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Isolation and characterization of cutaneous epithelial stem cells

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SUMMARY

The outer layer of mammalian skin is a multilayered epithelium that perpetually renews multiple differentiated lineages. During homeostasis, the maintenance of skin epithelial turnover is ensured by regionalized populations of stem cells that largely remain dedicated to distinct epithelial lineages including squamous, follicular, sebaceous, Merkel, and sweat glands. Cutting edge developments in this field have focused on i) stem cell activation cues derived from a number of extrinsic sources including neurons, dermal fibroblasts and adipocyte, and immune cells; and ii) characterization of epithelial stem cell homeostasis via hierarchal versus stochastic paradigms. The techniques outlined in this chapter are designed to facilitate such studies and describe basic procedures for cutaneous stem cell isolation and purification, which are based on leveraging their unique expression of surface proteins for simultaneous targeting and purifying of multiple subpopulations in adult skin. In addition, protocols for assessment of *in vitro* and *ex vivo* progenitor capacity, as well as techniques to visualize progenitor populations in whole skin are discussed.

1. Introduction

The perpetual renewal of mammalian skin is known to be maintained by permanently residing stem cells that are able to sustain at least five principal differentiated lineages: the interfollicular epidermis (IFE), sebaceous gland (SG), hair follicle (HF), Merkel cells and sweat glands (1–3). While it has long been accepted that skin homeostasis is dependent on the ability of stem cells to replenish the turnover of these mature epithelial lineages, it is the technological advances in the areas of skin stem cell isolation and genetic drivers over the last decade that has significantly enhanced our ability to effectively characterize progenitor niches in the skin. These findings have dramatically changed our view of the cutaneous epithelial stem cell landscape rendering a highly compartmentalized epithelium maintained by multiple classes of phenotypically distinct regional niches (2).

In some cases progenitor niches have been accessed using mouse genetics approaches and characterized under normal conditions to be long-lived and able to sustain the cellular input

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to certain epithelial structures including the interfollicular epidermis (4, 5), sebaceous gland (6, 7), hair follicle (8–11, 36), and touch dome (23, 24). In other cases, antibodies against cell surface proteins have been utilized to mark and isolate epithelial progenitors located in the IFE (3, 5, 12) and HF (13–16, 34). These efforts have facilitated our understanding of the relative proliferative capacity of progenitor pools as well as their capacity to regenerate IFE, HF, SG or Merkel lineages in surrogate assays.

In recent years, studies have elucidated that regulation of skin homeostasis by epithelial stem cells depends on several types of extrinsic activation signals. Treg expression of Notch family member Jagged1 induces HFSC differentiation during the hair cycle (29). Also, both FGF9 secretion by γδ T cells and CX_3CR1 and TGFβ1 secretion by cutaneous macrophages support skin homeostasis by inducing stem cell mediated hair follicle regeneration after injury (27–28). In alopecia areata, signaling by cytotoxic T lymphocytes disrupts hair follicle progenitor cells, stopping hair growth (30). Dermal adipocytes have also been shown to play a role in hair follicle stem cell activated regulation of the hair follicle growth cycle; they can both stimulate and inhibit hair growth through PDGF and BMP signaling (26, 31–32). Additionally, both bulge and touch dome stem cells require neuronal SHH signaling for their maintenance (25, 33). Collectively, these studies have illustrated that epithelial progenitors maintain skin homeostasis and respond to insult through a complex crosstalk with a variety of external cues.

