Assaying proliferation and differentiation capacity of stem cells using disaggregated adult mouse epidermis

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In this protocol, we describe how to isolate keratinocytes from adult mouse epidermis, fractionate them into different sub-populations on the basis of cell surface markers and examine their function in an *in vivo* skin reconstitution assay with disaggregated neonatal dermal cells. We also describe how the isolated keratinocytes can be subjected to clonal analysis *in vitro* and *in vivo* and how to enrich for hair follicle-inducing dermal papilla cells in the dermal preparation. Using these approaches, it is possible to compare the capacity of different populations of adult epidermal stem cells to proliferate and to generate progeny that differentiate along the different epidermal lineages. Isolating, fractionating and grafting cells for the skin reconstitution assay is normally spread over 2 d. Clonal growth in culture is assessed after 14 d, while evaluation of the grafts is carried out after 4–5 weeks.

INTRODUCTION

Stem cells in mammalian epidermis

The outer layer of adult mammalian skin, the epidermis, comprises a multi-layered epithelium, known as the interfollicular epidermis (IFE), with associated adnexal structures: hair follicles, sebaceous glands and sweat glands. In mouse skin the sweat glands are restricted to non-hair bearing regions, such as the paws, and therefore the differentiation potential of mouse epidermal stem cells is generally assessed in terms of contribution to IFE, hair follicles and sebaceous glands only. The connective tissue, or stroma, underlying the epidermis is known as the dermis. The dermis contains a variety of cell types, including fibroblasts, endothelial cells, nerve fibers and infiltrating T cells and macrophages[1]. Epidermal homeostasis depends on constant reciprocal signaling with the dermis, and maintenance of the hair follicle requires communication with specialized mesenchymal cells at the base of the hair follicle, known as dermal papilla cells[2,3].

The terminally differentiated cells of the IFE, hair follicle and sebaceous gland are unable to divide and are continually lost from the epidermis during adult life. It has therefore been appreciated for many years that the epidermis is maintained by stem cells, which not only self-renew, but also generate daughter cells that undergo terminal differentiation. Epidermal stem cell self renewal and differentiation are assayed using three different basic techniques[4,5]. One is to examine the clonal growth ability of individual cells in culture. This can provide a quantitative read-out of stem cell number and has been used to evaluate potential stem cell markers[6]. The culture conditions are permissive for IFE differentiation, but multi-lineage differentiation in culture is somewhat limited[7,8]. The second method is to use lineage tracing to monitor the behavior of mouse epidermal cells *in situ*, either under steady state conditions or in response to genetic modification or tissue damage[9,10]. Lineage tracing is a powerful approach to analyzing stem cell properties, the main limitations being the length of time and numbers of mice required when studying the effects of multiple genes. The third assay, like the first, uses disaggregated epidermal cells, but instead of placing them in culture tests the ability of the cells to contribute to, and maintain, the differentiated epidermal lineages in an *in vivo* skin reconstitution assay. Grafting cells for this type of assay requires some surgical skill. However, using skin reconstitution multi-lineage differentiation can be assessed and multiple parameters can be evaluated with fewer mice and in a shorter time than by *in situ* lineage tracing.

It is important to bear in mind that the behavior of a given population of epidermal stem cells may differ in the three different assay conditions. This is exemplified by stem cells that express the EGF receptor antagonist Lrig1[11,12]. Lrig1 was originally identified as a stem cell marker on the basis of single-cell gene-expression profiling of cultured human epidermal keratinocytes[12]. Selection of Lrig1 positive cells enriches for clonogenic mouse and human epidermal cells in culture; nevertheless, knockdown of Lrig1 leads to increased clonal growth by stimulating stem cell proliferation[11,12]. In mouse epidermal reconstitution assays, the Lrig1 positive population is capable of differentiating into IFE, hair follicle and sebaceous glands. However, *in situ* lineage tracing demonstrates that under steady state conditions the cells are bipotent, thus giving rise to IFE and sebaceous gland cells but not to the hair follicle. Several different stem cell populations have now been described in the epidermis; however, most have not been tested under the full range of assay conditions, and this can make it difficult to draw conclusions about the relative contributions of the different populations to epidermal homeostasis, lineage selection and tissue repair[5].

In this report we describe the protocol that we currently use to isolate stem cells from adult mouse epidermis and to examine their behavior in epidermal reconstitution experiments *in vivo*. The same isolation procedure yields cells that can be tested for clonal growth in culture. A refinement of the methodology makes it possible to not only examine different sub-populations of epidermal cells but also different dermal cell populations.

Development of the protocol

The procedure that we use to isolate adult epidermal cells is that of Morris[13] with minor modifications[14]. The *in vitro* clonal growth assay

is based on the assay developed for human epidermis by Rheinwald[15] and Green[16] and involves culturing the cells on a feeder layer of 3T3 cells[15–17]. When we applied the human culture methodology directly to adult mouse keratinocytes the colony-forming efficiency (CFE) of the mouse cells was ~100-fold lower than that of neonatal human epidermal cells and we therefore did not attempt to use CFE as a read-out of stem cell numbers[14]. Instead we used the technique to generate well-differentiated, immortalized lines of mouse keratinocytes with a success rate of close to 100% (refs.14,18).

We subsequently found that if we modified the culture medium by removing calcium ions from the base medium formulation (FAD) the CFE of adult mouse epidermal cells was improved substantially and could thus be used for meaningful comparisons between different epidermal cell populations[10]. The culture medium contains calcium ions present in the fetal calf serum, and so epidermal cells form adherens junctions and desmosomes and undergo partial stratification. This is in contrast to the culture conditions we use to grow human keratinocytes as a 'low calcium' monolayer; under those conditions by dialyzing the fetal calf serum or treating with a chelating agent we remove sufficient calcium ions to prevent intercellular junctions from forming[17].

It is important to bear in mind that there is considerable variation in the CFE of adult mouse epidermal cells from different inbred strains of mice[19]. In addition, all our cultures, regardless of mouse background, undergo spontaneous immortalization within eight to ten passages. Therefore, we carry out stem cell assays on cells freshly isolated from skin.

Quantitation of growth assays is an important consideration. The most rigorous method involves isolating cells from individual actively growing clones of human epidermal cells and examining their secondary clone-forming ability[20]. The original clones are then assigned retrospectively to one of three categories according to the type of secondary clone they form: holoclones have highest reproductive capacity; paraclones have a short replicative lifespan; and meroclones have intermediate characteristics[20]. It is incorrect to describe epidermal cell colonies as holo-, mero- or paraclones unless secondary clonal growth has been characterized.

Our original studies were designed to screen for cell surface markers to enrich for clonogenic human epidermal cells and we adopted a one step assay rather than measuring secondary clones. Clones containing fewer than 32 cells 14 d after plating consisted solely of cells that expressed the terminal differentiation marker involucrin and were scored as abortive clones; larger clones, containing a mixture of dividing and differentiated cells, were judged to have been founded by a putative stem cell population[6].

There is no single agreed method to evaluate epidermal cell CFE, and provided that the method is quantitatively rigorous and clearly defined other workers will be able to reproduce the experiments. The cut-off for defining a clone could be as low as two cells. Some assessment of clone size can also be made. Clones can be evaluated for the proportion of terminally differentiated cells they contain; this is straightforward because differentiating IFE cells are large, flat and lie on top of the actively cycling basal layer of cells. Furthermore, clonal behavior can also be evaluated in the context of a confluent, stratified sheet of epidermal cells by marking the test population (e.g., by GFP expression) and seeding them in the presence of an excess of unlabeled cells[21].

