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Techniques for the Reprogramming of Exogenous Stem/Progenitor Cell Populations Towards a Mammary Epithelial Cell Fate

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Abstract

This chapter considers the techniques necessary and required for the reprogramming of exogenous stem/progenitor cell populations towards a mammary epithelial cell fate. The protocols describe how to isolate cells from alternate mouse organs such as testicles of male mice and mix them with mammary cells to generate chimeric glands comprised of male and female epithelial cells that are fully competent. During the reformation of mammary stem cell niches by dispersed epithelial cells, in the context of the intact epithelium-free mammary stroma, non-mammary cells are sequestered and reprogrammed to perform mammary epithelial cell functions including those ascribed to mammary stem/progenitor cells. This therefore is a powerful technique for the redirection of cells from other organs/cancer cells to a normal mammary phenotype.

Keywords

Mammary gland; Survival surgery; Transplantation; Immunohistochemistry; Mouse mammary; Fat pad clearing; Reprogramming

1 Introduction

The capacity of any portion of the mouse mammary gland to produce a complete functional mammary outgrowth upon transplantation into an epithelium-divested fat pad is unaffected by the age or reproductive history of the donor [1, 2]. Likewise, through serial transplantations, no loss of potency is detected when compared to similar transplantations of the youngest mammary tissue tested. This demonstrates that stem cell activity is maintained intact throughout the lifetime of the animal despite aging and the repeated expansion and depletion of the mammary epithelium through multiple rounds of pregnancy, lactation and involution. These facts support our belief that mammary stem cells reside in protected tissue locales (niches), where their reproductive potency remains essentially unchanged through life. Disruption of mammary tissue removes the protection provided by the “niche” and

leads to a reduced capacity of dispersed epithelial cells (in terms of the number transplanted) to produce complete functional mammary structures. We were inspired to test this niche concept in the regenerating mammary gland because we had successfully rescued mammary stem/progenitor cells from transgenic mammary tissues where regenerative capacity had been halted. We achieved this by the ectopic expression of suitable transgenes which allowed generation of two models (WAP-Notch4/Int3 $\times$ WAP-Cre/Rosa26R and WAP-TGF β 1 $\times$ WAP-Cre/Rosa26R), in which some of a population of mammary epithelial cells that were incapable of proliferation, in vivo, were marked with a lacZ-reporter gene. We mixed the incompetent epithelial cells with normal wild type mammary epithelium and inoculated them into a cleared mammary fat pad and found that mixing them with normal wild type epithelial cells produced chimeric progeny during mammary gland regeneration. These results suggested that the mammary epithelial cells themselves in combination with the mammary fat pad and its stroma were essential components for a mammary stem cell niche.

The regenerative capacity of the mammary gland has been well documented [1–4]. Based on our understanding of the mammary niche, from our own work and the literature, we can hypothesize that mammary stem cells are stably maintained within specific microenvironments throughout the gland for life [5]. Mammary regeneration also occurs when dissociated epithelial cells from mammary glands are transplanted into cleared mammary fat pads, suggesting that complete mammary epithelial stem cell niches may be reconstituted de novo [6–8]. Stepwise dilution of dispersed mammary cells, or limiting dilution, results in a decrease in the percentage of inoculated fat pads that are rendered positive for mammary tree growth, implying reduction in the number of mammary epithelial stem cells [7, 9]. We hypothesize that the remaining cells encompass the epithelial signaling components and might support glandular regeneration if supplied with an extraneous source of stem/progenitor cells when injected into the mammary stroma.