As new biomarkers have been implemented to better define the profile of progenitor cell subsets in the IFE and HFs, the individual cell of interest becomes less frequent. This can be a major technical challenge to functional studies such as skin and hair reconstitution and clonogenic studies where a significant number of cells may be required. In this chapter, we will outline some basic methods for isolation and functional assessment of keratinocyte clonogenicity, multipotency and self-renewal capabilities from freshly isolated single cell suspensions of murine epidermal keratinocytes that have been subjected to FACS sorting. In particular, we will focus on clonogenic and skin and hair reconstitution assays. Methodologies to establish cultures of epidermal keratinocytes at clonal densities have been established for more than 3 decades and were developed by Rheinwald and Green, whose Colony Forming Efficacy (CFE) assay uses a feeder layer of mitotically-arrested mouse 3T3 fibroblasts (17). There have been many modifications added this method over the years (18). As such, we also describe new methodology that enhances the CFE assay by using serum-free media with additional extracellular matrix proteins. The development of the hair reconstitution assay (19, 20) revealed the shortcomings of *in vitro* assays, which typically do not account for stem cell potency. Importantly, we feel the ability to conduct skin and hair reconstitution assays from freshly isolated FACS-sorted keratinocyte subsets provides a robust platform to identify and distinguish unipotent, bipotent and multipotent epithelial progenitors.

We also include a protocol for whole mount skin immunolabeling, which enables the identification of less frequent cell types and the visualization of entire cell networks. This whole skin protocol can also be used with genetically labeled mice to visualize individual stem cells in their niches. This technique potentially allows for lineage-tracing studies in

three-dimensional space, which can facilitate studies to determine whether skin homeostasis results from a hierarchical (37) or stochastic (4, 35) stem cell function (38).

2. Materials

2.1. Skin cell isolation solutions

1. 2.5% Trypsin (Life Technologies) diluted to 0.25% in Hank's Balanced Salt Solution (HBSS) (Invitrogen).
2. 1X PBS, pH = 7.6 (Invitrogen), sterilized.
3. Fibroblast growth medium: DMEM (Invitrogen) supplemented with 10% Donor Bovine Serum (Invitrogen) and 2% Penicillin-Streptomycin (Invitrogen).
4. Collagenase Type I (Worthington Biochemical), 10 mg/ml stock solution in PBS.
5. DNAse I (Worthington Biochemical), 20,000 units/ml stock solution in PBS.
6. 70 μm cell strainer (Fisher Scientific).
7. Betadine 1% solution in water.
8. 70% EtOH solution.

2.2. Antibodies

1. α6 integrin (CD49f, BD Biosciences) (see Note 1).
2. Sca-1 (Ly6G, BD Biosciences)
3. CD34 (RAM 34, BD Biosciences)
4. CD200 (OX-2, BD Biosciences)

2.3. Clonogenic assay

1. Nunclon 6-well dishes (Fisher Scientific).
2. 4% Paraformaldehyde in PBS, pH 6.
3. Rhodamine B, 1% solution in H_2O (Sigma).

Classic CFE assay:

1. Complete FAD growth medium: 3 parts DMEM (Invitrogen), 1 part Ham's F12 Supplement (Invitrogen), 10% Defined Fetal Bovine Serum (HyClone), 10 ng/ml EGF (Peprotech), 0.5 mg/ml hydrocortisone (Sigma-Aldrich), 10^{-10} M cholera enterotoxin (Sigma-Aldrich), 5 mg/ml insulin (Sigma-Aldrich), 1.8×10^{-4} M adenine (Sigma-Aldrich), 100 U/ml penicillin (Invitrogen) and 100 mg/ml streptomycin (Invitrogen).
2. 3T3 fibroblasts (ATCC) mitotically arrested with either Mitomycin c (Sigma) or γ-radiation.
3. 0.25% trypsin/1 mM EDTA stock solution (Invitrogen).

Serum-free CFE assay:

1. Cnt-PR serum free medium (ZenBio).
2. VF coating medium (William's E medium without L-Glutamine (Gibco) with: 0.01mg/mL Fibronectin (Sigma), 0.03mg/mL Vitrogen Collagen (Advanced Biomatrix), 25 ug/mL Tenascin-C (R&D Systems) (See Note 2), 10% BSA, 1M HEPES, and 116mM CaCl2)

2.4. Skin reconstitution assay

1. Silicon culture chambers- Upper F2U #30–268; Lower F2L #30–269 (Renner Gmbh).
2. Surgical instruments including forceps, curved scissor, stapler and staple remover (all from Temin); sterile drapes, alcohol swabs, and anesthetics.
3. Immunodeficient mice Nude mice (NCR nude, male, 7–9 weeks old, supplied by Taconic or we prefer NSWNU-M (homozygote females) (see Note 3).
4. Small heating pad.

