed to the next step when everything is prepared.

NOTE: Make sure the tip of the needle never touches any other surface, even within the sterile field. It is helpful to rest the body of the syringe over a small container of ice to promote viability of the cells.

4.11. Place the recipient animal in the anesthesia chamber and turn on the machine.

4.12. When the animal is fully relaxed (does not react to tapping or gentle movement of the chamber), direct the anesthesia to the nose cone and transfer the animal to the sterile field.

NOTE: Extended duration of anesthesia is not well-tolerated by rats. Complete the procedure for each animal in 10 min or less.

4.13. Place the animal in a prone position (on its stomach) so the back of the head and the upper spine is accessible.

NOTE: Ensure adequate heat support for the animal all times and regularly assess the depth of anesthesia using a firm toe pinch.

4.14. Optionally, apply ophthalmic vet ointment to prevent drying of the eyes.

4.15. Clean the freshly-shaved area to remove excess hair. Use a circular motion and apply 70% ethanol (or another reagent, per institutional guidelines) to the skin, followed by an antiseptic (such as iodine), and repeat. Place a towel drape over the animal so only the region for the shaved area is exposed.

4.16. Ensure the animal remains unresponsive to deep stimuli with a firm toe pinch, and then proceed to the next step.

4.17. Make a small (2 cm) interscapular incision using a sharp surgical blade.

NOTE: The cut must be superficial, as the fat pad is located just beneath the skin.

4.18. Locate the medial blood vessel for orientation (**Figure 2B**).

4.19. Lift the skin on one side of the incision using forceps and hold it away from the fat pad while the transplant is performed (**Figure 2B**). Insert the needle into the graft site.

4.19.1. Optionally, move the tip of the needle inside the tissue and create a small pocket to collect the cells. Use a small, repetitive motion. Do not remove the needle.

NOTE: This step is recommended for first-time users of the protocol. Use extreme caution when creating a pocket, as the interscapular fat pad tissue is very delicate.

4.20. Carefully inject 40 µL of the cell mixture into the interscapular fat pad tissue. Remove the needle slowly.

4.21. Hold the tissue in place and allow the transplanted cells to settle for 3-5 s. Use an additional pair of forceps , if needed.

4.22. Remove the needle. Repeat the injection procedure (steps 4.18-4.21) at the second site of transplantation.

NOTE: The epithelium from one donor group can be injected into the same side of the fat pad in every animal, or alternating sides to prevent batch effects from the hand-dominance of the surgeon.

4.23. Close the surgical wound using wound clips or sutures , and then discontinue anesthesia.

4.24. Provide post-operative analgesic as indicated by institutionally-approved protocol.

4.25. Immediately move the animal to a recovery cage with the heat support. Monitor for signs of distress such as bleeding from the incision or trouble breathing.

NOTE: The animal should fully recover within 5-10 min. Refer to institutional guidelines for returning animals to the colony and post-operative monitoring after survival procedures.

4.26. Optionally, perform carcinogenesis studies at the graft site(s) by administering carcinogens to the recipient rats 3-4 weeks after transplantation.

NOTE: Typically, rat mammary carcinogenesis is performed using a chemical carcinogen treatment at 50-57 days of age. This treatment dictates the age of the transplant surgery (which must be done between 29-36 days of age) to allow enough time for the grafted cells to initiate growth of the mammary gland.

5. Assessment of epithelial outgrowth

5.1. Monitor the estrus cycle of rats through daily vaginal lavage and examine the cytology on a microscope slide. Begin 8-12 days before the endpoint of the study. Sacrifice all rats in the same stage. This is an optional step.

NOTE: The rat estrus cycle is 4-5 days. Allowing the animal to go through 1-2 full cycles will facilitate interpretation, as lavage slides from previous cycles can be used for comparison.

5.2. Sacrifice transplant recipient rats 6-8 weeks after transplantation, per institutional guidelines.

NOTE: Outgrowth is usually detectable 3-6 weeks after transplantation, but additional time may be required.

5.3. Place the animal in a prone position and clean the body with 70% ethanol. Lift the skin with forceps and make an incision along the vertebral column to expose the interscapular fat pad. Dissect the skin away from the tissue so the majority of the fat pad is visible.

5.4. Identify the medial blood vessel that separates the graft sites in the interscapular fat pad. Excise the entire pad as a single piece of tissue or cut along the blood vessel and remove sides individually.

5.5. Place the tissue on a positively-charged microscope slide for whole mount. Use 2 pairs of blunt forceps and gently spread the tissue to restore its original conformation on the slide.

NOTE: Rat mammary tissue is extremely delicate. The edges of the tissue may curl under itself. Always handle with care and hold in place until the tissue adheres to the slide (a few seconds).

5.6. Whole-mount at least one of the endogenous abdominal-inguinal mammary glands (with lymph nodes for orientation) for comparison.

5.7. Place the slides in 70% ethanol for 7-10 days to defat the tissue. Replenish ethanol as often as necessary to ensure the tissue does not dry out.

5.8. Prepare alum-carmine stain and process the slides when the tissue is sufficiently opaque. Allow the stain to cool before usage (**Supplemental File 4**).

NOTE: The stain can be prepared up to one day in advance of the fixation and rehydration steps. The solution can be stored at 4 °C and has limited potential for reuse.

5.9. Fix the tissue by placing the slides in 25% glacial acetic acid : 75% ethanol for 60 min.

5.9.1. Rehydrate the tissue through a series of 3 washes in a series of decreasing concentration of ethanol: 70% ethanol for 15 min, 50% ethanol for 5 min and dH_2O for 5 min.

5.10. Stain with alum carmine for 4-8 days. Check the back of the slides each day to determine if the stain has fully penetrated the tissue. Proceed to the next step when the staining is complete.

NOTE: Staining is complete when the thickest parts of the gland have a purple hue and no longer appear white.

5.11. Destain and dehydrate the tissue by transferring the slides through a series of increasing concentration of ethanol: 70% ethanol for 30 min, 95% ethanol for 30 min and 100% ethanol for 30 min.

5.12. Place dehydrated slides in xylene for 3+ days to clear the tissue. Transfer to mineral oil for long term storage.

5.13. After the slides have cleared, use low-powered light microscopy or high-resolution digital photography to acquire images of the slides for analysis. Ensure image acquisition parameters are consistent for all slides.

NOTE: Epithelial outgrowth must be clearly distinguishable.

5.14. Treat the presence of outgrowth as a binary outcome.

5.15. Calculate the mean number of transplanted epithelial cells that produced ≥1 mammary outgrowth in 50% of graft sites using the acquired images. Quantification other physical features, as needed.

REPRESENTATIVE RESULTS:

Donor and recipient mammary glands

The steps to isolate and prepare rat mammary epithelial cells for transplantation are shown in **Figure 1A**. At 4 weeks of age, the endogenous mammary gland of the donor rat has begun maturation and epithelium can be visualized on whole mounted slides stained with alum carmine (**Figure 1B**). One donor rat at this age will provide approximately 1 x 10^6 cells for transplantation. If the amount of donor tissue collected or subsequent mincing is insufficient, the yield of cells after collagenase digestion may be low. As such, it is important to collect as much mammary gland tissue as possible from the donors. Fully digested mammary gland tissue should have an oily appearance, with no visible pieces of tissue in the suspension (**Figure 1C**). Complete digestion of the mammary gland tissue from a single donor should result in the formation of a visible pellet that contains the mammary organoids, as shown in (**Figure 1D**). If a pellet is not visible, the assay may require optimization. In **Figure 2**, rat mammary gland and interscapular fat pad locations are shown. Results cannot be interpreted unless biological reference points are properly identified at the time of tissue collection (**Figure 2A**) and transplantation (**Figure 2B**). In this assay, the medial blood vessel of the interscapular white fat pad is used as a biological reference point.

Qualitative and quantitative assessment of epithelial outgrowth:

The presence, absence, or abundance of epithelium can be evaluated to determine success of the experiment, as well as the autonomy of effects related to experimental variables. For the latter, certain studies may require whole mounted slides with the endogenous abdominal-inguinal mammary gland of the host for comparison. As a preliminary measure, light microscopy can be used to document the epithelial outgrowth as a binary outcome. These data can be statistically analyzed to test the hypothesis that graft rejection is dependent on donor or recipient variables. A reciprocal transplantation experiment where each of these factors has 2 levels- for example, wildtype (WT) vs knockout (KO), will create 4 transplant groups for hypothesis testing (**Figure 3A**). The transplant groups, expressed as donor:recipient genotype, are: WT:WT, WT:KO, KO:KO, KO:WT. When isogenic or near-congenic animals are used, graft rejection is minor and occurs equally across the transplant groups. One advantage of multiple sites for transplantation within the interscapular fat pad is a reduction of recipient animals needed, since 2 donor cell types can be evaluated in a single host. Additionally, both sites can be used to test a single donor cell type at a higher incidence rate, using the same number of rats. Using genotype as an example, this is demonstrated in **Figure 3B**.

The epithelial outgrowth can also be analyzed using images of the slides that were previously acquired. An example of a whole mounted interscapular fat pad containing epithelial outgrowth at both graft sites (recorded as positive outcomes) is shown for an experiment using the mammary cell transplantation protocol described here (**Figure 3C**). Lack of epithelium in the interscapular fat pad across many samples may indicate a technical problem with the procedure and is treated as a negative outcome.