Several different strategies have been reported for reconstituting the epidermis *in vivo*, including injecting cells into a pocket formed between the epidermis and dermis[22] and seeding epidermal cells onto a tracheal scaffold[23]. The one that we use is based on the early studies of Yuspa *et al.*[24,25] and Fusenig *et al.*[26] and can readily be adapted to examine the properties of malignant epidermal cells[24]. A silicon chamber is inserted into a full thickness wound on the upper back of immunocompromized or isogenic host mice. A mixed single-cell suspension of epidermal and dermal cells is injected into the chamber[24]. When the chamber is removed 2 weeks after grafting a darkened area is clearly visible in the healing wound bed. This is where new hair follicles are beginning to form. Within the subsequent 2 weeks the follicles will emerge from the surface of the reconstituted tissue.

The skin reconstitution assay not only facilitates evaluation of the ability of different epidermal stem cell populations to self-renew and generate differentiated progeny but also allows clonal analysis. For this, we isolate the test epidermal cells from a mouse expressing eGFP under the control of the β-actin promoter and combine those with an excess of unlabeled, unfractionated epidermal cells[11]. If the eGFP-expressing cells comprise $<5\%$ of the total population the regions of GFP positive cells within the mature graft can be judged to represent individual clones[27]. The neonatal dermal cells used for grafting may contain a low level of contamination with epidermal cells, and therefore some hair follicles may form in the 'dermis only' controls[28]. To ensure that the hair follicles being examined are derived from the input epidermis one approach is to combine unlabelled dermis with epidermis from a mouse that is expressing a label such as dsRed[28].

Experimental design

We describe the fractionation of adult mouse epidermis on the basis of several different cell surface markers. As epidermal cells differentiate they enlarge and so we always select the cells with low forward and side scatter to exclude differentiated cells[6,10,11,29]. In making claims for the relative self renewal ability of different epidermal stem cell populations it is clearly essential to compare only undifferentiated cells. A common mistake is to compare the test stem cell population with a mixture of differentiated and basal cells.

Essentially all undifferentiated epidermal cells express the α6 integrin subunit and this is a valuable marker of the basal cell population[6,30]. From P19 onwards CD34 is an excellent marker for the bulge stem cell population[31,32]; in adult mouse epidermis CD34 positive cells can express either low or high levels of α6 integrin[32]. Sca-1 is reported to be a marker of IFE cells that have exited the stem cell compartment and are undergoing limited divisions before IFE differentiation[33,34]. After labeling disaggregated adult mouse epidermal cells with antibodies to α6, CD34 and Sca-1, six distinct populations of undifferentiated cells can be isolated[11,35]. CD34 is used to define the cells with high or low α6 integrin expression. The integrin high and low populations are then separated according to whether or not they express CD34 or Sca1. All six populations defined by this approach are clonogenic in culture, although they differ in their CFE, and can be further evaluated in the skin reconstitution assay[11,35].

Quantitative PCR (qPCR) can be used to confirm that fractionation on the basis of each marker has been achieved successfully, and also to examine expression of additional markers[11]. Both the high and low α6 expressing CD34+ populations are enriched for K15, a well-known bulge marker[36]. Plet1/MTS24 (ref. 37) is depleted in CD34 positive populations. Lgr5 is most abundant in the CD34

positive cells with high α6 levels, consistent with Lgr5 expression being confined to a sub-population of bulge cells[9]. In contrast, the Lgr5 family protein Lgr6 is expressed most highly in CD34–Sca-1 cells that express high α6 levels.

Some cell surface markers, such as Lrig1, are trypsin sensitive. To overcome this problem we have used thermolysin to generate epidermal cell suspensions[11]. The drawback is that cell viability is lower than when trypsin is used. Therefore, as an alternative, Lrig1-enriched populations can be selected in trypsinized cells on the basis that they express high levels of α6 and lack CD34 and Sca-1 (ref. 11). This approach has the further advantage that it can be used to isolate junctional zone stem cells from Lrig1 −/− epidermis[11].

In our protocol we combine adult epidermal cells with dermal cells from neonatal skin[24,28,38,39]. One marker that can be used to enrich for dermal papilla cells is CD133 (ref. 39). We describe the protocol for sorting dermal cells on the basis of CD133 expression; this can be combined with additional markers if required[28].

By combined *in vitro* and *in vivo* assays of the same starting population of epidermal cells it is possible to examine many different aspects of stem cell populations in a reasonable time frame. This can subsequently inform the decision to generate genetically modified mice for loss or gain of function studies and for lineage tracing, which takes many more months and is considerably more expensive.

MATERIALS

REAGENTS

- Cells: it is advantageous to use cells that subsequently can be tracked in the reconstituted tissue, e.g., GFP labeled (β-actin-eGFP, C57BL/6 jax strain, stock no. 003291). Epidermal cells can be isolated at any stage of the hair cycle using the protocol.
- Balcb/C Nude mice (CAnN.Cg-Foxn1nu/Crl). Use mice that are approximately 7 weeks old for grafting procedures (Charles River) **! CAUTION** All procedures involving live rodents have to be carried out in accordance with national and institutional regulations.
- 3T3 J2 feeder cells for keratinocyte cultures[16]
- DMEM (Invitrogen, cat. no. 41966-029)
- FAD – Ca (mixture of 3 parts Ca-free DMEM and 1 part Ca-free Ham's F12 with 1.8×10^{-4} M adenine) (custom made by either Invitrogen or PAA Laboratories)
- Dispase (BD Bioscience, cat. no. 354235)
- Collagenase, type I (Gibco, cat. no. 17100-017)
- Amniomax C100 basal medium (Invitrogen, cat. no. 17001-082)
- Amniomax C100 supplement (Invitrogen, cat. no. 17002-080)
- Adenine (Sigma Aldrich, cat. no. A3159)
- Penicillin and streptomycin (Invitrogen, cat. no.15070-063)
- Hydrocortisone (Fisher Scienific, cat. no. 35245-0010)
- Cholera enterotoxin (Enzo Life Sciences, cat. no. G117) **! CAUTION** may be irritating to the eyes, respiratory system and skin. Harmful in contact with skin, and harmful if swallowed. Dispose down the sink after diluting with large excess of water.
- EGF (Peprotech EC, cat. no. 100 15)
- Insulin (Sigma Aldrich, cat. no. I 5500)
- Videne antiseptic solution (Betadine) (Ecolab, cat. no. 469920)
- Ethanol (70%)
- Isoflurane (W&J Dunlops, cat. no. MBIS004) **! CAUTION** May be irritating to the eyes, respiratory system and skin. Use with proper ventilation.
- 5% wt/vol Norocarp (Norbrook Pharmaceuticals)
- Dulbecco's PBS without Mg and Ca (PAA Laboratories, cat. no. H15002)
- BSA Cohn V fraction (Sigma Aldrich, cat. no. A4503-100g)
- Trypsin (0.25%) without EDTA (Invitrogen, cat. no. 25050-014)
- Thermolysin diluted to 0.25 mg ml^{-1} in sterile PBS (Sigma Aldrich, cat. no. T7902)
- Fetal bovine serum (PAA Laboratories, cat. no. A15-151)
- Donor bovine serum (Invitrogen, cat. no. 16030-074)
- Trypan blue stain (Invitrogen, cat. no. 15250-061)
- Collagen type I from rat tail (BD, cat. no. 354236)
- Mitomycin C (Sigma Aldrich, cat. no. M0503-2 mg)
- Versene (Invitrogen, cat. no. 15040-033)
- Cell strainer 70 μm (BD, cat. no. 352350)
- 50-ml Falcon tubes (BD, cat. no. 352070)
- 15-ml Falcon tubes (BD, cat. no. 352096)
- 100-mm plastic cell culture dishes (Corning, cat. no. 430293)
- T75 tissue culture flask (BD Falcon, cat. no. 353133)
- Costar 6-well tissue culture plate (Corning Incorporated, cat. no. 3516)
- Nalgene bottle-top sterile filter units (Sigma Aldrich, cat. no. Z358215-12EA)
- Antibodies:
  - RPE conjugated anti-human integrin alpha 6 (CD49f) antibody (AbD Serotec, cat. no. MCA699PE; Clone NKI-GOH3)
  - Biotin-conjugated anti-mouse CD34 antibody (eBioscience, cat. no. 13-0341)
  - Alexa Fluor 647-conjugated CD34 antibody (eBioscience, cat. no. 51-0341-82)
  - Alexa Fluor 700 anti-mouse Ly-6A/E (Sca-1, Sca1) (eBioscience, cat. no. 56-5981-82)
  - Goat anti-Lrig1 polyclonal antibody (R&D Systems, cat. no. AF3688)
  - Anti-prominin-1 (APC–CD133) (eBiosciences, cat. no. 17-1331-81)
  - PE-conjugated streptavidin (Biosource, cat. no. SNN1007)
  - Alexa 647-conjugated donkey anti-goat antibody, highly cross-adsorbed (Invitrogen, cat. no. A-21447)
- 4′,6-Diamidino-2-phenylindole, dilactate (DAPI, dilactate) (Invitrogen, cat. no. D3571) **! CAUTION** Very toxic by inhalation; irritating to the eyes, respiratory system and skin. Possible risk of irreversible effects. Disposal should be by a chemical waste company.
- 7-AAD staining solution (BD Pharmingen, cat. no. 559925) **! CAUTION** Very toxic by inhalation; irritating to the eyes, respiratory system and skin. Possible risk of irreversible effects. Disposal should be by a chemical waste company.