2.5. Whole mount staining assay

1. 1X PBS, pH = 7.6 (Invitrogen).
2. Depilatory cream (Surgicream, American International Industries).
3. 4% Paraformaldehyde in PBS, pH 6.
4. Wash solution (3% Triton X-100 in PBS).
5. Antibody solution (20% DMSO, 0.05% Normal Serum, 1% Triton X-100 in PBS).
6. Aqueous mounting media containing a nuclear counterstain such as DAPI.

3. Methods

3.1. Epidermal keratinocyte isolation

1. Under a biological cabinet, submerge euthanized eight-week old mice in 1% Betadine for 2 min and wash in sterile H_2O. Submerge mice in 70% EtOH for 1 min and wash in sterile H_2O.
2. Under a biological cabinet, surgically excise the dorsal skin from using sterile forceps and scissors and float skin dermis side up in sterile PBS in a sterile Petri dish. Scrape away the subcutaneous fat and muscle using a sterile scalpel and forceps. During scraping, move skin to a new Petri dish containing fresh PBS as necessary (see Note 4).
3. Float skins epidermis side up in 0.25% trypsin (in Ca^{++} free HBSS) in a 10 cm Petri dish for 1.5 – 2 hours (see Note 5).

4. Aspirate trypsin and recover skins in 10 ml fibroblast growth medium. Gently detach epidermis from dermis using a scalpel and mince epidermal scrapings into small pieces.

5. Transfer scrapings into a sterile 100 ml bottle and add 30 ml fibroblast growth medium and a stir bar. Recover epidermal cells with gentle stirring for 30 min and filter cells through a 70 μm cell strainer into a 50 ml Falcon tube. Spin cells for 10 min @ 1000 rpm. Wash cells in 10 ml PBS, spin and resuspend cells in 10 ml fibroblast growth medium and spin for 10 min @ 1000 rpm.

6. For antibody labeling (see below), resuspend cell pellets at 5 – 10 × 10^6 cells/ml in fibroblast growth medium. Typically 10 – 12 × 10^6 viable basal keratinocytes are harvested from a single dorsal skin.

3.2. Preparation of highly inductive dermal fibroblasts

1. Surgically excise the dorsal skin from euthanized postnatal day 1–2 mice using sterile forceps and scissors (see Note 6).

2. In a dry Petri dish lay skins flat dermis side down with no folded edges. Slowly pour in ice-cold 0.25% trypsin/1 mM EDTA and avoid getting the tops of the skins wet. Incubate skins overnight at 4°C.

3. Remove skins, one at a time, from trypsin and place on dry p150 plate, dermis side up. Flatten it again and use fine forceps to separate epidermis from dermis, starting at one edge of skin and flipping the dermis up and off the epidermis, which should stay on the plate.

4. Transfer dermis one at a time to a plate containing 10 ml of media on ice.

5. For 8 dermises, use 0.5 mL collagenase stock solution (10 mg/mL in H_2O) plus 12 mL HBSS in a 50–100 ml sterile beaker. Transfer dermises into the beaker and mince into small pieces using sharp scissors.

6. Transfer to a 250 ml flask with a magnetic stir bar. Stir @ 37°C for 30 min (Optional: For the last 5 min add 20 μl of DNAse I (stock at 20000 U/ml in PBS)

7. Dilute 3 – 4 fold with media and filter through sterile gauze or 70 μm filter. Rinse the flask with 5 ml media and pass through the filter.

8. Spin the cells at 1500 rpm for 5 min at 4C

9. Resuspend and wash the pellet once in HBSS

10. Count cells, use @ 8 × 10^6 fresh dermal cells per graft, cryopreserve or plate at 1 × 10^6 cells per 10 cm dish for later use.