The outcome of reciprocal transplantation experiments can be further used to distinguish effects that are autonomous or non-autonomous to mammary epithelial cells. To test the hypothesis that an effect is driven by processes in the mammary epithelial cells (cell-autonomous) or influenced by the host/microenvironment (non-autonomous), concordance of donor cell

phenotype to that of the host (endogenous) phenotype is treated as a dichotomous outcome. In an example such as a carcinogenesis assay, tumor incidence can be analyzed as binary response data, and logistic regression analysis used to determine if the donor, host, or donor-host interaction contributes significantly to the tumor incidence rate at the transplant site. If the effect is driven by properties of the donor epithelium, a similar transplantation outcome can be observed across recipient groups, irrespective of the host's condition. If donor epithelium develops as if it were endogenous to the host, due to a contribution of the host's genotype or treatment, the effect may be non-autonomous. In both situations, the transplantation groups where donor epithelial cells matched the host (self:self) should be interpreted as controls, and conclusions supported by statistical analyses.

To demonstrate results of autonomous and non-autonomous effects on transplanted epithelium, an illustration has been provided (**Figure 4A**), along with slides from reciprocal transplantation of wild type and *Cdkn1b* knockout rat mammary epithelium experiments. Results of this study suggested non-mammary cell-autonomous effects[18] (**Figure 4B**)**.** For reciprocal transplantation outcomes classified as a binary response, the likelihood of the outcome (e.g., concordance of phenotype to host epithelium, or successful outgrowth in quantitative assays) being dependent on categorical variables (e.g., donor or recipient genotype) can be tested by building a logistic regression model for main effects and interaction terms.

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of donor mammary glands. (**A**) Overview of the procedure to extract mammary gland tissue from donor animals and recover organoids for transplantation. (**B**) Whole-mounted endogenous abdominal-inguinal mammary glands of a 4-week old rat (typical age of transplant donors), after alum carmine staining. At 4 weeks of age, the mammary epithelium was in the process of expanding, but the ductal tree does not fully penetrate the fat pad, as evidenced by proximity to the central lymph nodes. (**C**) The consistency of the minced mammary gland is shown after chopping (pink) in a 60 mm dish on ice, with an appropriate amount of DMEM/F12 media to keep it moist. The adjacent images provide a comparison of the minced epithelium after transferring the slurry to Collagenase Digestion Media and when the digestion is complete, 90-120 minutes later. (**D**) The pelleted epithelial organoids and layer separation are visible after centrifugation.

Figure 2: Identification of boundaries for tissue collection and mammary epithelial cell injection. (**A**) Locations of rat mammary glands for tissue collection are shown. The approximate location of endogenous abdominal-inguinal mammary glands harvested from donors and recipients is outlined. (**B**) After shaving and making a superficial incision, the white interscapular fat pad of a transplant recipient is exposed. Top image: the medial blood vessel (yellow arrow) is visible in the center of the incision. Bottom image: the skin on one side of the incision is lifted to show the width of the IS fat pad underneath the skin, relative to the medial blood vessel (yellow arrow). Donor epithelium is injected underneath this flap into the fat pad.

Figure 3: Reciprocal transplantation schema. (**A**) Typical experimental design for reciprocal transplantation is shown, using genotypes as an example. Testing a single experimental variable,

such as gene knockout (KO) relative to wildtype (WT) donor epithelium, creates 4 transplantation recipient groups. (**B**) Example design for a single recipient animal receiving 2 injections of donor material. Multiple sites for grafting are accessible when using the interscapular white fat pad because of the presence of a blood vessel along the midline. Separate preparations of wild type and knockout donor epithelium (or other test conditions) can be injected to the left and the right of the blood vessel. (**C**) Representative whole mounted IS fat pad tissue from a transplant recipient is displayed in the same orientation as in the example presented in (**B**). Mammary epithelial outgrowth is visible at two sites of transplantation on a whole-mounted slide with alum-carmine stained tissue. The interscapular fat pad was excised as a single piece 6 weeks after transplantation. Additional time for epithelial development may be needed but may also facilitate overgrowth and difficulty distinguishing individual donor grafts. Common biological artifacts may be visible: MS = muscle, TP = transplanted epithelium, BF = brown adipose fat tissue.

Figure 4: Mammary cell autonomous and non-autonomous results analysis. (**A**) Simulation of results that may be observed when there are significant contributions of autonomous and non-autonomous effects on the phenotype of donor epithelium. In this example, the endogenous abdominal-inguinal glands from the host are used as a comparison. Concordance of the phenotype of donor epithelial outgrowth, as compared to the endogenous gland, can be used as a reference, but should not be used to exclusively determine effects. (**B**) Donor mammary epithelium outgrowth is shown at the site of transplantation, adjacent to images of the endogenous gland for all 4 groups in a reciprocal transplantation experiment. Non-autonomous effects on mammary epithelial observed following knockout of a single gene, *Cdkn1b*, suggesting the host's microenvironment affects the developing rat mammary gland[18]. Scale bars represent 5 mm.

Table 1: Items requiring advance consideration at each step. This list is designed to be used as a reference when preparing an experiment and should not be considered exhaustive. Reagents may only be necessary for specific applications of the protocol, based on the inclusion/exclusion of optional steps. In any experiment, these items must be accessible without delay once the procedure is started.

Supplemental File 1: Serum-Free Collagenase Digestion Media Preparation.

Supplemental File 2: Monodispersion Mixture Preparation.

Supplemental File 3: Inactivation Solution Preparation.

Supplemental File 4: Alum-Carmine Stain Preparation.

DISCUSSION:

This protocol describes a mammary epithelial cell transplantation technique optimized for working with rats. Isolated mammary epithelial organoids from donor rats (3-5 weeks of age) are grafted into the interscapular white fat pad of recipient rats (also 3-5 weeks of age). Results can be interpreted as little as 4-6 weeks later, using light microscopy to examine the grafted tissue;

however, the optimal amount of time between transplantation and sacrifice must be determined prior to implementing a full experiment. If too little or too much time has passed, the results will neither be interpretable nor meaningful. To optimize the protocol, analyze the outgrowth in a small set of animals 6-8 weeks after transplantation. If the transplanted epithelium is present, but underdeveloped, increase the length of time. If the grafts are well-developed but overlapping features of the epithelium interfere with the analyses, consider reducing the number of weeks for epithelial outgrowth. If the amount of time cannot be shortened (e.g., in carcinogenesis experiments), it is advised to inject the same type of donor epithelium into both transplantation sites (one on each side of the medial blood vessel), as outcomes cannot be interpreted from individual sides with 100% certainty. If any type of interaction (autocrine, paracrine) is suspected, it is strongly advised to include additional control animals injected with the same type of donor epithelium into both transplantation sites. Critical steps in the procedure include proper quantification of donor cells after enzymatic digestion, and uniform mixing with brain homogenate. Extra care must be taken at these steps to ensure the number of transplanted cells is consistent across recipient animals. Also, during injection, make sure that the grafted tissue mixture is not leaking out of the interscapular fat pad. 3. Excising the interscapular fat pad at endpoint of the experiment. The entire pad can be removed as a single piece, but care must be taken if choosing to separate the 2 sides of the interscapular fat pad, cutting only after identifying the medial blood vessel. It can be difficult to determine the side from which outgrowth originated, especially when one graft has overgrown into the other, making removal as a single piece more ideal.

A common modification of the procedure is the addition of a carcinogenic treatment of the recipient rat[25,29]. The grafted tissue can retain the susceptibility to carcinogenesis that was possessed by the donor rat[25], or, conversely, the donor tissue can adopt the susceptibility of the host[30]. These effects can only be determined when using the interscapular fat pad as the site of transplantation, because the endogenous mammary glands remain intact and function as a positive, internal control.

When using mammary gland organoids for transplantation, absence of epithelial outgrowth may be due to problems with the donor cell preparation or injection procedure. Graft rejection can also occur when the recipient and the donor strain are not congenic, causing an immune response in the host. In such cases, the recipient immune system recognizes the donor tissue as non-self, initiates an immune response, and the grafted tissue fails to grow. To reduce the risk of graft failure when donor and recipient are on different genetic backgrounds, a minimum of 6, and, ideally, more than 10 generations of backcrossing are recommended to prevent challenges that can affect result interpretation. At 6 backcross generations, most grafts will grow out, but a minority might still fail. When troubleshooting donor cell preparation as the cause of graft failure, consider whether the enzymatic digestion was too harsh, cells were kept at too high or too low of temperatures, sources of contamination, optimization of donor cell numbers used for the assay, or other protocol deviations affecting cell viability and outgrowth.

Single mammary stem cells have been shown in the mouse to be able to reconstitute a functional mammary gland, illustrating that the addition of hormonal support is not necessary for the

primary outcome. The addition of brain homogenate significantly improves the outcome of transplantation by serving as a structural matrix for the donor cells andreducing the risk of migration transplant rejection[3,34-36]. When combined with brain homogenate, the minimum number of mammary epithelial cells required for transplantation is reduced more than 10-fold, as compared to alternatives[3]. Importantly, admixture of syngeneic brain homogenate has not been shown to affect the phenotype of transplanted epithelium, and has produced consistent outcomes in mammary carcinogenesis and susceptibility studies for over 40 years .