EQUIPMENT

- Flow sorter (e.g., MoFlo; Dako Cytomation)
- Swinging bucket bench top centrifuge (Eppendorf, cat. no. 5702)
- Hemocytometer (VWR, cat. no. 720-0104)
- Sterile scissors, scalpels and forceps (Harvard Instruments)
- Reflex wound clip applier for 7-mm clips (Harvard Instruments, cat. no. 726060)
- Reflex wound clip remover forceps (Harvard Instruments, cat. no. 726064)
- Reflex wound clips 7 mm (Harvard Instruments, cat. no. 726062)
- Sterile beakers (250 ml)
- Contura Cordless clipper (Harvard Instruments, cat. no. 340243)
- Gilson pipettes (VWR)
- Integra Pipetboy (VWR, cat. no. 612-0926)
- Basic anesthetic set-up with a flow cell for isoflurane (e.g., VetTech)
- Insulin syringe for analgesic (VWR, cat. no. 613-4904)
- 1-ml disposable syringe (BD Plastipak, cat. no. 30013)
- 19 × G 2″ needle (Terumo, cat. no. NN-1950R)
- 6-mm Silicon chambers (Silicon culture FK L—culture area 0.18 cm^2) (Renner GmbH)
- 6-mm Stiefel biopsy punch (Schuco International, cat. no. BC-BI-2000)
- Eppendorf tubes
- Sterile disposable 5-, 10- and 25-ml pipettes for pipetboy (VWR)
- Stuart Rotator SB2

REAGENT SETUP

Sterilization of tissue Prepare a series of solutions for sterilizing the skin samples. Dilute 20 ml Betadine in 180 ml sterile dH_2O in a sterile 250 ml beaker and prepare with two sterile beakers containing 70% ethanol and one sterile beaker containing sterile PBS. Ethanol and diluted betadine can be stored at room temperature (RT; ~22 °C).

Fetal bovine serum The serum used for culturing epidermal keratinocytes is batch tested to ensure that it supports keratinocyte proliferation and stem cell expansion. Store batch tested serum at −20 °C.

FAD – Ca basal medium Supplement the medium with penicillin (100 IU ml^{-1}) and streptomycin (100 μg ml^{-1}). The medium can be stored

at 4 °C for several weeks if fresh glutamine (450 mg liter^{-1}) is added immediately before use.

FAD low Ca complete medium Immediately before use, supplement FAD – Ca basal medium with 10% batch-tested fetal bovine serum, hydrocortisone (0.5 μg ml^{-1}), cholera enterotoxin (10^{-10} M), EGF (10 ng ml^{-1}) and insulin (5 μg ml^{-1}). Store complete medium at 4 °C for up to 1 week.

PBS with BSA Add 0.5 g of BSA to 500 ml PBS and sterile filter using a Nalgene bottle-top sterile filter unit to obtain a 0.1% solution. The sterilized solution can be stored at 4 °C for up to 6 months.

Trypsin–dispase solution for dermal preparation Prepare this solution by combining 0.25% trypsin without EDTA with Dispase at a 1:1 ratio. Trypsin (0.25%) without EDTA and dispase should be stored at −20 °C.

▲ **CRITICAL** Prepare trypsin–dispase solution fresh at the start of the experiment.

0.25% Collagenase for dermal preparation Prepare this solution by adding 0.125 g of Collagenase type I to 50 ml of FAD – Ca basal medium. Store Collagenase type I at 4 °C. ▲ **CRITICAL** Prepare fresh solution at the start of each experiment.

PROCEDURE

Removing mouse back skin ● TIMING 15 min for six mice

1| Kill mouse by using an approved technique. We kill mice by cervical dislocation.

2| Clip the hair off the entire back skin against the direction of hair growth in order to cut the hair as short as possible.

3| Remove the back skin from the mouse with sterile dissection tools.

4| Place the back skin flat in a 100-mm cell culture dish (**Fig. 1a**). The tissue can be kept on ice for 5–6 h without affecting the quality of the cell preparation.

Digestion of mouse back skin ● TIMING 30 min for six mice

5| Start the preparation by sterilizing the tissue to eliminate any potential contamination. Prepare one sterile beaker with 10% betadine (~200 ml), two sterile beakers with 70% ethanol and one beaker with sterile PBS. The tissue is bathed in the betadine solution for 2 min, in each beaker with 70% ethanol for 1 min and then in sterile PBS for 1 min.

6| Place the back skin flat in a petri dish, dermal side facing up (**Fig. 1b**).

7| Scrape the dermal side of the skin with a sterile scalpel to remove adipose tissue and the muscle (**Fig. 1c**).

▲ **CRITICAL STEP** The quality of the cell preparation is determined by how well the dermal side is prepared.

8| Float the tissue epidermal side up in 10 ml trypsin in a petri dish either overnight at 4 °C or for 2 h at 37 °C (**Fig. 1d**). Alternatively, if cells are being prepared for flow cytometric analysis of trypsin sensitive antigens the tissue digest can be carried out using thermolysin for 1–2 h at 37 °C.

▲ **CRITICAL STEP** There is considerable batch to batch variation in purchased thermolysin, and each batch will therefore need to be tested for its activity. The following steps in the preparation of epidermal cells are the same whether trypsin or thermolysin is used.

! CAUTION Digestion with thermolysin reduces cell viability to a greater extent than trypsin digestion.

Isolation of cells from mouse back skin ● TIMING 2 h for six mice

9| Transfer the floating tissue to the inside of the lid of the petri dish epidermal side up (**Fig. 1e**).

10| Pressing one edge of the tissue down on the lid with forceps, use a sterile scalpel to scrape the epidermis off the dermis without exerting excessive force (**Fig. 1f**).

? TROUBLESHOOTING

11| Discard the dermis and mince the epidermis using two scalpels.

12| Transfer the minced epidermis into a sterile 50 ml Falcon tube in 8 ml of the trypsin solution that was used for the digestion step.

13| Repeatedly pipette the tissue suspension up and down until the small clumps disaggregate (~30–60 s).

14| Add 16 ml FAD low Ca complete medium and pour the suspension through a 50 μm cell strainer. The tissue remaining in the cell strainer should, for the most part, be separated hairs (**Fig. 1g**).