Employing tissue-targeted, Cre-lox-mediated, conditional activation of a reporter gene, our laboratory has obtained evidence of the formation of a previously unrecognized mammary epithelial cell population that may originate from differentiating cells during pregnancy. This population does not undergo cell death during involution following lactation and persists throughout the lifetime of the female mouse. In transplantation studies, these cells show the capacity for self-renewal and contribute significantly to the reconstitution of resulting mammary outgrowths. In limiting dilution assays, it was found that these cells could generate both luminal and myoepithelial lineages, generate out-growths comprising both lobule and duct-limited epithelial outgrowths, and differentiate into all of the cellular subtypes recognized within the murine mammary epithelium. We have named these cells parity-induced mammary epithelial cells (PI-MEC). They undergo self-renewal, are multipotent, and contribute progeny directly to the formation of secretory acini in subsequent pregnancies. Therefore, PI-MEC represent LacZ⁺ lobule-limited, pluripotent epithelial progenitors, one of three distinct multipotent cell types previously identified in the mouse mammary gland [7, 9]. To determine whether LacZ⁺ PI-MEC could develop from uncommitted cells from another adult tissue upon interaction with a mammary microenvironment, comprised of signaling mammary epithelial cells and the mammary fat pad stroma, we mixed testicular cells (from seminiferous tubules), hematopoietic cells (from

bone marrow) and thymus cells from adult whey acidic protein promoter (WAP)-Cre/Rosa26 R mice with limiting dilutions of mammary epithelial cells and inoculated them into epithelium-cleared mammary fat pads. The mammary outgrowths generated in the transplanted glands through pregnancy, lactation, and involution in the hosts were “phenotypically” normal. Therefore during the reformation of mammary stem cell niches by dispersed epithelial cells, in the context of the intact epithelium-free mammary stroma, non-mammary cells must be sequestered and reprogrammed to perform mammary epithelial cell functions including those ascribed to mammary stem/progenitor cells. This is therefore a powerful technique for the redirection of cells from other organs/tumors to a normal mammary phenotype. Figure 1 provides a summary illustration of the normal mammary niche and its disruption and incorporation of non-mammary stem/progenitor cells. Further studies have demonstrated our ability to also reprogram cells from alternate germ layers, to reprogram mouse embryonic stem cells, and both mouse and human cancer cells to a non-tumorigenic mammary fate. Below we describe our experimental methods and strategy.

2 Materials

2.1 Mice

The transgenic WAP-Cre/Rosa26 R mice were engineered and typed as described by Wagner et al. (Fig. 2) [10]. Female Nu/Nu/NCR mice were used as hosts for the transplantation studies. All mice are housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The National Cancer Institute Animal Care and Use Committee approved all experimental procedures.

2.2 Media and Solutions

1. Complete Media: DMEM with glutamine, PenStrep 1×, HiFBS 10 %, EGF 10 ng/ml, insulin 4 µg/ml.
2. Carnoy’s solution: 60 % ethanol, 30 % chloroform, 10 % acetic acid (*see* Note 1).
3. 4 % paraformaldehyde: 40 g/l in 1× PBS. Start at 800 ml PBS. In laminar flow hood heat to 60 °C, adding 1 N NaOH until powder goes into solution. Cool and adjust pH to 7.4.

2.3 Tissue Culture Supplies

1. DMEM media for mixing with collagenase and pronase.
2. 250 ml disposable Erlenmeyer flasks for shaking cells in incubator.
3. 60 mm dishes for mincing cells.
4. Scalpels (any size blade).
5. Centrifuge.
6. 10 ml pipets.

7. 10 cc syringes.
8. 19 G needles.
9. T-75 flasks.
10. Trypsin.
11. DNase.
12. 40- μ m filters.
13. Trypan blue stain.
14. Hemocytometer.

2.4 Surgical Instruments

1. Deweckers scissors.
2. Watchmakers forceps.
3. Bent angle forceps.
4. Micro dissection scissors.
5. Low temperature cautery.
6. Cork board.
7. Tie downs made from string to immobilize mouse during surgery (*see* Note 2).
8. Microscope for visualizing animal (or magnifying headset) (*see* Note 3).
9. Light source (*see* Note 4).
10. Wound clips (9 mm).
11. Wound clip applicator.