Some may argue that the interscapular fat pad is not representative of the endogenous mammary fat pad because of the anatomical distinctions: the proximity of the IS fat pad to brown adipose tissue, potential differences in blood vessel density resulting in differences in exposure to hormones or the presence of prominent lymph nodes in the inguinal-abdominal fat pad, which may expose the epithelium to different levels of cytokines. Although this has not been specifically tested in rats, both of these depots are subcutaneous and develop prior to visceral adipose[37,38]; in human adipose, greater molecular differences exist across adipose regions, and the heterogeneity within groups is not fully understood[38,39]. An additional factor to consider is that the white interscapular and mammary fat pads share $Myf5^{+}$ mesenchymal precursor lineage, but differ in the number of cells derived from that population[40]. Notwithstanding, there is sufficient evidence to suggest the white interscapular fat pad provides a microenvironment similar to that of the lower mammary gland. Mammary epithelium recombined with its own mesenchyme develops a typical mammary pattern[20], an effect that is well-document in rodent studies and supports the observations in human adipose tissue[19,20,24,41,42]. Above all, the primary determinants of mammary epithelial transplantation success in both rats and mice are the size and integrity of the fat pad[43,44]. In using this technique, many breast cancer susceptibility studies have proven that functional mammary tissue can be effectively and routinely generated when transplanted into the white interscapular fat pad[18,30,45].

Because of the high compatibility, the epithelial outgrowth is amenable to mammary cell-autonomous and non-autonomous factors and will respond to hormonal manipulation of the recipient rats, for example, to promote differentiation or functional secretion of milk. Transplantation of organoids is often used to study factors that affect mammary gland development and/or carcinogenesis. Organoids can be further digested to single cell suspensions to facilitate quantitative interpretation of results. While the method described in this paper can be adapted to graft intact sections of mammary gland tissue (as is commonly performed in mice), the enzymatic dissociation steps allow more detailed conclusions to be made. Since preclearing the endogenous mammary fat pad in the rat is not feasible, this is currently the only method allowing for grafting of rat mammary epithelium.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

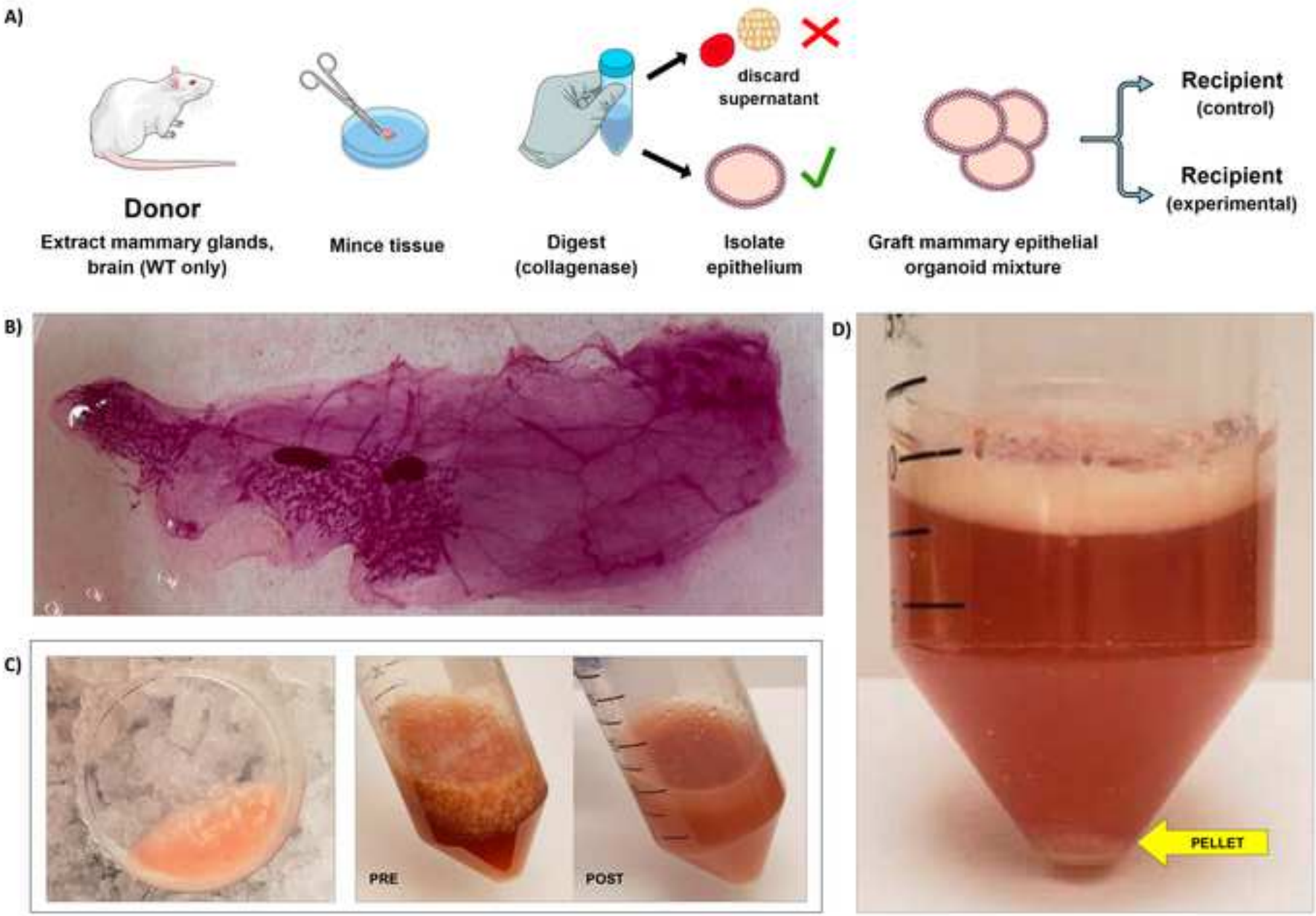

Figure 2

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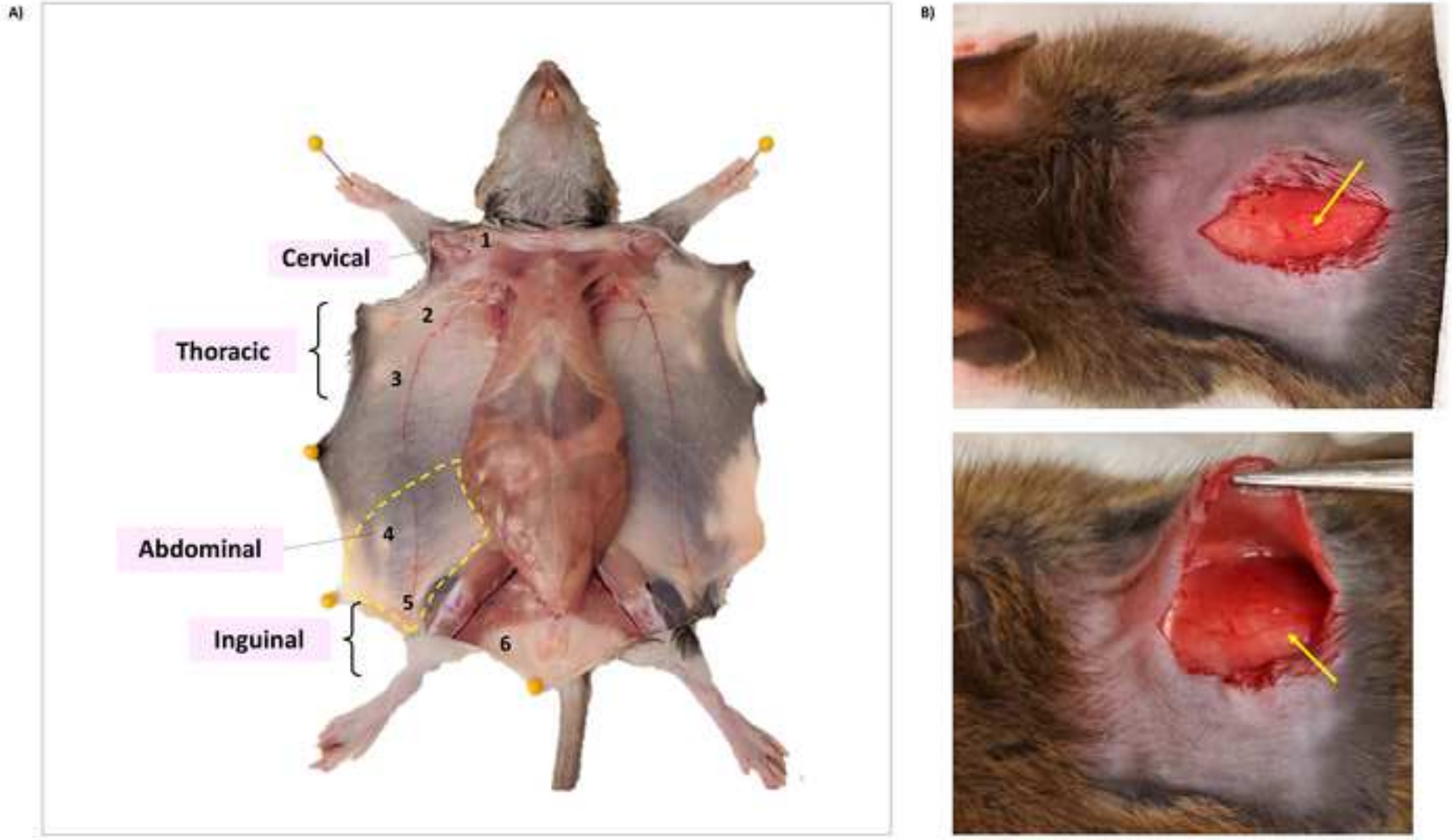