3.3. Antibody labeling and FACS analysis

1. Select an appropriate panel of antibodies for the target cells of interest (Please see Table 1 for examples of published progenitor marker profiles). When possible, select antibodies directly conjugated to fluorescent dyes.

2. Count cells and aliquot equal amounts into experimental and control (single-stained and unstained tubes) in complete growth medium.

3. Incubate antibodies at concentrations according to manufacturer guidelines for 30 min to 1 hour in complete growth medium on ice (see Note 7). Spin and wash cells, resuspend in growth medium supplemented with DAPI or an alternative nuclear stain.

4. Conduct sorting (see Note 8). Typically, 10 – 20 × 10^6 viable $\alpha6^+$ basal keratinocytes can be sorted from a single dorsal skin.

3.4. Clonogenic analysis

Clonogenic assays typically require fewer cells compared to skin and hair reconstitution assays, and harvested cells from a single animal will usually suffice. Cells can be grown with a confluent layer of mitotically arrested 3T3 fibroblasts in complete FAD growth medium or defined serum free medium in the absence of fibroblasts. We include the both the classic protocol for the CFE assay that uses a feeder layer of mitotically-arrested mouse 3T3 fibroblasts, as well as our CFE assay that uses serum-free media with additional extracellular matrix proteins. The classic protocol was optimal to perform the CFE assay with human keratinocytes. While human keratinocytes form colonies in high Ca^{++} conditions, mouse keratinocytes differentiate when exposed to high Ca^{++}. The recent availability of low Ca^{++} media enables our ability to perform the CFE assay with human and mouse keratinocytes in serum-free conditions and without co-culture with a feeder layer.

Classic CFE assay in FAD medium:

1. Preparing the 6-well plates one day in advance will allow the fibroblasts to fully attach, spread and condition the growth medium. Plate 1 × 10^6 mitotically arrested 3T3 fibroblasts in 3 ml complete FAD growth medium per well.

2. The next day harvest human keratinocytes from a single mouse dorsal skin and FACS sort desired keratinocyte subpopulations as described above. The FACS instrument can be optimized towards purity and accuracy in counting since cell numbers will be in excess. Propidium iodide or DAPI should be used to exclude dead cells. Many of the dead cells are post-mitotic suprabasal cells that are sensitive to the 70% ethanol washes during cell harvesting. Use either the FACSAria Automated Cell Deposition Unit (ACDU) function or manually seed 1 × 10^3 (a range of 0.5 to 2 X10^3 cells can be used) sorted keratinocytes per well.

3. Incubate cells for 2 weeks at 32°C in a humidified incubator with 5% CO_2 and change the medium every 48 hours.

4. After 2 weeks, aspirate off the culture medium and replace with 3 ml Versene per well. After 1–2 min at room temperature, detach the feeder layer by repeated pipetting of Versene over the plate (keratinocytes will not detach). Gently wash plates 2 times with PBS (take care not to detach colonies).

5. Fix for 1 hour at room temperature with 4% PFA. Gently wash plates 2 times with PBS.

6. Stain wells with Rhodamine B for 1 hour (see Note 9) at room temperature (just enough Rhodamine solution to cover the cells is sufficient). Aspirate off Rhodamine solution and gently wash wells 3 times with PBS.

7. Aspirate off the final wash and allow wells to completely dry by turning plates upside down. Afterwards, plates can be imaged and colonies may be manually counted. Typically the total number of colonies as well as the number of colonies greater than 4 mm in diameter are counted and compared between keratinocyte subpopulations.

Serum-free CFE assay:

1. Add 1mL VF coating medium to each well of 6-well plate and incubate at 37°C for 1 hour. Aspirate VF coating medium and add CnT medium to wells.