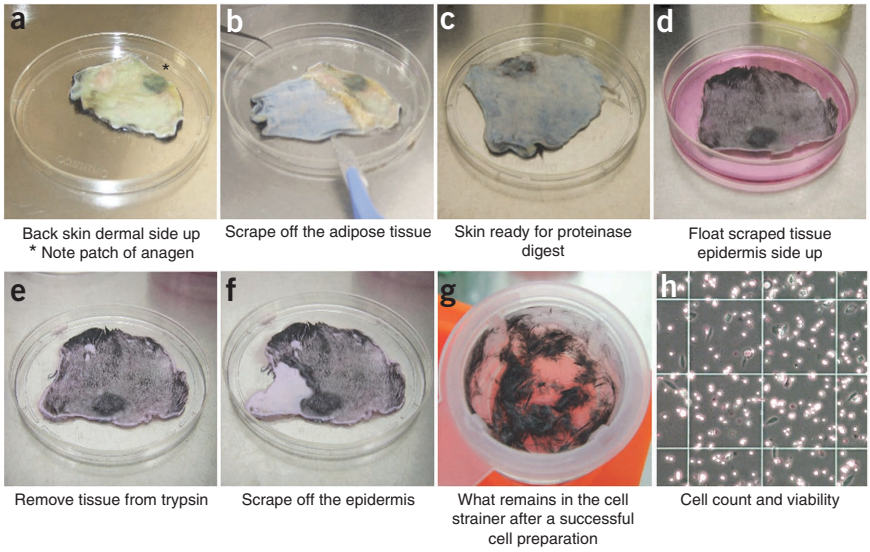

Figure 1 | Preparation of single cell suspension of epidermal cells from mouse back skin. (**a**) Back skin in petri dish, dermal side up. Asterisk indicates patch of actively growing (anagen) hair follicles. (**b**) Scraping off adipose tissue with a sterile scalpel. (**c**) Once the adipose layer has been removed the skin is almost transparent. (**d**) Skin floating epidermal side up in trypsin. (**e**) Following trypsinization, skin is placed epidermal side up in a sterile petri dish. (**f**) Partial detachment of epidermis from underlying dermis. (**g**) A good tissue preparation will release the entire epidermis as single cells and only hair remains in the cell strainer. (**h**) Cells are examined under a microscope using a hemocytometer to establish cell yield and cell viability. Appropriate institutional regulatory board permission was obtained to use the mouse that was the source of tissue for this and all subsequent figures.

▲ **CRITICAL STEP** It is essential to use medium without calcium when preparing the cell suspension with thermolysin as calcium stabilizes adherens junctions and thus reduces the efficiency of generating a single cell suspension.

15| Spin down the cells at 500*g* for 8 min at room temperature. When samples are harvested from multiple mice they can be kept on ice and then centrifuged simultaneously.

16| Aspirate the supernatant.
▲ **CRITICAL STEP** Be very careful when aspirating the supernatant and leave 2–3 ml above the cell pellet.

17| Transfer the cell pellet in 8 ml FAD low Ca complete medium to a 14 ml sterile Corning tube and centrifuge at 500*g* for 8 min.

18| Resuspend the pellet in 4 ml PBS supplemented with BSA.
▲ **CRITICAL STEP** Be careful not to let the keratinocytes settle after resuspension, as this will lead to an underestimation of cell number.

19| Determine cell number and viability using a hemocytometer by adding 50 µl Trypan blue to 50 µl cell suspension (**Fig. 1h**).
? TROUBLESHOOTING

20| Spin down the cells and resuspend at a dilution of 5×10^6 cells per ml in PBS supplemented with BSA for flow cytometry. Alternatively, resuspend in FAD low Ca complete medium if plating in culture (see **Box 1**). Do not proceed with flow cytometry for grafting purposes if the cell yield is below 10^7 cells. The cells can instead be flow sorted for cell culture assays or for the isolation of RNA.
? TROUBLESHOOTING

Labeling cells for flow cytometry ● TIMING 2 h
21| Prepare sterile 14 ml tubes for labeling cells. Prepare tubes for single color controls and unlabeled cells to allow proper compensation when carrying out multicolor flow cytometry.

22| Resuspend cells at a concentration of 5×10^6 cells per ml in PBS supplemented with BSA.

23| Add antibody to the resuspended cells. The concentration of antibody needs to be determined by dilution experiments (see **Table 1** for suggested concentrations). In order to isolate a sufficient number of stem cells for grafting purposes we recommend labeling up to 2×10^7 cells with primary antibodies. See **Table 2** for anticipated cell yields.

24| Incubate for 1 h at 4 °C with gentle agitation.

25| Spin down the cells and resuspend in 5 ml of PBS with BSA—repeat this step twice.

BOX 1 | CLONAL GROWTH OF MOUSE KERATINOCYTES

1. Prepare 1–2 × T75 cell culture flasks of 3T3 J2 feeders cells per epidermal subpopulation to be plated 7 d before isolating epidermal cells by flow cytometry[10].
   (i) Prepare feeder cells by seeding 1.25 × 10^5 cells in a T75 flask in DMEM supplemented with 10% donor bovine serum and penicillin–streptomycin (complete DMEM).
   (ii) Change the medium every second day.
2. The day before the isolation of epidermal sub-populations prepare 6-well plates with mitotically inactivated J2 3T3 feeder cells.
   (i) Mitotically inactivate the required number of J2 3T3 cells by treating them with 4 μg ml^{-1} Mitomycin C for 2 h in complete DMEM.
   (ii) Immediately after adding Mitomycin C to the J2 3T3 feeders, coat the surface of 6-well plates with collagen type I by incubating wells with 1 ml of 40 μg ml^{-1} rat tail Collagen type I in PBS for 2 h at 37 °C.
   (iii) After 2 h of Mitomycin C treatment wash each of the T75 cell culture flasks twice with 8 ml of PBS.
   (iv) Trypsinize J2 3T3 feeder cells by incubating with 2.5 ml of Trypsin (0.25%) diluted 1:5 in versene until cells detach from the culture plastic.
   (v) Transfer cells to a 50 ml tube in 5.5 ml complete DMEM.
   (vi) Pellet cells by centrifugation (500*g*) at room temperature and resuspend cells from 1 T75 cell culture flask in 12 ml complete DMEM.
   (vii) After 2 h wash the collagen-coated wells with PBS.
   (viii) Add 2 ml of mitotically activated J2 3T3 feeder cells per well and incubate overnight at 37 °C.
   (ix) On the day of cell sorting of epidermal sub-populations aspirate the complete DMEM from the 6 well plate and add 2 ml FAD low Ca complete medium to each well.
3. Add the flow sorted epidermal cells to the wells at a known cell density. (Hint) It is important to resuspend the cells carefully before seeding to ensure that equal numbers of cells are seeded in replicate plates.

! CAUTION The optimal number of cells needs to be determined empirically as the assay requires that the formed colonies will not have merged in 2 weeks.

The CFE is highly influenced by mouse strain (up to 50-fold differences in CFE) and the sub-population of epidermal stem cells studied[19]. A seeding density of 2,500 cells per well works for most strains and cell populations. Epidermal cell colonies should be obvious 8–10 d after plating, as feeder cells at the edge of the colonies are dislodged. The colonies are heterogenenous in appearance. Colonies may contain highly proliferative, tightly packed cells (**Fig. 6a**) or may appear more disorganized and contain a higher proportion of differentiated cells (**Fig. 6b**). For quantification of CFE, cells are seeded as technical triplicates. Independent cell isolates for three to five different mice are analyzed.

4. Culture cells at 32 °C in 8% CO_2 and 5% O_2. Adult mouse epidermal cells grow better at 32 °C than at 37 °C.
5. Change the medium three times per week.
6. Fix the cells in 2% neutral buffered saline or paraformaldehyde for 10 min at room temperature.
7. Rinse wells using PBS and stain the colonies using a mixture of 1% Rhodamine B and 1% Nile blue in water for 20 min at room temperature.
8. Rinse thoroughly with water.
9. Allow wells to dry and assess CFE by counting colonies and measuring colony sizes. The size of individual colonies can be quantified using Volocity or ImageJ software to analyze high quality scanned images of wells or culture dishes[40]. The smallest colonies that can be scored in this way contain 10–20 cells. The range of colony size is shown in **Figure 6c–e**.