Figure 3

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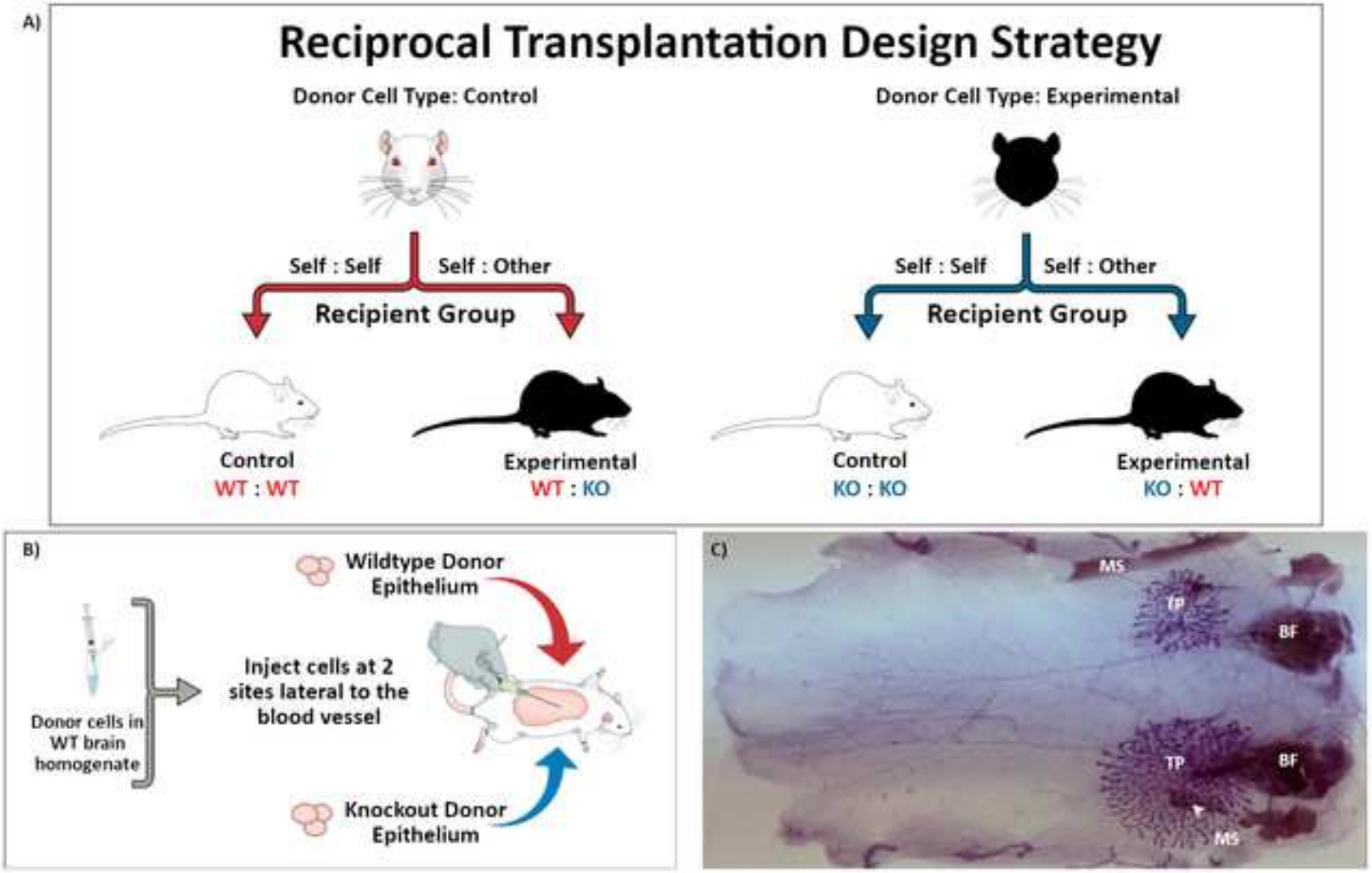

Figure 4

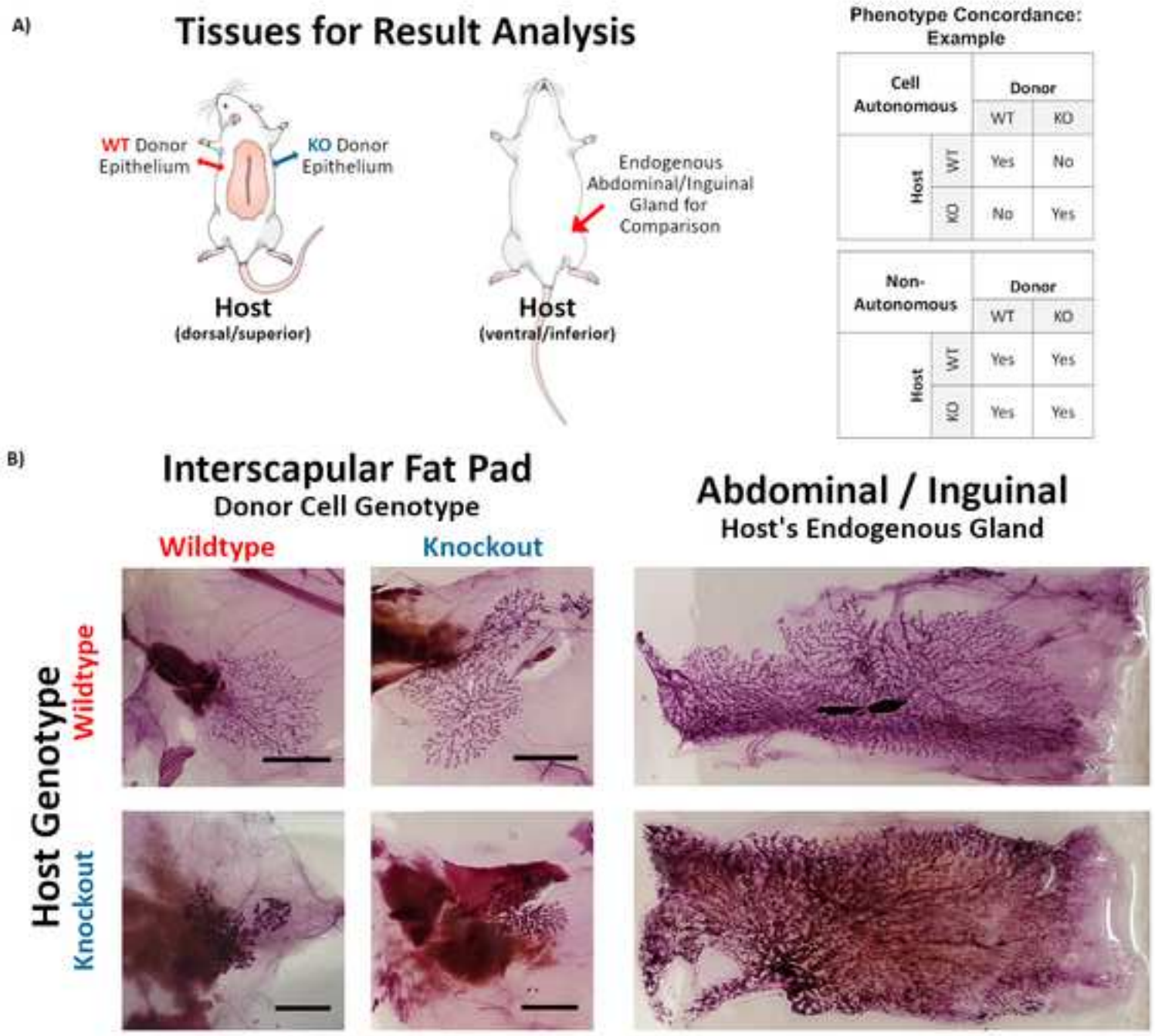

Table 1: Items requiring advance consideration at each step.