2. Harvest mouse or human keratinocytes from a single mouse dorsal skin and FACS sort desired keratinocyte subpopulations as described above. The FACS instrument can be optimized towards purity and accuracy in counting since cell numbers will be in excess. Propidium iodide or DAPI should be used to exclude dead cells. Many of the dead cells are post-mitotic suprabasal cells that are sensitive to the 70% ethanol washes during cell harvesting. Use either the FACSAria Automated Cell Deposition Unit (ACDU) function or manually seed 1 × 10^3 (a range of 0.5 to 2 X10^3 cells can be used) sorted keratinocytes per well.

3. Incubate cells for 2 weeks at 32°C in a humidified incubator with 5% CO_2 and change the medium every 48 hours.

4. After 2 weeks, aspirate off the culture medium, gently wash with PBS (take care not to detach colonies) and fix in 4% PFA for 1 hr at room temperature.

5. Gently wash with PBS and stain wells with Rhodamine B for 1 hour (see Note 9) at room temperature (just enough Rhodamine solution to cover the cells is sufficient). Aspirate off Rhodamine solution and gently wash wells 3 times with PBS.

6. Aspirate off the final wash and allow wells to completely dry by turning plates upside down. Afterwards, plates can be imaged and colonies may be manually counted. Typically the total number of colonies as well as the number of colonies greater than 4 mm in diameter are counted and compared between keratinocyte subpopulations.

3.5. Skin and hair reconstitution assay

1. Clip hair with electric clippers, if necessary, and clean the dorsal skin with 1% Betadine and place anesthetized mice on heating pad.

2. Use scissors to make a small incision on the back of the mouse (approximately 1 cm in diameter). Better areas for chamber placement are interscapular or suprapelvic. Do not make incisions directly on the spinal protrusion.

3. Assemble the upper and lower grafting chambers together and insert through the incision so that the rims of the chamber are under skin (see Note 10).
4. Secure the chamber to the skin with surgical stapler clips (two staples is usually enough).
5. Allow the chamber to adhere to the dorsal surface overnight prior to implanting cells.
6. Mix the desired number of epidermal cells and 2×10^6 dermal cells together as a slurry in HBSS. We have successfully implanted 1×10^5 to 6×10^6 epidermal cells per graft. Spin cells at 1000 rpm for 5 min, resuspend the pellet in 100 μl HBSS and store on ice until use.
7. Gently mix cell suspensions before pipetting entire aliquot into chamber of hat, through the hole on top.
8. Replace each mouse in individual cages (on belly and away from the spout of the water bottle).
9. After one week (see Note 11), anesthetize mice and remove staples and gently tug on chamber to release it from mouse's back. Use tweezers to loosen skin around edge of chamber. Grafted area may be moist and oozy, leave it be and replace mouse in cage, as before.
10. Chambers are retained, cleaned (soak overnight in soapy water) and autoclaved for reuse.
11. Grafts are usually biopsied at 5 – 10 weeks post-grafting. Hair usually appears after approximately 2 – 3 weeks.

3.6. Skin whole mount immunolabeling

1. Shave the hair of the dorsal skin of euthanized mouse with electric clippers and apply depilatory cream to the shaved dorsal surface for 6 minutes. Rinse well with warm water and pat dry with paper towel.
2. Surgically excise the dorsal skin using forceps and scissors. Place the excised skin on a flat surface dermis side up. Quickly scrape away the subcutaneous fat and muscle using a scalpel. Do not allow the skin to dry. Transfer the scraped skin to a Petri with PBS and scrape off residual adipose tissue.
3. Secure dorsal skin epidermis side up onto a microscope slide using four small binder clips. Ensure that the skin is stretched and flat. Place slides into a container containing cold 4% PFA (up to 3 skins per 200 mls 4% PFA). Refrigerate at 4°C overnight.
4. Use a scalpel to remove the tissue from the slide, cutting a rectangle just inside the bounds of the clips. Cut this into two narrow strips and nick the top right corner of each strip to indicate the epidermal side.
5. Place strips into 50mL falcon tubes containing 45–50mL wash solution for 4 hours with shaking, changing solution every hour.

