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## Isolation and characterization of cutaneous epithelial stem cells

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### SUMMARY

During homeostasis, adult mammalian skin turnover is maintained by a number of multipotent and unipotent epithelial progenitors located either in the epidermis, hair follicle or sebaceous gland. Recent work has illustrated that these various progenitor populations reside in regionalized niches and are phenotypically distinct from one another. This degree of heterogeneity within the progenitor cell landscape in the cutaneous epithelium complicates our ability to target, purify and manipulate cutaneous epithelial stem cell subpopulations in adult skin. The techniques outlined in this chapter describe basic procedures for the isolation and purification of murine epithelial progenitors and assessing their capacity for *ex vivo* propagation.

### 1. Introduction

The perpetual renewal of mammalian skin is known to be maintained by permanently residing stem cells that are able to sustain three principal differentiated lineages: the interfollicular epidermis (IFE), sebaceous gland (SG) and hair follicle (HF) (1, 2). In addition, recent studies identified Merkel cell mechanoreceptors residing in specialized epithelial structures termed touch domes in the hairy skin as a fourth lineage maintained by keratinocyte progenitors (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 work over the last decade that has significantly enhanced our understanding the location and function of multiple stem or 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).

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In some cases progenitor niches have been labeled using mouse genetics approaches and characterized under normal conditions to be long-lived and able to sustain the cellular input to certain epithelial structures including the interfollicular epidermis (4, 5), sebaceous gland (6, 7) and hair follicle (8-11). 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). 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. Collectively, these studies have illustrated the role of epithelial progenitors during skin homeostasis as well as their ability to respond to skin insult.

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 (17). While there have been many modifications added to this method over the years (18), we observe the highest success rates when maintaining Rheinwald and Green's principle of using a feeder layer of mitotically-arrested mouse 3T3 fibroblasts. 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.

## 2. Materials

### 2.1. Skin cell isolation solutions

1. 0.25% trypsin/1 mM EDTA stock solution (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  $\mu$ m cell strainer (Fisher Scientific).
7. Hank's Balanced Salt Solution (HBSS) (Invitrogen).

8. Betadine 1% solution in water.
9. 70% EtOH solution.

## 2.2. Antibodies

1.  $\alpha 6$  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. 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),  $1.8 \times 10^{-4}$  M adenine (Sigma-Aldrich), 100 U/ml penicillin (Invitrogen) and 100 mg/ml streptomycin (Invitrogen).
2. Cnt-57 serum free medium (CELLnTEC).
3. 3T3 fibroblasts (ATCC) mitotically arrested with either Mitomycin c (Sigma) or  $\gamma$ -radiation.
4. 0.25% trypsin/1 mM EDTA stock solution (Invitrogen).
5. Nunclon 6-well dishes (Fisher Scientific).
6. 0.5 mM EDTA (Fisher Scientific), a.k.a. Versene.
7. Rhodamine B, 1% solution in H<sub>2</sub>O (Sigma).

## 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 2**).
4. Small heating pad.

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<sup>1</sup>The use of directly conjugated antibodies is recommended whenever possible.

<sup>2</sup>In our hands, hairless immunodeficient mouse strains such as Nude are more amenable to skin grafting procedures.

### 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<sub>2</sub>O. Submerge mice in 70% EtOH for 1 min and wash in sterile H<sub>2</sub>O.
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 using multiple dishes with new PBS as necessary (see **Note 3**).
3. Float skins epidermis side up in 0.25% trypsin/1 mM EDTA in a 10 cm Petri dish for 1.5 – 2 hours (see **Note 4**).
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  $\mu$ m 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  $\times$  10<sup>6</sup> cells/ml in fibroblast growth medium. Typically 10 - 12  $\times$  10<sup>6</sup> 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 5**).
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.

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<sup>3</sup>We typically use three Petri dishes with clean PBS per skin.

<sup>4</sup>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.

<sup>5</sup>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.

5. For 8 dermises, use 0.5 mL collagenase stock solution (10 mg/mL in H<sub>2</sub>O) 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  $\mu$ l of DNase I (stock at 20000 U/ml in PBS))
7. Dilute 3 - 4 fold with media and filter through sterile gauze or 70  $\mu$ m 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 @  $8 \times 10^6$  fresh dermal cells per graft, cryopreserve or plate at  $1 \times 10^6$  cells per 10 cm dish for later use.

### 3.2. 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 9**). Spin and wash cells, resuspend in growth medium supplemented with DAPI or an alternative nuclear stain.
4. Conduct sorting (see **Note 10**). Typically,  $10 - 20 \times 10^6$  viable  $\alpha 6^+$  basal keratinocytes can be sorted from a single dorsal skin.

### 3.3. 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, although the clonogenic efficiency is generally lower. Serum free medium can also be used in control

<sup>9</sup>Depending on affinity and purification, antibody labeling concentrations typically range from 0.25 – 1.0  $\mu$ g per  $1 \times 10^6$  cells.

<sup>10</sup>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 a larger size nozzle (100  $\mu$ m) 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  $1 \times 10^6$  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.

experiments to facilitate assays that quantify the number of adherent cells shortly after plating (12 - 16 hours).

1. Preparing the 6-well plates one day in advance will allow the fibroblasts to fully attach, spread and condition the growth medium. Plate  $1 \times 10^6$  mitotically arrested 3T3 fibroblasts in 3 ml complete FAD growth medium per well.
2. The next day harvest mouse 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  $1 \times 10^3$  (a range of  $0.5$  to  $2 \times 10^3$  cells can be used) sorted keratinocytes per well. 3. Incubate cells for 2 weeks at  $32^\circ\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$  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. Stain wells with Rhodamine B for 1 hour (see **Note 6**) 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 (take care not to detach colonies).
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.

### 3.4. 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 7**).
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.

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<sup>6</sup>The cells can be stained from 1 hour to 1 week in Rhodamine B.

<sup>7</sup>Prior to implanting the chambers, make sure there is a hole in top half of hat. A small hole punch can be used.

6. Mix the desired number of epidermal cells and  $2 \times 10^6$  dermal cells together as a slurry in HBSS. We have successfully implanted  $1 \times 10^5$  to  $6 \times 10^6$  epidermal cells per graft. Spin cells at 1000 rpm for 5 min, resuspend the pellet in  $100 \mu\text{l}$  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 8**), 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.

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<sup>8</sup>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 <sup>+</sup>	5
IFE	$\alpha 6^{\text{bright}}\text{CD}71^{\text{dim}}$	12
IFE & HF Infundibulum	$\alpha 6^+\text{CD}34^-\text{Sca-1}^+$	16
IFE Touch Dome	$\alpha 6^+\text{CD}34^-\text{Sca-1}^+\text{CD}200^+$	3
Junctional zone	Lrig1 <sup>+</sup>	5
HF Isthmus & Infundibulum	$\alpha 6^+\text{MTS}24(\text{Plet-1})^+$	15
Sebaceous duct	Blimp1 <sup>+</sup>	7
HF Isthmus	$\alpha 6^{\text{low}}\text{CD}34^-\text{Sca-1}^-$	16
	Lgr6 <sup>+</sup>	11
HF Bulge	$\alpha 6^{\text{dim+bright}}\text{CD}34^+$	13, 14
	Krt15 <sup>+</sup>	21
Lower HF bulge & hair germ	Lgr5 <sup>+</sup>	10
Hair germ	P-cadherin <sup>+</sup>	22