Step Needed	Items on ice	Thawed/Pre-warmed	Items near surgeon
1. Preparation of mammary gland epithelium	60 mm dish Aliquot of DMEM/F12		Sterile surgical tools 70% Ethanol
2. Donor brain extraction	Aliquot of media in 15 or 50 mL tube (approx. 1-2 mL/donor) Labeled 15 mL tube for each donor, suitable for homogenization		Balance for weighing brain, within sterile field Foil Pipette and tips (1000 µL, or electronic with 5 mL serological pipettes) Mechanical homogenizer
3. Enzymatic digestion of donor glands	Sufficient DMEM/F12 for washes DNAse I	Serum-free digestion media Monodispersion Mixture Inactivation Solution	Lab scale (g) Incubator/shaker 50 mL tube (labeled) for each donor 10 mL (or greater) syringe for sterile-filtering collagenase digestion media 20-40 µM filters Aliquot of media to pre-wet filter(s) 50 mL tube(s) for collecting filtered enzyme solution Sterile scissors for cutting disposable pipet tips Large beaker for collecting supernatant, or vacuum line for aspirating
4. Transplantation	Donor epithelium + brain homogenate mixture Aliquot of DMEM/F12 to prime syringes		Hamilton syringes – 1 per donor genotype/condition Scale Sterile surgical supplies (scalpel/scissors, multiple forceps) Wound clips/sutures Gauze 70% ethanol or isopropranol Beta-dine or iodine Analgesic Heat support for recipient animals Paper towels or delicate task wipes

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2 µM syringe filters	Fisher Scientific	09-715G	sterile-filtering collagenase digestion media
1.5 - 2.0 mL microcentrifuge tubes (sterile)	Fisher Scientific	05-408-129	containing resuspended cells and/or brain homogenate mixture
100 µM cell strainers	Corning	431752	filtering brain homogenate
100 uL gastight syringes with 25 gauge needles	Hamilton	81001 & 90525	For injecting graft mixture into recipient animals (1 per donor genotype/condition)
1000 uL pipette tips + pipette	-	-	transferring cells/mixtures/tissue
15 mL polypropylene tube	Falcon (Corning)	352196	brain homogenate mixture storage, or cell : homogenate mixture for transplantation
40 µM cell strainers	Corning	431750	filtering organoids after washing the cell pellet
50 mL polypropylene tubes	Fisher Scientific	05-539-6	for collagenase digestion of donor mammary gland tissue
60 mm dishes	Thermo Scientific	130181	for mincing tissue
Alum Potassium Sulfate	Sigma-Aldrich	243361/237086	staining mammary gland whole mount slides
Anesthesia vaporizer for veterinary use	-	-	follow institutional protocol
Beta-dine or iodine	-	-	
Borosilicate glass culture tube for homogenization	Fisher Scientific	14-961-26	for homogenization of brain (use appropriate tube for homogenizer)
Carmine	Sigma-Aldrich	C6152/1022	staining mammary gland whole mount slides
Cell counting apparatus	-	-	
Clean animal cages for recovery	-	-	follow institutional protocol
Collagenase Type 3	Worthington Biochemical Corp.	LS004183	enzymatic digestion of minced mammary gland tissue from donor rats
deionized water	-	-	for chemical solutions
DMEM/F12	GIBCO	11320033	for mincing tissue, collagenase digestion media and resuspending epithelial cell mixtures
EDTA	-	-	monodispersion mixture
Ethanol, 200 Proof	Decon Labs	2705/2701	mammary whole mount slide fixative, mammary whole mount slide washes, cleaning surgical incision sites (diluted)
Fetal Bovine Serum (FBS)	Hyclone	-	inactivation solution
Gauze	-	-	
Glacial acetic acid	Fisher Scientific	A38-212	use for mammary whole mount slide fixative (1:4 glacial acetic acid in 100% ethanol)
HBSS	GIBCO	-	monodispersion mixture
Heating pads	-	-	follow institutional protocol
Ice buckets (x2)	-	-	
Incubator with orbital rotation	-	-	must be capable of maintaining 37°C, shaking at 220-225 RPM (for collagenase digestion of mammary tissue)
Isoflurane anesthesia	-	-	follow institutional protocol
Light microscope or digital camera	-	-	visualizing whole mounted mammary epithelium and/or acquiring images
Mechanical homogenizer	Fisher Scientific	-	TissueMiser or alternative models
Mineral oil, pure	Sigma-Aldrich/ ACROS Organics	8042-47-5	long-term storage of cleared mammary gland whole mounts
Oxygen tanks for anesthesia vaporizer	-	-	follow institutional protocol
Paper towels or delicate task wipes	-	-	
Positively-charged microscope slides	Thermo Scientific	P4981-001	mammary gland tissue whole mounts
Postoperative analgesic	-	-	Institutional protocol
Scale			body weight measurements of animals, proper dosing of pain medication
Shaver	-	-	electric clippers, or other
Staining jars	-	-	minimum of 1 per chemical wash, size appropriate for the number of slides, glass preferred
Sterile field drapes	IMCO	4410-IMC	used during transplantation
Sterile scissors and forceps x3 (autoclaved)	-	-	autoclave surgical tools used for donors and recipients
Syringes: 5 mL (or greater)	-	-	for sterile filtration of collagenase digestion media
Trypsin	Worthington		monodispersion mixture
Waste collection receptacle for liquids (poured or aspirated)	-	-	
Wound clip applier, clips, and removal tool	Fine Science Tools	12020-00	Closing the skin incision over the interscapular white pad pad
Xylenes	Fisher Scientific	X3S-4	clearing mammary gland whole mount slides after staining

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please ensure the Introduction contains all of the following with citations:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

We have ensured the Introduction contains all of the above items. The a) overall goal has been clarified and explicitly stated; b) rationale is explained in paragraphs 4-5; c) advantages over techniques (with citations) are discussed in paragraphs 5-6; d) context is provided throughout, and emphasized in the concluding paragraph (lines 149-156 of the expanded, tracked-changes manuscript), which incorporates e) aspects of the rat model organisms and the interscapular fat pad that should be considered when deciding to use this technique.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

This has been done.

4. We cannot have paragraph of text in the protocol section. Please number all action steps and move the details to the introduction/discussion section.

Thank you for this clarification, the text within the protocol section was removed and all action steps appropriately revised for simplicity.

5. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

Thank you for this helpful suggestion. We agree that the simplification of steps has improved the manuscript. The revised Protocol has more concise steps, with fewer actions in each.

6. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

We have revised the manuscript to remove personal pronouns.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described

in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However only one can follow one step.

Thank you for this editorial feedback; we have implemented this suggestion throughout the entirety of the Protocol and revised the text to use the imperative tense.

8. The Protocol should contain only action items that direct the reader to do something.

All descriptive text has been removed or simplified into notes under the numbered, actionable steps.

9. Please ensure you answer the “how” question, i.e., how is the step performed?

Thank you for this suggestion. We identified many instances in the Protocol that were improved by adding how the step is performed.

10. Lines 136-143, 159-166, 191-197, 202-206, 291-294, etc. please convert to numbered action step, note or move to the discussion.

The nonactionable summaries of the major steps in the Protocol have been converted to numbered actions, moved to the discussion or added as notes.

11. 1.3: How is this done? Volume of the isotonic medium?

The volume of the medium has been clarified in Step 1.10 of the revised manuscript.

12. 4.1: what is the anesthesia used in your experiment? Concentration, etc? How do you check the depth of anesthesia? Do you apply any vet ointment on the eyes? DO you shave the animal prior to the surgery? Any age sex, strain specificity?

In our experiment, inhalation anesthesia (isoflurane) is used. Liquid isoflurane is vaporized by a VetEquip Mobile Laboratory Animal Anesthesia System and comprises 2.0-2.5% of oxygen flowing at a rate of 2.0 L per minute. We have incorporated mention of the anesthetic agent in Part 4 of the procedure; the detailed parameters are fairly standard but are highly specific to approval of the protocol by an Institutional Animal Care and Use Committee (IACUC) or other animal welfare governing body and therefore, cannot be explicitly recommended. The VetEquip Laboratory Animal Anesthesia System Manual (http://www.vetequip.com/pdfs/LAAS%20Manual.pdf) provides a framework for developing an inhalant anesthesia protocol that can assist researchers who do not have one in place: for example, the manual indicates the typical range of for rodents is 0-5% and the flow rate is adjustable from 0-4 L per minute. This range is particularly important because it provides the reader with a range of parameters to discuss with their IACUC (or other) committee and accommodates any adjustments that might need to be made for age, sex, and strain of the laboratory rats used.

Objective measures (ie: assessing depth of anesthesia) are included as a note beneath Step 4.13. Due to the very short nature of this survival procedure, ophthalmic ointment is not required per our IACUC-approved protocol; it could be deemed necessary by the IACUC at other institutions. We added this as an optional step (4.14).

Shaving is typically performed under anesthesia to minimize stress to the animal. This can be done on the day of surgery (outside the sterile field) or 24 hours in advance, when body weight measurements

are obtained to calculate dosages for analgesic medication. We have made changes in Step 4 to reflect these options.

13. 4.2: What do you start off here? Shaving?

Thank you for directing our attention to the lack of clarity in the start of the transplant procedure. The entire section has been revised and 4.1 begins with weighing of the animal. The changes in Step 4 reflect the fact that shaving and weighing can be performed up to one day in advance, but must occur outside of the sterile field.

14. 4.4: Do you also use iodine-based scrub?

Yes, we do use iodine-based scrub, as detailed in our IACUC-approved protocol. We amended this in the Protocol. Step 4.15 describes this action and accommodates the differences in preoperative washing procedures that exist across institutions.

15. 4.7: How do you visually identify the same?

Step 4.7 is now divided into smaller, concise steps that comprise 4.18-4.22 with references to Figure 2B.

16. 4.7: How much of the cell mixture is injected, how is this done, what is the desired side of the fat pad?

The volume has been specified at the injection step (4.20) and also in the preparation of the final mixture, when donor cells are mixed with 50% brain homogenate (step 3.16 with note). Both sides of the interscapular fat pad are typically injected. We are not aware of left-right asymmetry in outgrowth, rather, outgrowth is mostly related to the length of elapsed time between transplantation and sacrifice (assessment of epithelial outgrowth). A preferred experimental design strategy includes randomization of "side" assignments with respect to the control and experimental variables. For example: an investigator is testing a hypothesis related to the function of a gene using the technique described herein, but injects all of the "knockout" donor cells into the right-side of recipient animals' interscapular fat pad. Similarly, the investigator injects all of the wildtype (control) epithelium to the left of the medial blood vessel in recipients' fat pads. We have clarified the protocol to avoid procedural side preferences and promote interpretation of the outgrowth in a note under Step 4.22.