6. Place strips into a 5mL tube containing antibody solution. Add an appropriate panel of antibodies for the target cells of interest. Incubate with shaking for 48–72 hours.
7. Wash in 50mL falcon tubes containing 45–50mL wash solution for 4 hours with shaking, changing solution every hour. Place strips into a 5mL tube containing antibody solution and secondary antibodies to the chosen panel.
8. Wash in 50mL falcon tubes containing 45–50mL wash solution for 4 hours with shaking, changing solution every hour.
9. Arrange the strips on a slide so that the nick is on the upper right-hand corner. Mount strips onto a slide with approximately 100 μL aqueous mounting media containing a nuclear stain such as DAPI.

4. Notes

1. The use of directly conjugated antibodies is recommended whenever possible.
2. We have seen that adding Tenascin-C to the VF coating medium augments clonogenic capacity.
3. In our hands, hairless immunodeficient mouse strains such as Nude are more amenable to skin grafting procedures.
4. We typically use three Petri dishes with clean PBS per skin.
5. After 1.5 hours of trypsin digestion, check the skins for detachment by gently scraping the epidermis with a scalpel. If the epidermis is easily removed then no further digestion is required. If the epidermis does not detach, check again every 15 minutes.
6. Euthanized pups are washed in sterile water once, followed by two washes in 70% EtOH. EtOH is removed completely and clean pups are placed in sterile Petri dish in the hood. When processing multiple pups, place detached skins in PBS until all skins are harvested.
7. Depending on affinity and purification, antibody labeling concentrations typically range from 0.25 – 1.0 ug per 1×10^6 cells
8. A high-speed sorting device is required, i.e. BD FACSAria; however, many factors contribute to a successful cell sort that require consideration. The length of the trypsin digest required to separate the epidermal and dermal compartments renders the isolated keratinocytes fragile to further mechanical stress. Keratinocytes also have a tendency to aggregate and, although it is possible to pre-filter the sample and gate out most aggregates electronically, they threaten the number of events that can be analyzed and sorted due to clogging of the sample line. To account for these issues, we prefer at larger size nozzle (100 μm) in order to obtain an uninterrupted sort. This on the other hand decreases the sheet pressure and limits the drop drive to around 24.000 drops/s on the BD FACSAria system. The resulting events that can be analyzed per second therefore

are around 8000. If the population of interest is 3% of the total sample, 240 target events can be identified of which between 5 −15% will be aborted electronically as they present a conflict to the purity of the sample. Thus, acquiring 1×10^6 target cells would require more than 1.5 – 3 hours of efficient sorting time. If more cells are required, it may be necessary to sacrifice the animals and harvest the cells at multiple time points in order to maintain viable cells.

9. The cells can be stained from 1 hour to 1 week in Rhodamine B.

10. Prior to implanting the chambers, make sure there is a hole in top half of hat. A small hole punch can be used.

11. If necessary, the grafting caps can be left on the skin for 2 weeks.

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Table 1.

Markers of epithelial progenitor cells in adult mouse skin

Progenitor location	Markers	References
IFE	Lrig1^{+}	5
IFE	α6^{bright}CD71^{dim}	12
IFE & HF Infundibulum	α6^{+}CD34^{-}Sca-1^{+}	16
IFE Touch Dome	α6^{+}CD34^{-}Sca-1^{+}CD200^{+}	3
Junctional zone	Lrig1^{+}	5
HF Isthmus & Infundibulum	α6^{+}MTS24(Plet-1)$^{+}$	15
Sebaceous duct	Blimp1^{+}	7
HF Isthmus	α6^{low}CD34^{-}Sca-1^{-}	16
	Lgr6^{+}	11
HF Bulge	α6$^{dim+bright}$CD34^{+}	13, 14
	Krt15^{+}	21
Lower HF bulge & hair germ	Lgr5^{+}	10
Hair germ	P-cadherin^{+}	22