3 Methods

3.1 Single Cell Dissociation Procedure

1. Excise the two inguinal (#4) mammary glands from the donor mouse.
2. Mince the glands into 1–2 mm pieces in DMEM (20 ml/mg tissue), containing 0.1 % collagenase and 0.1 % hyaluronidase, using scalpels and sterile technique.
3. Shake the mixture at 100 rpm for approx. 90 min at 37 °C, until the pieces are dissipated.
4. Centrifuge the mixture at 100 $\times g$ for 5 min at room temperature.
5. Save the fatty layer on top and the pellet.
6. Resuspend the pellet in 10 ml DMEM containing 1.25 % pronase, using 10 ml/g of original tissue.
7. Shake the cells at 100 rpm for 15 min at 37 °C.

8. Again, centrifuge the cells at $100 \times g$ for 5 min at room temperature.
9. Wash the cell pellet in $3 \times$ DMEM.
10. Resuspend the pellet in 10 ml DMEM.
11. Filter the cells through 40 μ m cell strainer.
12. Use 5 μ l for trypan blue viability stain.
13. Count the cells using a hemocytometer.

3.2 Clearing of Mammary Fat Pad

Mammary fat pad clearance and transplantation is performed on female mice between 3 and 4 weeks of age.

1. Anesthetize the mice with an intraperitoneal (IP) injection of ketamine–xylazine (120–130 μ l at 10 mg/ml ketamine and 1 mg/ml xylazine).
2. Once the mouse is unconscious, secure it to a surgical table with string tie downs.
3. The ventral surface is further anesthetized with a topical solution such as Sensorcaine solution (*see* Note 5)
4. Expose the mammary fat pads by cutting and folding back the ventral skin. Use a cautery tool to block blood vessels in the inguinal (#4) fat pads, and also to disconnect these fat pads from nearby tissue.
5. “Clear” the fat pads off endogenous epithelium by surgically removing the proximal portion (from the nipple to the lymph nodes) (Fig. 3).
6. In order to transplant the tissue fragments (1–2 mm² of mammary tissue) create a small cavity in the fat pad with watch-maker forceps.
7. For cell inoculations, use a Hamilton syringe with a fine gauge (e.g., 30 G) needle to inject 10 μ l of cell preparations into each cleared fat pad.
8. Once the transplant is in place, pull back the ventral skin over the exposed area and close the wound using metal clips.
9. Place the mouse on a heating pad in a “recovery cage” with non-stick bedding, and monitor them until they are fully recovered.
10. Remove the metal clips 7–10 days post-surgery.

3.3 Sperm/Germ Cell Dissociation Procedure

Excise the testes from WAP-Cre/Rosa26 R males following the protocol in Bellve et al. [11] with a few modifications:

1. De-capsulate the testes to remove the tunica albuginea.
2. Place them in DMEM containing 0.5 mg/ml collagenase and incubate at 33 °C in a shaking water bath for 15 min at 120 rpm.

3. Centrifuge the suspension at $120 \times g$ for 5 min to pellet the seminiferous chords.
4. Wash the top, fatty layer, and the pellet twice in DMEM to facilitate removal of the interstitial tissue.
5. Place the dispersed seminiferous chords in 20 ml DMEM containing 0.5 mg/ml trypsin and 1 g/ml DNase, and incubate as above.
6. Shear the remaining cell aggregates by pipetting 10–12 times.
7. Recover the cells by centrifugation and wash as above.
8. Resuspend the cells in 10 ml DMEM containing 0.5 % BSA and filter them through a 40- μ m filter to remove any remaining clumps.
9. Determine the viability by Trypan blue exclusion, and count the cells in a hemocytometer. Average yields of $15\text{--}20 \times 10^6$ cells are usually obtained per procedure.