17. 5: After how many days do you perform this procedure? How is the tissue collection performed?

Assessment of epithelial outgrowth is typically performed 6-8 weeks after transplantation. We have included the optional first step of monitoring the estrus cycle of rats prior to sacrifice and provide an enhanced description of the tissue collection in the revised manuscript.

18. Please consider moving all the buffer/solution recipes to a table. Please upload the tables individually to the editorial manager account as .xlsx file and refer the tables wherever needed. Please include concentrations and volume used.

We have removed buffer/solution recipes from the protocol and included them as individual supplemental files. The files also include fixed equations that will perform calculations based on the user's input, eg:) total number of donor animals used in the experiment.

19. Please move all the materials to the materials table.

All materials are moved to the materials table.

20. Please use complete sentences throughout the protocol section.

We have thoroughly reviewed the updated manuscript and corrected any instances of such errors.

21. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Less than 2.75 pages are highlighted as requested.

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The images in Figure 4B were specifically acquired for this manuscript and we certify that the images have not been previously published.

23. For all images obtained with a microscope, please include a scale bar.

The figure has been revised to include scale bars.

24. As we are a methods journal, please ensure the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

25. Please do not abbreviate the journal titles in the references section.

The reference section has been revised accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this protocol, Shunkwiler et al describe a transplantation method where rat mammary cells are transplanted into the interscapular white fat pad of recipient hosts. The authors describe the

preparation of cells to be transplanted from donor rats (clumps of cells or monodispersed cells), and include clear images of this process. The transplantation procedure is then described, in addition to guidelines on how to analyze the transplanted tissue by carmine staining. Representative images of transplanted tissues are provided, which are clear and helpful. Overall, the protocol is relatively clear, and the figures well presented. More specific points are provided below:

Major Concerns:

1) While the protocol will be of interest to some scientists, the fact that there are less transgenic rat strains available, in addition to increased husbandry costs, will likely limit the uptake of this protocol by mammary gland biologists. While the authors briefly highlight the advantages of using rats compared to mice for breast cancer research (lines 81-85), it would be beneficial to highlight in the introduction more recent studies that exemplify the utility of the rat model in this setting (for example some of the studies cited later on in the discussion e.g. Ding et al 2019).

We thank for the reviewer for drawing attention to this fact. Although the timeline of developing genomic resources for mice and rats is decades apart, genetic engineering technology that facilitated popularity of the mouse model organism is now being used to genetically engineer rats for similar studies. Neither of these organisms is superior to the other in breast cancer research, but there are strengths and limitations in any model. With respect to ER+ breast cancer, the homogeneity of rat mammary tumors is advantageous. Rats are known to develop tumors that morphologically and histologically resemble primary adenocarcinoma of the human breast as a result of various exposures, include ionizing radiation or chemical carcinogens. Importantly, rat mammary tumors are hormone-dependent, and the normal mammary tissue is very sensitive to ovarian and pituitary hormones. Lobuloalveolar growths will develop in the mammary gland of hypophysectomized/ovariectomized rats, but this is not always observed in mice. The unique physiological aspects of the rat mammary gland are advantageous to studies on ER+ breast cancer susceptibility. Transplantation methods are valuable for studying host and cell autonomous effects, and in rats, transplantation into the white interscapular fat pad is a reliable way to perform this assay.

For those who use rat models of ER+ breast cancer, the mammary clonogen transplantation model facilitates investigations of host:cell autonomous effects, and the technique we describe is a reliable way to perform transplantation.

To provide clarity in the Introduction of this manuscript, we revised lines 80 - 95 to describe critical advantages of the rat model organism for modeling mammary gland development and ER+ disease.

2) In this protocol, mammary cells are transplanted as a mix with brain homogenate, which the authors say improves take rates (Line 529 - 530). A potential concern is that this may impact/modify the phenotype observed with specific genetic KO cells, or response to carcinogenic exposures. Can the authors comment as to what the average take rates are without including the homogenate? In addition, can the authors comment as to how this might compare to studies using matrigel instead? While including matrigel is also imperfect, it has been extensively used in mouse transplantation assays and it's effect on mammary stem cell potency and carcinogenic susceptibility is likely better characterized.

The initial study published by Dr. Gould in 1977, and many thereafter, have reported consistency in the outcome of transplantation experiments using brain homogenate. The addition of this extract has been

shown to reduce the minimum amount of cells needed for transplantation experiments more than 10-fold, which has obvious implications on the number of animals used for this procedure. In addition, recent work has shown that using the same brain homogenate across genotypes did not lead to significant differences in outgrowth potential across multiple experiments. These results suggest the brain homogenate merely acts as a universal growth enhancer and does not affect the differentiation of cells or phenotypic outcome of transplantation assays.

Discussion and comparison to the matrigel assay protocol is beyond the scope of this protocol. To address the concern of this reviewer we will refer to our previously publications that describe flow cytometric-assisted cell sorting (FACS) isolation of specific rat mammary epithelial cell populations for cultivation in matrigel assays that quantified formation of 'alveolar units' in vitro. These clonogenic structures resemble alveolar buds, but do not resemble a full mammary tree as appearing in in vivo transplantation assays, suggesting materiel may not be permissive for full differentiation of rat MEC clonogenic cells. We are not aware of any comparative studies on matrigel and brain homogenate in rat MEC transplantation analysis.

3) As noted by the authors (lines 535-536) the interscapular fat pad may not be representative of the endogenous mammary gland fat pad due to differences in cellular/hormonal micro-environment. Could the authors comment more specifically on these differences? Have there been any studies directly comparing the impact of genetic KOs and/or carcinogenic susceptibility in cells transplanted into the different sites i.e. do they give similar results?

It is requested by 2 reviewers to discuss the potential differences of the inguinal (endogenous) versus the interscapular white fat pads. On the anatomical level the IS fat pad is different from the inguinal white fat pad because of its proximity to brown adipose tissue, which may expose transplanted cells to a slightly different endocrine milieu. Another anatomical distinction is the proximity to a string of lymph nodes present in the inguinal, but not in the IS fat pad, which could expose the endogenous mammary epithelium to higher levels of cytokines. On the molecular/cellular level differences exist between subcutaneous and intra-abdominal white fat, as evidenced by gene expression profiles[8]. Although it has been shown that the IS white fat pad and inguinal fat pad of rodents are not entirely derived from the same progenitor pool, they do share the $Myf5^{+}$ lineage (REFs provided in manuscript).

The question then becomes if these differences affect mammary epithelial development and/or carcinogenesis. We are not aware of any functional studies in rats that specifically address this question. However, the effect of the stroma and site of transplantation on epithelial outgrowth has been studied in mice. These studies have shown that grafts from the same donor will successfully develop and resemble mammary epithelium when transplanted into subcutaneous white adipose with estrogen and progesterone support, but to a lesser extent in perirenal and interscapular brown adipose tissue, suggesting a more mammary growth-permissive environment in the subcutaneous white adipose tissues (REFs provided in manuscript). It is possible that the IS white fat pad is influenced by regional proximity to differently-specialized adipose tissue (possible differences in glucocorticoid and estrogen responsiveness), but this has not been studied (to our knowledge) in rats. There appear to be some differences in success rates for tumor and mammary epithelial grafts based on the transplantation site in mice, but the predominant factor of successful graft growth was intactness of the fat pad (REFs provided in manuscript). To summarize, given the longstanding success of this technique in rats and the fact that both the interscapular white fat pad and inguinal/abdominal mammary fat pads are

subcutaneous in origin, these white adipose depots are more similar than different in supporting mammary gland development.

We incorporated a clear, concise discussion on this topic in the manuscript in the third and fourth paragraphs of the discussion (lines 774 - 793).

4) The authors mention that different cells can be injected either side of the medial blood vessel in the fat pad. How often to they observe overgrowth between the two regions, and can they give an indication as to how long after transplantation that this is more likely to occur? Can the authors comment on the risk of paracrine interactions between the different transplanted cells (e.g. WT vs Mutant) that might complicate observed phenotypes?

We thank the reviewer for this constructive feedback. Length of time to overgrowth depends on the variable being tested. In our experience, gene knockout of *Cdkn1b* in the recipient caused hyperproliferative conditions that equally affected the donor graft tissue of both genotypes. Despite proliferative conditions as extreme as we observed in this study, the grafted tissue injected on both sides of the blood vessel was still discernible at necropsy performed 8 weeks post-transplantation. The length of time after transplantation must be empirically determined for every rat strain and condition tested using this method **prior to implementation of a full experiment**. 6-8 weeks after transplantation is a good starting point to assess the epithelial outgrowth and whether the time needs to be shortened or lengthened. We added statements to Step 5.2 of the protocol and the first paragraph of the discussion (lines 948-963 of the expanded, tracked-changes manuscript) to emphasize these points.