26| Resuspend the cells at 5×10^6 cells per ml in BSA supplemented PBS.

27| Add Alexa–RPE conjugated secondary antibody or streptavidin and incubate for 20 min at 4 °C with gentle agitation. The concentration of antibody/streptavidin needs to be determined in dilution experiments (see **Table 1** for suggested concentrations).

TABLE 1 | Antibodies used to select epidermal and dermal subpopulations.

Antibody	Clone name	Company	Ab vol (μl)/10^6 cells (100 μl total)	Conjugate
ItgA6	GOH3	Serotec	4	Alexa647/RPE
CD34	RAM34	eBioscience	5	Biotin/Alexa647
Sca1	D7	eBioscience	1	RPE/Alexa700
CD133	13A4	eBioscience	5	APC
Lrig1	Goat polyclonal	R&D	10	
Streptavidin		Biosource	1	RPE
Donkey anti-goat	Highly cross adsorbed	Invitrogen	1	Alexa 647

28| Spin down the cells and resuspend in 5 ml of PBS with BSA—repeat this step three times.

29| Resuspend the cells at 10^7 ml^{-1} in BSA supplemented PBS and filter using a 50-µm cell strainer to remove any cell clumps.

30| In order to be able to gate out (exclude) dead cells, add DAPI or 7-AAD to the cell suspension ~5 min before sorting.
▲ **CRITICAL STEP** Do not add DAPI to control samples.
A number of live-dead dyes can be used instead of DAPI, but it is important to choose a dye that does not have major spectral overlap with one of the chromophores used on the secondary antibodies or streptavidin.

TABLE 2 | Expected yields of live cells per mouse from successful epidermal and dermal cell preparations.

Type of prep	Cell yield
Adult back epidermis	10–20 × 10^6
Neonate epidermis	3 × 10^6
Neonate dermis	20 × 10^6

Flow cytometric isolation of mouse epidermal cell populations ● TIMING 2 h (the speed of these steps is determined by the available flow sorter, as this has a big influence on the events (cells) that can be analyzed per second)
31| Analyze all single color controls to carry out adequate color compensation and ensure isolation of pure cell populations.

32| Set up the sorting parameters as follows: when flow sorting epidermal keratinocytes use a 100 µm nozzle and a sheath pressure of 30 p.s.i. This will allow the cells to be sorted at a speed of 10–12,000 events per second. Basal keratinocytes are characterized by their low side and forward scatter. First select these cells for further analysis (**Fig. 2a**), by excluding cell debris and differentiated cells. In order to minimize the number of cells that stick to each other, subsequently gate on pulse width (**Fig. 2b**). Finally, exclude dead cells based on the uptake of DAPI or 7-AAD (**Fig. 2c**).

33| Select subsequent parameters based on the antibodies used for selecting stem cell populations. For ItgA6, Sca1 and CD34 follow option A. The described parameters enable the identification and isolation of six distinct epidermal cell populations in trypsinized suspensions. For Lrig1 and CD34 follow option B. The described sorting technique allows isolation of bulge (CD34+) and junctional zone (Lrig1+) stem cells from thermolysin-dissociated cell suspensions.
(A) ItgA6, Sca1 and CD34

(i) View events based on CD34 along the *y* axis and ItgA6 along the *x* axis. Select basal cells based on ItgA6 expression and gate into ItgA6 high and low cells based on the two distinct CD34 positive populations (**Fig. 2d**—Gate I and II).

(ii) View events based on CD34 along the *y* axis and Sca1 along the *x* axis. The ItgA6high cells can be divided into three populations based on Sca1 and CD34 expression (**Fig. 2e**). Hair follicle stem cells are positive for CD34 (Population 1), cells in the junctional zone are negative for CD34 and express low levels of Sca1 (Population 2), whereas interfollicular epidermal cells and cells in the infundibulum express Sca1 (Population 3). The ItgA6low cells can likewise be divided into three populations based on

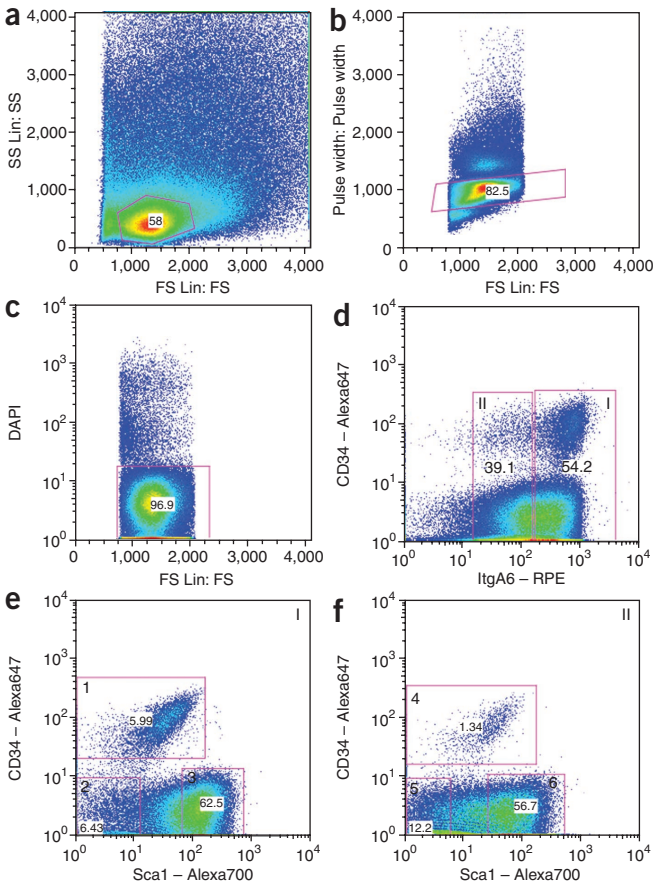

Figure 2 | Isolation of cells based on expression of CD34, Sca1 and Integrin alpha 6. (**a**) Epidermal cells are gated by their low forward and side scatter. (**b**) Single cells are identified based on their pulse width. (**c**) Live cells are identified based on exclusion of DAPI. (**d**) Cells are gated according to high (I) or low (II) α6 integrin levels based on the two distinct populations of CD34 expressing cells. (**e**) Three distinct populations are identifiable in the ItgA6 high population when visualizing CD34 and Sca1 levels. Population 1 represents hair follicle stem cells; population 2 is enriched for junctional zone stem cells; and population 3 contains committed interfollicular epidermal cells. (**f**) Three distinct populations are identifiable in the ItgA6 low population when visualizing CD34 and Sca1 levels. Population 4 represents suprabasal hair follicle stem cells; population 5 is enriched for stem cells in the hair follicle isthmus; and population 6 contains committed interfollicular epidermal cells.

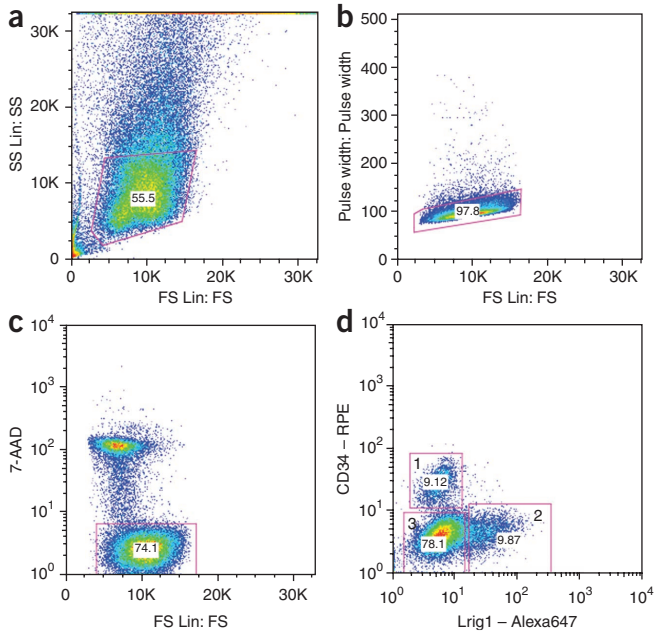

Figure 3 | Isolation of epidermal cells based on sorting for CD34 and Lrig1 expression. (**a**) Cells disaggregated with thermolysin have a slightly different forward and side scatter profile from cells disaggregated with trypsin (compare **Fig. 2a**). Basal cells are selected based on low forward and sideward scatter. (**b**) Single cells are selected based on pulse width. (**c**) Live cells are isolated on the basis of 7-AAD exclusion. Note lower viability than in trypsin-disaggregated preparations (compare **Fig. 2c**). (**d**) Three discrete populations can be identified: (1) hair follicle bulge stem cells, (2) junctional zone stem cells and (3) other basal epidermal cells.