3.4 Mammary Cell Isolation

Mammary epithelial cells are isolated using a standard protocol employed for primary cell culture:

1. On day one, place the excised mammary glands in a 60 mm petri dish with a small amount of collagenase (1 mg/ml in complete media).
2. Mince the glands with scalpels into 1–2 mm fragments.
3. Transfer the fragments into a 50 ml conical tube containing 10 ml collagenase (1 mg/ml in complete media) per two glands.
4. Place the tubes in a tissue culture incubator at 37 °C overnight.
5. On day 2, Triturate the fragments through a 10 ml pipette three times to dissociate the cell aggregates.
6. Centrifuge the resulting suspension for 10 min at $100 \times g$ to recover the cells.
7. Resuspend the cells in 10 ml complete media.
8. Shear the resuspended cell aggregate ONCE only through a 19-G needle (*see* Note 6).
9. Recover the cells by centrifugation for 10 min at $100 \times g$.
10. Resuspend the cells in 15 ml complete medium, transfer them into a T-75 flask and place it in a tissue culture incubator.
11. Carry out differential trypsinization to remove fibroblasts after 3–4 days (*see* Note 7).
12. Collect cells from primary mammary cultures after 4–7 days on plastic culture flasks.

3.5 Mixing Experiments

Setup the exogenous stem/progenitor cell populations from Wap-Cre/Rosa26 transgenic mice in the following manner (Fig. 4).

1. Mix 50,000 cells from normal FVB or Balb/C mice in a culture tube with 50,000 stem/progenitor cells from Wap-Cre/Rosa26 mice (*see* Note 8).
2. For ES cells use stem/progenitor cells at 1000 and 10,000 cells and mix with 50,000 mammary cells (*see* Note 9).

3.6 Cell and Tissue Transplantation

The surgical techniques used to clear the mammary epithelium from the no. 4 fat pad of 3-week old host mice, and the subsequent transplantation of tissue fragments or cell suspensions, have been described in detail [4, 6–8].

1. Anesthetize the mice and clear the gland as described above immediately before insertion of the transplanted fragment or cell suspension.
2. Inject the cell suspensions in 10 μ l volumes with a Hamilton (Reno, NV) syringe equipped with a 30-G needle.
3. Using correct ratio (1:1 or less depending upon starting material) mix mammary and progenitor cell populations diluted to a final volume of 10 μ l.
4. Mix cells well before inoculation into animal.
5. Draw cells into syringe.
6. While holding the cleared fat pad with forceps, gently insert needle at an angle and slowly inject 10 μ l cell mixture.
7. Remove the needle and gently set the flap of skin over the gland.
8. Repeat this procedure for each gland.
9. Close the mouse with wound clips post inoculation.
10. Cut the fragments to be implanted into 1–2 mm pieces.
11. Use watchmaker forceps to create a small pocket in cleared gland (*see* Note 10).
12. Gently place the fragment into the hole.
13. Close the flap of skin over transplanted gland.
14. Staple the mouse closed upon completion of both inguinal glands.
15. Place the implanted females with males 6–8 weeks following implantation to initiate pregnancy and secretory development.

3.7 Mammary Gland Whole Mounts and X-Gal Staining

3.7.1 Paraformaldehyde Fixation and X-Gal Staining

1. Excise the cleared/implanted inguinal fat pad from the mouse and place on a glass slide.

2. Fix the gland with 4 % paraformaldehyde (PFA) at room temperature for 1–1.5 h.
3. Permeabilize the gland in 0.01 % Nonidet P-40 in PBS overnight at 4 °C.
4. Place the glands (on the glass slides) in 5 ml of X-gal mixture in a 50 ml conical tube, and add 125 µl (1× final concentration) of X-gal substrate.
5. Wrap the tubes in aluminum foil and incubate for 24–30 h at 37 °C in an incubator. After staining, wash the glands 2 × 30 min in 1× PBS.
6. Dehydrate the glands by stepwise treatment with ethanol, starting with 2 × 70 % for 30 min, and then 2 × 100 % for 60 min.
7. Once dehydrated, clear the glands in xylene for 2 h.
8. Whole mount the glands on slides using mounting medium and cover slips. They are now ready for image analysis (Fig. 5).