Various autocrine and paracrine signals from and between the main mammary epithelial cell populations (namely luminal and basal) are known to influence normal mammary gland development. When transplanting tissue from different experimental conditions, there is a risk of paracrine interactions, for example, if the outcome of the experiment depends on tissue from both experimental conditions being present. In cases when paracrine interaction is suspected between the grafted cells of the different experimental condition, the experiment can be repeated using a design strategy where both sides of the interscapular fat pad are transplanted with tissue from the same genotype or other variable. For carcinogenesis experiments, the grafted tissue remains in the animal much longer than other applications and it could become unclear from which side a tumor arose. Therefore, we recommend to always graft tissue of from the test condition of each donor group <u>two times</u> (a single site on either side of the medial blood vessel) in a small number of animals for carcinogenesis experiments. We have updated the first paragraph of the Discussion to include this recommendation (lines 958 – 963 of the revised, tracked-changes manuscript).

Minor Concerns:

1) Some of the text is highlighted in yellow - I assume this was accidentally left in the draft uploaded?

The yellow highlighted text is a formatting requirement of the journal.

2) In the abstract (lines 37-41) the authors state that:

"common, but limiting, feature is that transplanted epithelial cells are strongly influenced by the surrounding stroma; to avoid this….. must be "cleared" to remove host mammary epithelium prior to transplantation."

Clearing removes endogenous epithelium but cells are still injected into the surrounding stroma, so this paragraph as it stands is inaccurate.

Thank you for noticing this detail, we have corrected the statement so it conveys the proper meaning. This section of the abstract now reads:

“A common, but limiting, feature is that transplanted epithelial cells are strongly influenced by the surrounding stroma and outcompeted by endogenous epithelium; to utilize native mammary tissue, the inguinal-abdominal white fat pad (a preferred site of transplantation in rodents), must be “cleared” to remove host mammary epithelium prior to transplantation. A major obstacle when using the rat model organism is that clearing the developing mammary tree in post-weaned rats is not efficient.”

3) Lines 56- 57:

"Normal mammary gland development occurs in response to intricate hormonal signaling at the onset of puberty"

This sentence doesn't reflect that mammary gland development in fact begins during embryogenesis. Thus, it might be more accurate to say "normal pubertal development" or "normal mammary gland ductal development" instead.

Thank you for this suggestion. We have revised the opening sentence of the Introduction as follows:

“Postnatal mammary gland development and ductal morphogenesis are processes largely influenced by hormonal signaling at the onset of puberty.”

4) Line 83 - 85:

"In particular, the rat mammary epithelium, when compared to the mouse, exhibits a higher level of branching, dependence on ovarian hormone signaling, and is flanked by a thicker layer of stroma"

Could the authors point to relevant references supporting this?

References supporting this statement have been added.

5) Line 329, step 4.9:

"Provide post-operative analgesic, as indicated by protocol"

While guidelines may vary from country to country, generally for short surgeries such as the one described analgesics should be provided ~10 minutes prior to surgery so that the drugs are already in the system when the animal wakes up

This detail has been clarified in Steps 4.7 and 4.24 of the revised manuscript.

6) Line 344:

"menstrual'.

Normally the term 'Estrus' is used instead when referring to the rodent cycle?

The laboratory rat has an estrous cycle of 4 to 5 days with 4 distinct phases, bilateral ovulation and does not shed the endometrium. We have ensured that the appropriate term 'Estrous cycle' is used throughout the manuscript.

7) Line 470:

"An arrow indicates the medial blood vessel".

The arrow is missing in figure 3B.

This legend for this Figure 3B has been revised to more accurately explain this panel.

8) Figure 4B, 4 left panels - this is slightly confusing as it stands. Does the 'wildtype' and 'knockout' text under the heading 'Interscapular' refer to the transplanted cells?

We have added text to Figure 4B to clarify the image.

Reviewer #2:

This manuscript describes a method of transplanting rat mammary epithelial cells into the interescapular white fat pad. Although the description is largely clear, I have the following suggestions for further improvement:

Major Concerns:

The advantage and disadvantage of the use of the interscapular white fat pad compared to the use of the white fat pads that naturally contain mammary epithelial cells have to be clearly described. Authors are trying to defend their method, and they appear trying to avoid detailed discussion about how the hormonal environment specific to the interscapular white fat pad may affect the assay and biological interpretation of the outcomes. Authors only state that they and other labs have used the interscapular white fat pad in previously published studies.

Authors should refer to the safe stopping points, where materials can be frozen for short- or long-term storage. Steps instructing on-ice storage of materials (such as lines 153 and 189) should refer to the longest possible periods of storage time.

We are grateful for this reviewer's reminder to denote safe stopping points. The revised manuscript includes maximum time allowances for enzymatic digestion steps and the storage of materials on ice. The lines referenced (153 and 189) are now written as Steps 1.11 and 2.8, respectively, and the stopping point details have been clarified. There is no permanent stopping point in the protocol we use, but we thoroughly discussed the inclusion of an option to freeze donor cells and brain homogenate, to create a stopping point after collagenase digestion that accommodates situations where the breeding of donor rats is not perfectly aligned, and donor tissue has to be collected on different days (Step 2). Although it should be possible, we have not tested this technique with stored materials, and cannot recommend it in the protocol.

The roles and importance of the brain extract (lines 164-165) are not sufficiently described. Should the extract be prepared freshly from the donor animals, or it can be stored frozen and/or prepared from other animals in advance? How the quality of the extract affect the success rate of transplantation?

Thank you for raising this interesting point. We have expanded the discussion on the importance of brain extract in Paragraph 5 (lines 992-1006 in the tracked-changes manuscript) and agree with the reviewer that this has enhanced the manuscript by highlighting one of the most critical aspects of the protocol. The revised Protocol makes it more clear to those considering its use that the brain is traditionally extracted from donor rats immediately after harvesting the mammary gland tissue. Technically, the brain of any rat that is matched by age, sex and genotype to the rest of the donors could potentially be used, but we have not investigated this hypothesis. We appreciate this reviewer's attention to detail and suggesting of timesaving strategies, but do not agree that advance harvesting would benefit the Protocol. Donor rat mammary glands must be harvested fresh, and subsequent extraction of the brain takes a mere 3-5 minutes. The benefits of the brain homogenate are likely not dependent on the viability of cells and/or the "quality" of the extract (which we assume is analogous to purity) and in our experience, do not diminish with temporary storage on ice (< 90 min).

This point was also raised by reviewer 1, and we have included the response below for your convenience:

"The concern regarding use of brain homogenate is certainly understandable. Fortunately, one of the major benefits of this technique is the quantitation it affords. The initial study published by Dr. Gould in 1977, and many thereafter, have reported consistency in the outcome of transplantation experiments using brain homogenate. The addition of this extract has been shown to reduce the minimum amount of cells needed for transplantation experiments more than 10-fold, which has obvious implications on the number of animals used for this procedure. In addition, recent work has shown that using the same brain homogenate across genotypes did not lead to significant differences in outgrowth potential across multiple experiments (REFs provided in manuscript). These results suggest the brain homogenate merely acts as a universal growth enhancer and does not affect the differentiation of cells or phenotypic outcome of transplantation assays.

Discussion and comparison to the matrigel assay protocol is beyond the scope of this protocol, but to address the concern of this reviewer we will refer to our previously publications that describe flow cytometric-assisted cell sorting (FACS) isolation of specific rat mammary epithelial cell populations for cultivation in matrigel assays that quantified formation of 'alveolar units' in vitro. These clonogenic structures resemble alveolar buds, but do not resemble a full mammary tree as appearing in in vivo transplantation assays. In addition, we are not aware of any comparative studies on matrigel and *in-vivo* rat MEC or tumorigenesis analysis. "

Why the media should be filtered through a 20 uM filter after addition of collagenase, even though collagenase is sterilized by passing 0.2 uM filter?

This typographical error has been corrected. Collagenase Digestion Media is sterilized using a 0.2 µM filter and is written only once in the revised manuscript.

It is somewhat puzzling that Authors do not mention to cell viability throughout the manuscript even though preparation of single-cell suspension from animal tissues will certainly damage cells. There is no mention to the typical, or minimum required, cell viability and a method to determine it.

We appreciate this concern regarding cell viability, however, it is not critical to the majority of applications of this protocol. Cell viability is tested in cell monodispersion applications because there are multiple, harsh enzymatic digestions that could affect the viability of single cells. This critical step has been added to 3.15.2 for mono dispersed cells.

For the organoid transplantation option, brief centrifugation and filtration of organoids after a 1.5-2h collagenase digestion results in clumps of cells for which cell viability cannot be accurately determined. Since the collagenase media for all groups is prepared in a single batch and the samples are prepared fresh all at once on the day of surgery, we assume that any effect on viability would be evenly distributed across experimental groups.

Line 328: Which method (including choice of sedatives and antibiotics) is recommended to close the surgical wound?

The protocol we use involves only inhaled (isoflurane) anesthesia and the NSAID carprofen for pain relief. The transplantation technique is minimally invasive, with a small, superficial incision, and does not require antibiotics because it is performed under sterile conditions. Even though these are commonly used in veterinary and laboratory procedures, guidelines for anesthesia and medications are subject to approval by the institution.

Line 355: Once tissues adheres - to what and how? Should a specially coated pathological glass slides be used?

-The Analysis of Epithelial Outgrowth has been revised in the current version of the Protocol. The mammary gland whole mounts adhere to positively-charged (poly-L-lysine coated) microscope slides and has been clarified in Step 5.5.

Figures 1C and 2B are not informative. It is difficult to identify what are shown there.