Sca1 and CD34 expression (**Fig. 2f**). Suprabasal hair follicle stem cells are positive for CD34 (Population 4), cells in the isthmus are negative for CD34 and express low levels of Sca1 (Population 5), whereas interfollicular epidermal cells and cells in the infundibulum express Sca1 (Population 6).

(B) Lrig1 and CD34

(i) View the double-labeled populations and select hair follicle stem cells based on CD34 expression and stem cells from the junctional zone based on Lrig1 expression (**Fig. 3a–d**).

? TROUBLESHOOTING

34| Isolate the required populations of epidermal stem cells by flow sorting the cells into sterile tubes containing BSA-supplemented PBS.

35| Spin cells down at 500*g* for 5 min and resuspend in a small volume of sterile HBSS for grafting. After flow cytometry cells can be used for a number of purposes in addition to grafting, including isolation of RNA or testing the clonal growth potential of cells *in vitro* using standard mouse keratinocyte culture conditions (**Box 1**).

? TROUBLESHOOTING

Isolation and sorting of dermal cells (generation of a single cell suspension from the dermis of embryonic or neonatal mice) ● TIMING up to 5 h depending on collagenase incubation time

36| A good dermal cell preparation is essential for successful grafts. In this and subsequent steps, creation of a single cell suspension of dermis from embryonic day 16.5 (E16.5) to post natal day 2 (P2) mice is described. Before dissection, prepare a 100-mm culture dish with 10 ml of trypsin–dispase digestion solution. The dermis of five mice can be combined into one 100-mm dish for faster processing or a single dermis can be processed using a 60-mm tissue culture dish with 6 ml of trypsin–dispase digestion.

▲ **CRITICAL STEP** Dermal cell preparation should be carried out in one session, from beginning to end, for best results.

▲ **CRITICAL STEP** It is important to use mice that are no older than P2. Although the dermis of older mice may support hair formation in grafts, the efficiency of collagenase digestion is greatly decreased and as a result the dermal preparation is of poor quality. See **Table 2** for expected dermal cell yield per P2 mouse.

37| Clean and sterilize all instruments using an autoclave and 70% ethanol before dissection.

38| Kill animals according to ethically approved protocols. We use cervical dislocation.

39| Clean the mice by submerging them in betadine solution, 70% ethanol solution and then sterile 1× PBS.

40| Remove the limbs, the tail and the head of each embryo or neonate using scissors and forceps.

41| Make an anterior to posterior incision in the skin on the ventral side of the mouse.

42| Grip a region of the anterior side of the incision with forceps and hold the mouse with another pair of forceps. The skin will separate from the rest of the mouse when the two pairs of forceps are pulled in opposite directions.

▲ **CRITICAL STEP** Digestion of skin with enzymes occurs more efficiently if the dermal side of the skin is scraped of all debris with a scalpel before incubation with trypsin and dispase. However, do not scrape too hard.

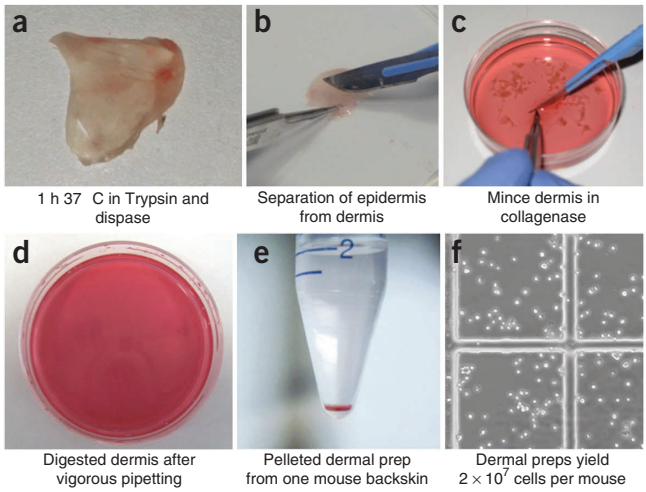

Figure 4 | Isolation of cells from neonatal dermis. (**a**) Mouse skin is incubated in trypsin and dispase. (**b**) The epidermis is separated from the dermis. (**c**) The dermis is minced in 0.25% collagenase and incubated for at least 1 h at 37 °C. (**d**) The dermis is disaggregated by vigorous pipetting. (**e**) After washing, the cell pellet should have no floating debris. (**f**) Cells viewed under a hemocytometer should have high viability and look heterogeneous in size and morphology.

43| Incubate the dissected skin by floating it on the dispase–trypsin solution in a 100-mm dish for 1 h at 37 °C (**Fig. 4a**). Up to five neonatal mice can be put into a single 100 mm culture dish with 15 ml of typsin–dispase digestion solution.

44| After 1 h of incubation the epidermis should easily separate from the dermis (**Fig. 4b**). After the epidermis is peeled it can be further processed to isolate up to 3 × 10^6 epithelial cells per mouse for additional analysis.

45| Mince the dermis into small pieces (**Fig. 4c**) in a solution of 0.25% collagenase/FAD +Ca. Incubate the minced dermis for at least 1 h at 37 °C. Mincing the dermis allows for more efficient digestion.
▲ **CRITICAL STEP** Incubation times with collagenase can be varied depending on the age of mouse, with incubation times of up to 2 h for P2 mice.

46| Vigorously pipette the collagenase/minced dermal solution up and down with a 10ml pipette until a single cell suspension is achieved (**Fig. 4d**).
? TROUBLESHOOTING

47| Filter the dermal cell suspension with a 70 micron filter.

48| Use 5 ml of FAD +Ca to rinse the 100-mm plate and transfer the entire solution to a 15-ml conical tube and centrifuge for 4 min at 500*g*. Make sure suspensions are shaken before centrifugation.
The pelleted cells should be compacted tightly at the bottom of the conical tube (**Fig. 4e**).
▲ **CRITICAL STEP** A pellet that is not fully compacted is difficult to wash.
? TROUBLESHOOTING

49| Resuspend cells in culture medium for grafting. If desired, flow cytometry can be used before grafting to sort CD133+ and CD133– dermal papilla populations (see **Box 2**).

50| Determine cell number in a hemocytometer (**Fig. 4f**).
? TROUBLESHOOTING

Skin surface grafting: grafting cells onto the backs of immunocompromized nu/nu mice ● TIMING 20 min per mouse
51| Prepare a mixture of cells in FAD low Ca complete medium in an Eppendorf tube for each graft. The tube will contain 5 × 10^6 dermal cells, 10^5 GFP-expressing flow-sorted keratinocytes and 3×10^6 unfractionated, GFP-negative epidermal keratinocytes. This ratio of GFP positive to negative cells allows the determination of relative clonal contribution of different sorted populations to reconstituted epidermis[27]. Keep cell suspension on ice until needed for the grafting procedure.

52| Sterilize equipment and reagents.
▲ **CRITICAL STEP** The grafting procedure must be carried out aseptically.