3.8 Preparation of Mammary Gland Whole Mounts for Immunostaining

1. Spread the whole inguinal gland on a glass slide and fix in 4 % PFA overnight at 4 °C as above.
2. Process the gland and stain with X-Gal as above.
3. Rinse the stained glands repeatedly in PBS.
4. Fix the glands in Carnoy's fixative for 1–2 h at room temperature.
5. Clear the glands in 100 % ethanol, 2 × 60 min.
6. Complete the clearing of the glands by placing them in xylene (before whole-mount analysis).
7. For histological examination, X-Gal-stained whole mounts are re-embedded in paraffin, sectioned at 6.0 µm, and counter-stained with nuclear fast red.
8. Immunocytochemistry is carried out on deparaffinized sections.

3.9 DNA Extraction and PCR

PCR analysis is performed for identification of male DNA in chimeric reprogrammed populations.

1. Extract the genomic DNA from mammary tissue using an appropriate DNA isolation kit. Perform PCR analysis with WAP and Cre primers as described by Wagner et al. [10, 12], Y6 primers described by Peters et al. [13] and standard mouse GAPDH primers [14].
2. Reaction times are as follows:
3. 95 °C for 5 min.
4. 30 cycles:
 - a. 95 °C for 30 s.

- b. 55 °C for 30 s.
 - c. 72 °C for 30 s.
 - d. 72 °C for 10 min.
 - e. 4 °C until removed.
5. Amplified DNA is loaded into 1.25 % agarose gels containing ethidium bromide (0.5 µg/ml), electrophoresed at 60–100 V, and visualized under UV light (Fig. 6).

4 Notes

1. When making this solution always start with ethanol and add chloroform second, acetic acid is added last.
2. Tie downs can be made by looping string through a 200 µl pipet tip that has had the end cut off. The loop allows the hand or foot to be held and the remaining string out of the back of the pipet tip can be pinned down with a pushpin.
3. Using either a microscope or magnifying glasses allows a clearer surgical view and more precise cautery/incision/implantation. The use of these is strongly recommended.
4. An alternate light source is useful to make the surgical area bright enough to see without straining one's eyes. This is also strongly recommended, but care must be used as this can cause the mouse to dry out quickly if the light is too close to the animal.
5. These are available through a veterinary pharmacy.
6. This procedure is only performed ONCE.
7. Fibroblast levels should be reduced before collection of the epithelial cells by differential trypsinization.
8. This number represents a limiting dilution of mammary populations that allows for competition from alternative stem/progenitor cells in reforming mammary niches.
9. For ES cells, the number of stem/progenitor cells is greatly reduced due to the ES cells capacity for tumor formation when transplanted into the mammary fat pad.
10. Instruments must be kept sterile during the survival surgical procedure.

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Fig. 1. Schematic presentation of niche and occupation by non-mammary stem cells. The *left side* shows a normal mammary microenvironment occupied by mammary stem cells. The *right side* shows the occupation of reforming mammary niches by non-mammary stem cell populations. We have found that PI-MECs cannot usually make cap cells

Fig. 2.
Wap-Cre/Rosa 26stop model. The diagram illustrates the removal of the stop sequence upon activation of Cre driven by a Whey Acidic Protein promoter (WAP) in the mammary gland (Wagner et al. [12])

Fig. 3.

Fat pad clearing. The mammary fat pad (*blue*) is exposed by cutting open the ventral skin. To clear endogenous epithelium, the lymph nodes and blood vessels (*red*) are severed with a cautery tool, and the proximal portion of the gland is cut away. Cells or tissue fragments can be transplanted into the remaining fat pad

Fig. 4. Experimental design. A schematic presentation of the cell mixing experiment and subsequent second-generation transplantation. WAP-Cre/Rosa26-fl-stop-LacZ marked testicular cells from seminiferous tubules can combine with wild type mammary epithelial cells to regenerate a mammary gland

Fig. 5.
Whole mount. Image of a cleared whole mount from a chimeric gland, illustrating how the testicular cells have been reprogrammed to a mammary fate

Fig. 6. Expression of male-specific Y6 transcripts in regenerated mammary gland post-transplantation of marked testicular cells. PCR detection of the chimeric outgrowth and control cell populations to identify the presence of male cell contribution in the mammary cell populations