We have revised the legends of Figures 1 and 2 to more clearly explain the panels. We have also referenced these figures specifically at Steps 1.8, 1.11 and 4.18-19 in the Protocol to directly connect the images with the associated actions.

Explain the figures to this reviewer and show the updates in the caption and protocol text

Figure 3 refers to a KO strain, but the target genes is not mentioned. In the absence of the KO target information, it is difficult to understand the experiment presented in this figure.

This figure was inspired by our recent publication involving reciprocal transplantation of rat mammary epithelium in a genetically engineered rat model. This rat model has a deletion mutation to knockout the gene *Cdkn1b*, encoding the cyclin-dependent kinase inhibitor 1B/p27^{Kip1} protein: an important regulator of cell cycle progression (Ding, Shunkwiler *et al.*, PLoS Genetics, 2019). In normal human breast tissue, a hormone-responsive p27^{+} progenitor cell population has been identified and related to risk of developing breast cancer. Using this rat model, we characterized the effect of p27 regulation on the mammary progenitor pool as it pertains to breast cancer susceptibility and incorporated the reciprocal transplantation technique described in this Protocol. One of the unique advantageous of the rat model organism is the hormone-responsiveness of both normal mammary tissue and tumors; a major conclusion of the study resulted from the transplantation experiments, which showed that the

mechanism of action of p27 is non-autonomous to the mammary epithelium and caused in part by the endocrine environment of the (p27 knockout) host.

Figure 3 is based on this type of application, but for the general purposes of the protocol, the figure is not intended to be specific and could reflect any gene. We describe this in better detail in the revised Discussion and Fig Legend.

Lines 535-538 discuss justification of the use of the interscapular fat pad (versus fat pads that naturally contain mammary gland). Papers arguing that the interscapular fat pad I not representative of the endogenous mammary gland fat pads due to different hormonal and cellular microenvironment. It is requested to cite adequate representative literature supporting this argument. Authors' double-negative statement, "we do not disagree with that," does not help Readers to understand whether such a problem does actually exist (and Authors admit it) or this argument itself is a controversial one. If Authors admit that the interscapular fat pad has different hormonal and cellular microenvironment from the fat pads naturally containing mammary epithelial cells, then Authors are expected to provide meticulous discussion about whether the convenience of the interscapular fat pad outweighs these biological differences and in which occasions such differences are negligible or manageable to obtain meaningful data.

Thank you for this recommendation, which was similarly noted by the other reviewer. The potential concerns surrounding alternative sites of adipose for mammary epithelial transplantation is mentioned in the introduction, but we have updated the Discussion to provide molecular details. As there has not been a specific investigation of the mammary and interscapular fat pad microenvironment reported in the literature, it is not possible to compare. However, as we mentioned to the other reviewer, the adipocytes in these 2 tissues are both subcutaneous and share $Myf5^{+}$ progenitor lineage. Gene expression profiling of human adipose has previously shown the largest differences in function exist between groups of subcutaneous adipose tissue and visceral/omental depots within the abdomen. For convenience, we have provided our response to the other review below:

“It is requested by 2 reviewers to discuss the potential differences of the inguinal (endogenous) versus the interscapular white fat pads. On the anatomical level the IS fat pad is different from the inguinal white fat pad because of its proximity to brown adipose tissue, which may expose transplanted cells to a slightly different endocrine milieu. Another anatomical distinction is the proximity to a string of lymph nodes present in the inguinal, but not in the IS fat pad, which could expose the endogenous mammary epithelium to higher levels of cytokines. On the molecular/cellular level differences exist between subcutaneous and intra-abdominal white fat, as evidenced by gene expression profiles. Although it has been shown that the IS white fat pad and inguinal fat pad of rodents are not entirely derived from the same progenitor pool, they do share the $Myf5^{+}$ lineage (REFs provided in manuscript).

The question then becomes if these differences affect mammary epithelial development and/or carcinogenesis. We are not aware of any functional studies in rats that specifically address this question. However, the effect of the stroma and site of transplantation on epithelial outgrowth has been studied in mice. These studies have shown that grafts from the same donor will successfully develop and resemble mammary epithelium when transplanted into subcutaneous white adipose with estrogen and progesterone support, but to a lesser extent in perirenal and interscapular brown adipose tissue, suggesting a more mammary growth-permissive environment in the subcutaneous white adipose tissues (REFs provided in manuscript). It is possible that the IS white fat pad is influenced by regional proximity

to differently-specialized adipose tissue (possible differences in glucocorticoid and estrogen responsiveness), but this has not been studied (to our knowledge) in rats. There appear to be some differences in success rates for tumor and mammary epithelial grafts based on the transplantation site in mice, but the predominant factor of successful graft growth was intactness of the fat pad (REFs provided in manuscript). To summarize, given the longstanding success of this technique in rats and the fact that both the interscapular white fat pad and inguinal/abdominal mammary fat pads are subcutaneous in origin, these white adipose depots are more similar than different in supporting mammary gland development.

We incorporated a clear, concise discussion on this topic in the manuscript in the latter half of the of the discussion (paragraphs 4-6).

Serum-Free Collagenase Digestion Media (10 mL/donor)

NOTE: User-defined fields are denoted by an asterisk (*).

1) Prepare a single batch of Digestion Media for all of the donors. Aliquot once prepared.
2) Determine the **minimum amount of DMEM/F12** needed to digest material for all animals
3) Add 0.01g of **Collagenase Type 3** per mL of DMEM/F12.
4) Mix by inverting. Solution will turn dark brown.
5) Sterile filter by passing the solution through a 0.2 µM filter. Keep the filtrate.
6) Aliquot 10 mL of the filtrate a 50 mL Falcon tube labeled for each animal
   NOTE: Up to 20 mL can be digested in each tube, if necessary
   NOTE: Scale if needed
7) Store at 4°C or on ice (less than 3 hours) to prevent degradation of the enzyme.

***Total # Donor Animals**

4

Minimum volume of DMEM/F12 (mL)

40

Total Volume of DMEM/F12 + extra (mL)

44

Type 3 Collagenase (g)

0.44

Monodispersion Mixture: (2 mL HBSS/donor, plus 10% extra)

NOTE: Always make fresh (on the day of transplantation). This solution cannot be frozen for long-term storage.

NOTE: Trypsin should be sterile-filtered or added to the mixture after filtration (Step 5), in a sterile field.

1) Determine the **total volume of HBSS (mL)** needed to digest all of the donor tissue
2) Calculate the **volume of EDTA** needed using for a final concentration of 6.8 mM and add to the mixture.
    2.1) Use the formula C1V1 = C2V2 or the EDTA calculator on the right.
3) Calculate the **volume of Trypsin stock** needed. Thaw or warm to 37°C within 10 minutes of use.
    3.1) Use the formula C1V1 = C2V2 or the Trypsin calculator on the right.

NOTE: Make all of the Monodispersion Mixture in one batch. Use a tube/glassware that can accommodate the final volume.

4) Add EDTA to HBSS. Mix by inverting.
5) Add Trypsin. Mix by inverting.
6) Sterilize by passing the mixture through 0.2 µM filter. Keep the filtrate.
7) Store at 4°C or on ice until needed (maximum time: 60 minutes)
8) Warm to 37°C 10-15 minutes before use to prevent degradation of the trypsin.

DO NOT ADD DNAse I enzyme until the Collagenase Digestion of donor mammary tissue is complete.

User-defined values are marked by an asterisk (*). The table will auto-populate results.

Total # Donor Animals

1

Minimum Volume of HBSS (mL)

2

Total Volume of HBSS + 10% extra (mL)

2.2

Total Volume of EDTA (mL)

0.1496

Volume of Trypsin stock (mL)

0.22

EDTA Calculator for a final concentration of 6.8 mM

*Enter the molarity of the EDTA stock:	100
Total Volume of HBSS (mL):	2.2
Final concentration of EDTA (mM):	6.8
Total Volume of stock EDTA to add (mL):	0.1496

Trypsin Calculator for a final concentration of 0.025%

*Enter the percent composition of the Trypsin stock:	0.25
Total Volume of HBSS (mL):	2.2
Final composition of Trypsin (%):	0.025
Dilution Factor:	10
Volume of Trypsin to add (mL):	0.22

Inactivation Solution: (4 mL/donor, plus extra)

***Always make fresh (on the day of transplantation).**

*Use only thawed FBS.

1) Determine the total volume of **DMEM/F12 (mL)** needed. Prepare under sterile conditions.
2) Make all of the Inactivation Solution in one batch.
   NOTE: Use a tube/glassware that can accommodate the final volume.
3) Supplement the final mixture with **10% Fetal Bovine Serum (FBS)**.
4) Store at 4°C or on ice until needed. Warm to 37°C 15 minutes before use.
   NOTE: This solution cannot be frozen and stored.

Total # Donor Animals

1

Total Volume of DMEM/F12 (mL)

4

Add 10% extra

4.4

Volume of FBS (mL)

0.44

Alum-Carmine Stain for Mammary Whole-Mounted Slides

1) Add the following to 500 mL dH20:
    2.5 g alum-potassium sulfate
    1.0 g carmine
2) Dissolve the powder by bringing the solution to a boil, with mixing. Ensure a continuous boil of at least 20 minutes. The solution may darken slightly.
3) Slowly filter the mixture to remove undissolved particulates.
4) Adjust final volume up to 500 mL, if needed.

Store at 4°C