53| Use isoflurane (at a minimum alveolar concentration (MAC) value of 1–1.34) to anesthetise mice by inhalation under an increasing concentration of isoflurane.

54| Subcutaneously inject Norocarp 4 mg kg^{-1} as analgesic. This constitutes post surgery pain relief. As the volume needed is very small we advise that a 1:20 dilution of 5% wt/vol Norocarp is prepared in sterile deionized water and 30 µl is used per animal.

BOX 2 | FLOW SORTING CD133+ AND CD133- DERMAL PAPILLA POPULATIONS

● TIMING UP TO 3 H (THE SPEED OF THESE STEPS IS DETERMINED BY THE FLOW RATE OF THE FLOW CYTOMETER AND ANTIBODY INCUBATION TIMES)

1. Wash the cell pellet twice by resuspending the pellet in 1× PBS and then spinning at 500*g* for 4 min at room temperature.
2. Resuspend the cell pellet to a concentration of 1×10^7 cells ml^{-1} using Amniomax C100 media.
3. Label the dermal preparation using 5 µl of APC–CD133 antibody (eBiosciences) per 1×10^6 cells in 100 µl of Amniomax C100.
   (a) While staining for CD133 rotate the dermal suspension at 4 °C for 1 h.
   (b) Wash the stained cells twice with 1× PBS and centrifugate at 500*g* for 4 min.
   (c) Resuspend the cell pellet at 1×10^7 cells ml^{-1}.
   (d) Add 2.5 µl ml^{-1} of DAPI 5 min before analysis and sort.
   ▲ CRITICAL STEP 1×10^6 cells should be set aside for each flow cytometry control sample. A control is stained for DAPI alone and CD133–APC alone in this flow sorting protocol.
   (e) A dermal preparation analyzed on a digital flow cytometer will have a characteristic forward and side scatter profile. Set the gates on the main population (**Fig. 7a**).
   ▲ CRITICAL STEP The quality of the dermal preparation can be gauged at this point because the gated population should be at least 65% of the entire FS/SS analysis. If not, abandon the experiment as the sort will take too long and will not yield sufficient cells.
   (f) Measure doublets by the pulse width/forward scatter plot. Single cells should be at least 97% of the gate.
   (g) Gate single cells (**Fig. 7b**)
   (h) Dead cells take up DAPI and are analyzed by the DAPI/forward scatter gates. Using a digital flow cytometer the DAPI-negative population will include a subset of cells with low forward scatter (FSC) and side scatter (SSC) (red arrow, **Fig. 7c**). These are blood cells and can be ignored because they are CD133 negative (red arrow, **Fig. 7d**).
   (i) Gate DAPI-negative cell population (**Fig. 7c**).
   ▲ CRITICAL STEP An acceptable preparation should have at least 90% DAPI-negative cells.
   (j) APC–CD133 positive cells appear as a 'shoulder' population above the negative cells when flow sorting using a Moflo. These cells when analyzed or sorted with a digital flow cytometer appear as a distinct cell population (**Fig. 7d**).
   ▲ CRITICAL STEP The CD133 population is roughly 10–15% of the dermal population. Careful planning and pilot experiments are required to ensure that sufficient CD133 positive cells can be isolated for grafting by flow cytometry. Our experience is that 1.5×10^5 APC–CD133 positive cells per P2 mouse is attainable with a good quality cell preparation.
   (k) Spin down sorted populations at 500*g* for 4 min and resuspend in 50 µl of medium to insert into chamber grafts with epithelial cell populations.

55| While keeping the mouse anesthetised make a 6-mm full thickness wound with a biopsy punch on the upper back just behind the shoulder blades.
▲ CRITICAL STEP When using the biopsy punch it is important to take care not to damage the underlying muscle. An alternative is to use the biopsy punch to mark the area and subsequently remove the skin using scissors.

56| With a pair of forceps lift the surrounding tissue from the muscle fascia. This will allow insertion of the silicon chamber under the skin surface.

57| Insert a 6-mm silicon chamber into the 6-mm wound. Folding the silicon chamber into quarters facilitates this step.

58| Use two wound staples to secure the chamber on the back of the mouse (**Fig. 5a**).

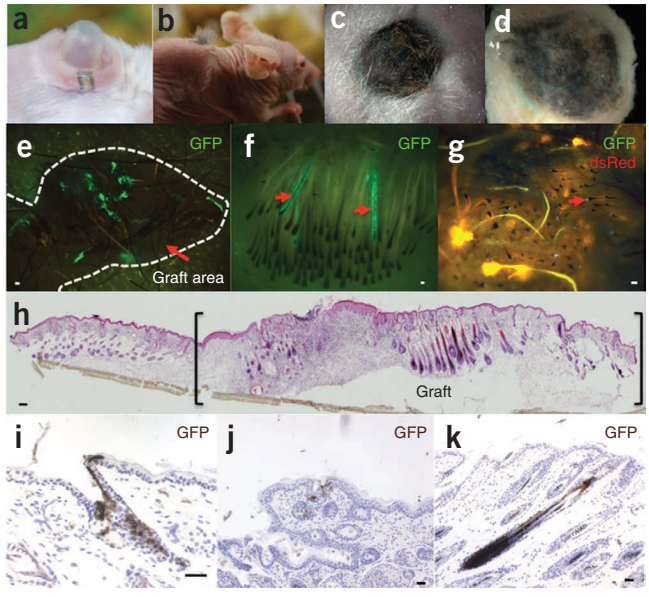

Figure 5 | Whole mount and histological analysis of *in vivo* reconstituted skin. (**a**) Grafting chamber in place. (**b**,**c**) Appearance of ectopic hair follicles on grafted nude/Balb/C mouse 4–5 weeks from the start of the experiment. (**d**) View of dermal side of graft. (**e**–**g**) GFP positive cells are seen contributing to the graft when viewed from the dermal side. (**g**) In this graft, the epidermal cells are expressing dsRed, whereas a sub-population of dermal papilla cells (arrows) express GFP. (**e**,**f**) Arrows mark GFP positive areas (**h**) H and E-stained section of a graft showing the reconstituted skin (bracketed) and adjacent host skin (left hand side). (**i**–**k**) Sections of grafts stained with antibodies to GFP showing the contribution of GFP-positive cells to the graft. Scale bars 50 µm.

BOX 3 | PROTOCOL FOR GFP STAINING PARAFFIN EMBEDDED TISSUE

1. Embed the grafted material in paraffin and cut 5 µm sections.
2. Deparafinize in a series of Xylene × 2, 100% EtOH × 2, 95% Ethanol, 80% Ethanol and twice in water by incubating in each solution for 1 min.
3. Use enzymatic antigen retrieval: incubate with 0.5 U proteinase K (Novocastra) for 20 min.
4. Block endogenous biotin molecules using avidin–biotin block according to the manufacturer's instructions (Novocastra).
5. Incubate with 10% bovine serum diluted in PBS for 45 min.
6. Incubate with chicken anti GFP antibody (Abcam, ab13970) diluted 1:2,000 in 10% bovine serum in PBS for 1 h.
7. Wash twice with 0.1% Triton X100 supplemented PBS for 5 min.
8. Incubate with biotinylated rabbit anti chicken antibody (Jackson ImmunoResearch) diluted 1:250 in 10% bovine serum in PBS for 30 min.
9. Wash twice with 0.1% Triton X100 supplemented PBS for 5 min.
10. Incubate with HRP-conjugated streptavidin (Novocastra) diluted 1:250 in 10% bovine serum in PBS for 30 min.
11. Wash twice with 0.1% Triton X100 in PBS for 5 min.
12. Develop with DAB (Novocastra) and hematoxylin counterstain (Novocastra).

59| Prepare the 100-µl cell suspension in a 1-ml syringe with a 19-G needle and slowly inject into the silicon chamber. Use a pair of forceps to steady the needle when penetrating the surface of the silicon chamber and injecting the cells.
▲ **CRITICAL STEP** Be careful to avoid touching the muscle when inserting the needle.

60| Allow the mouse to recover on a heat pack. When the mouse has recovered fully from anesthesia transfer to a cage with paper clippings rather than saw dust. This will help keep the site of surgery clean.

61| After 1 week, cut off the top of the silicon chamber with scissors. This allows the wound to dry out and facilitates healing.
? TROUBLESHOOTING

62| After 2 weeks, anesthetise the mouse and remove the wound staples and the silicon chamber. Push down on the wound bed inside the chamber to release it from the sides of the chamber.

63| Hair should penetrate the grafted area 3–4 weeks after grafting (**Fig. 5b,c**). Kill the animals 5 weeks after grafting and cut out the graft including a margin (~2 cm diameter) of surrounding skin.

64| Examine both sides of the graft under a dissecting microscope (**Fig. 5d**). If desired and appropriate, fluorescence illumination can be used to look at areas of special interest (e.g., expressing GFP). Significant contribution from grafted cells can be visualized (**Fig. 5e–g**).
? TROUBLESHOOTING

65| Prepare paraffin sections of areas of special interest (**Fig. 5h**). Examine paraffin embedded sections using antibodies to GFP (**Box 3**) in order to identify areas with significant contribution from GFP-expressing epidermal cells (**Fig. 5i–k**).
? TROUBLESHOOTING

● **TIMING**
Steps 1–4, Removing mouse back skin: 15 min
Steps 5–8, Digestion of mouse back skin: 30 min plus incubation time
Steps 9–20, Isolation of cells from mouse back skin: 2 h
Steps 21–30, Labeling cells for flow cytometry: 2 h
Steps 31–35, Flow cytometric isolation of mouse epidermal cell populations: 2 h
Steps 36–50, Isolation and sorting of dermal cells: 5 h
Steps 51–59, Skin surface grafting: grafting cells onto the backs of immunocompromized nu/nu mice: 20 min
Steps 60–65, Evaluation of graft take: at least 5 weeks

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10	Hair/epidermis does not separate from the dermis	The dermis has not be scraped properly before trypsinization	Rescrape the dermal side of the tissue and trypsinize for an additional 45 min at 37 °C
19	Low cell yield	The dermal side has not be prepared properly before trypsinization	Abort grafting and use cells for *in vitro* experiment instead
		The sample has not been pelleted properly	
		The trypsin used contained EDTA	
		Calcium was added to the sample after thermolysin digestion	
	Low cell viability	The trypsin used contained EDTA	Use trypsin without EDTA
		The cells have been kept for too long in trypsin solution after disaggregation	Reduce trypsinization time after disaggregation
20	Small pellet—too few cells	Cells have not been pelleted properly by centrifugation	Make new cell preparation
33	The flow profiles do not look the same as in the figure	Different flow sorters will acquire data in different ways and the profiles will accordingly look different	Isolate RNA or perform *in vitro* colony forming efficiency assays from the different populations to ensure that cells have been sorted appropriately
	As above	A different fluorescent conjugated antibody has been used This will result in a different profile	Purchase the correct antibody or validate cells as above
35	Cells are dead after sorting	The sheath pressure is too high	Lower the sheath pressure
46	Clumping of dermal cells	Insufficient pipetting and insufficient collagenase digestion	Carry out additional PBS washes and increase collagenase digestion time
48	Cells not forming tight pellet	Cells were not thoroughly resuspended before centrifugation	Resuspend pellet and centrifuge immediately
50	Low dermal cell yield	Cell preparation of poor quality	Use younger mice for easier digestion of dermis Increase collagenase digestion times
61	The chamber is loose	A wound clip has fallen out	Reanesthetize the mouse and reapply the wound clip
64	No hair follicles in grafts	Cell preparations were of poor quality	Increase the quality of both dermal and epidermal cell preparations
65	Hair follicles in the graft but no GFP-expressing cells	The sorted epidermal cell population is not of sufficient quality	Cell viability after flow cytometry should at least be in the range of 90–95%

ANTICIPATED RESULTS

The analysis of surface grafts can be difficult because unlike embryonic hair follicle development hair follicles in surface grafts do not form in an orderly pattern (**Fig. 5a–d**). In addition, nude mice do have hair follicles, making it

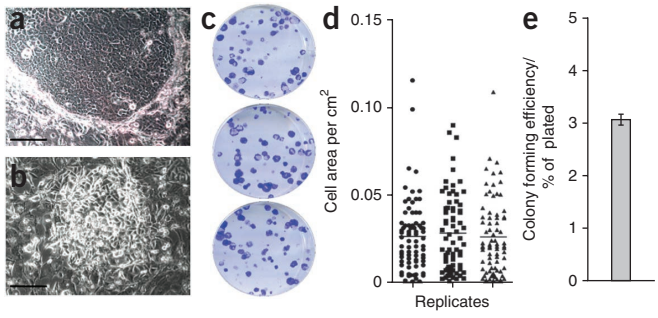

Figure 6 | Measurement of epidermal colony forming ability in culture. (**a**,**b**) Phase contrast micrographs of two morphologically distinct colonies. (**a**) Colony containing highly proliferative, tightly packed cells of undifferentiated morphology. (**b**) Colony containing loosely packed cells with high proportion of rounded, differentiated cells. (**c**) Technical replicates of adult epidermal mouse keratinocytes grown for 2 weeks before fixation and staining. (**d**) Size range of individual colonies in **c** measured using ImageJ. (**e**) CFE of cells plated in **c**, determined as the average % plated cells that formed colonies. Standard deviation of the triplicate samples is indicated by error bar. Scale bar 250 μm.

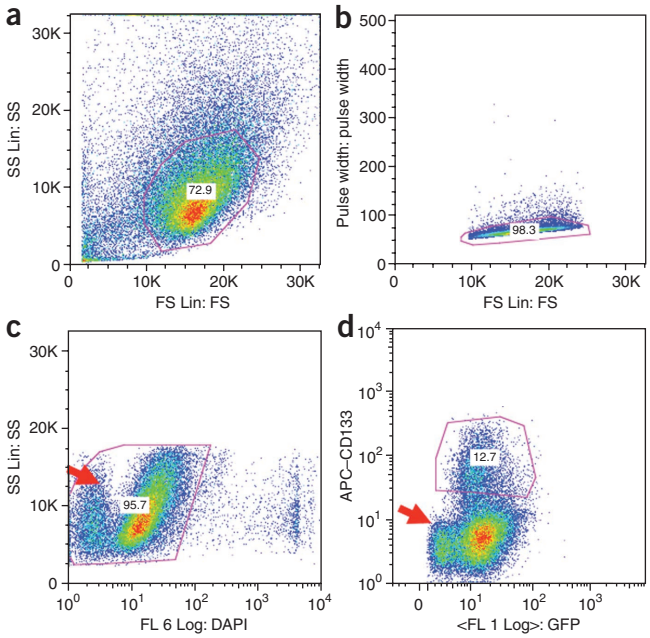

Figure 7 | Flow cytometric analysis of dermal single cell suspension stained with APC–CD133 antibody. (**a**) Gating on the basis of forward and side scatter. (**b**) Selection of single cells on the basis of pulse width. (**c**) Gating live cells on the basis of DAPI exclusion. Blood cells characterized by low side scatter are indicated with an arrow. (**d**) Gating CD133 positive cells. Note that blood cells in the preparation (red arrow) are CD133 negative.

difficult to distinguish between ectopic hair follicles generated from grafted cells and endogenous nude/Balb/C hair follicles (**Fig. 5f**). The latter problem is addressed by labeling surface graft cells with reporter genes such as GFP (**Fig. 5e–g**). Labeled cells can be easily analyzed under a fluorescent dissecting microscope to determine their contribution to the graft (**Fig. 5e–g**). Immunostaining for marker proteins can be carried out on histological sections to the graft (**Fig. 5i–k**).

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