Chapter 8

Isolation of Mouse Hair Follicle Bulge Stem Cells and Their Functional Analysis in a Reconstitution Assay

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Abstract

The hair follicle (HF) is a dynamic structure readily accessible within the skin, and contains various pools of stem cells that have a broad regenerative potential during normal homeostasis and in response to injury. Recent discoveries demonstrating the multipotent capabilities of hair follicle stem cells and the easy access to skin tissue make the HF an attractive source for isolating stem cells and their subsequent application in tissue engineering and regenerative medicine. Here, we describe the isolation and purification of hair follicle bulge stem cells from mouse skin, and hair reconstitution assays that allows the functional analysis of multipotent stem cells.

Key words Hair follicle stem cell, Bulge, Hair reconstitution assay, Regeneration

1 Introduction

Stem cells (SC) have the unique capacity to self-renew and to differentiate into more mature cell types; these properties allow stem cells to play a crucial role in the homeostasis of adult tissues and organs as well as during healing and regenerative responses following injury. Characteristics of epithelial stem cells are quiescence and slow cycling, persistence during the lifetime of the organism, having a distinct biochemical makeup, and localized in a protected reservoir [1]. Studies have revealed that adult stem cells reside in specific niches within various tissues.

Cell lineage tracing studies have demonstrated that the hair follicle bulge, a specialized structure of the skin located in the mid to upper portion of the follicle at the arrector pili muscle attachment site [2], harbors stem cells necessary for hair follicle regeneration and maintenance. These cells express specific markers, such as keratin 15 [3] and CD34 [4] in the mouse. The stemness of HFSC has been demonstrated by reconstitution assays where these cells regenerate de novo hair follicles in vivo, by repopulating all lineages of cells in the nascent hair follicles with HFSC progeny.

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Moreover, similar to native hair follicles, these de novo hair follicles go through cyclic regression and regeneration that rely on the function of the isolated hair follicle bulge stem cells.

Morris et al. first established a method for skin cell isolation in 1990 [5]. Subsequently, several methods to test the hair-forming ability of the isolated cells were developed, including the patch assay [1, 5, 6] and the silicone chamber assay [7, 8] described in details here. Liang et al. [9] compared these methods and concluded that the patch assay offered the following advantages over others: easy to perform, requires a manageable number of cells, shorter assay times, and cost-effective. However, the patch assay also suffers some shortcomings: uncontrolled hair shaft orientation and inability of the de novo hairs to cycle properly. The chamber assay, on the other hand, is labor-intensive, requires longer assay times and cell numbers that are at least an order of magnitude higher than the Patch assay. However, the chamber assay results incorrectly oriented hairs capable of normal cycling for an extended period of time. In this chapter, we describe these two assays that are most commonly used for reconstitution of the hair follicle, so that researchers are able to choose an assay according to their needs.

2 Materials

2.1 Hair-Follicle Cell-Isolation Reagents

1. C57/BL mice, 6–8 weeks (Jackson Laboratory, Sacramento, CA).
2. Scissors.
3. Tweezers.
4. Oster Golden A5 hair clipper with #40 blade (Fisher, NC 9378235).
5. Povidone iodine prep solution 16 oz (More Medical, Farmington, CT, 11602).
6. 70% ethanol.
7. 100 mm × 20 mm CELLSTAR TC Petri dish (BioExpress, Kaysville, UT, T-2881-2).
8. #22 blade, sterile (Fisher, 08-918-5C).
9. MooreBrand® scalpel handle #4 (More Medical, 30976).
10. 1× DPBS, no calcium, no magnesium (Invitrogen, 14190-144).
11. Gentamicin (Gibco, Grand Island, NY, 15750-060).
12. 0.25% trypsin/saline without EDTA (Invitrogen, 25200-056).
13. SMEM (Bio-Whittaker, Walkersville, MD, 12-1260).
14. 70 μm cell strainer (BD Falcon 35-2350).
15. 50 mL conical tube.

2.2 FACS Reagents

1. Antibody-staining buffer: 1× PBS + 2% FBS.
2. CD49f-PE (BD Pharmingen, San Diego, CA, 555736).
3. CD34-FITC (BD Pharmingen, 553733).
4. DAPI, FluoroPure™ grade (Invitrogen, D21490).

2.3 Mouse Neonatal Dermal Cell Isolation Reagents

1. Postnatal C57BL/6 Mice day 0–2.
2. Scissors (Geoge Tiemann & Co., Hauppauge, NY, 110-1250SS or equivalent).
3. Tweezers (Geoge Tiemann & Co. 160-55PP or equivalent).
4. Collagenase type I (Invitrogen, 17100-017).
5. DNase (Roche, 104159).
6. 1× DPBS, no calcium, no magnesium (Invitrogen, 14190-144).
7. 100× AB/AMY (Invitrogen, 14190-144).
8. 40 μm cell strainer (BD Falcon 35-2340).
9. 100 μm cell strainer (BD Falcon 35-2360).
10. 0.25% trypsin/saline without EDTA (Invitrogen, 25200-056).
11. Dispase (Invitrogen, 17105-041).
12. $CaCl_2$-free Eagles minimal essential medium (Sigma, St. Louis, MD, M8167).
13. Fetal calf serum (Hyclone, SH30037.03).
14. Chelex (Sigma, C7901-500G).
15. 1 M solution of $CaCl_2$ (Sigma, C-7902).
16. 200 mmol/L L-Glutamine (Invitrogen, 25030-081).
17. 1 M Tris–Cl (Sigma, T3038).
18. 5 M NaCl (Sigma, S-5150).
19. Dithiothreitol (DTT) (Sigma, 43815-1G).
20. Glycerol (Sigma, G5516).
21. Distilled water (Invitrogen, 15230-147).

2.4 Reconstitution Assay Reagents

1. Nude/nude mice (6 weeks, Jackson Laboratories).
2. 1 mL TB syringe with 25 G needle (BD, 309626).
3. DMEM/F12(1:1) medium (Gibco, 11039-021).
4. Alcohol swabs (Kendall Webcol, Brooksville, FL, alcohol prep 70% isopropyl alcohol).
5. Tattoo ink (Permanent Black Animal Identification and Marking Systems, Inc., Hornell, NY).
6. Silicone chamber dome sets (*see* **Note 18**).
7. Eye ointment (Puralube ointment, E. Fougera & Co., Melville, NY 11747).

8. Curved scissors.
9. Tweezers.
10. Heating pad.

3 Methods

3.1 Hair-Follicle Cell Isolation

1. Sacrifice 6- to 8-week mice by cervical dislocation or CO_2 euthanasia (*see* **Note 1**).
2. Clip dorsal fur on the entire back. Place mice in a jar with enough Betadine to cover them. Roll the jar in your hands until the mice are completely wet. Pour out the Betadine and rinse with distilled water until the water is clear. Repeat the process.
3. Add 70% ethanol to cover the mice and let them soak for 5–10 min. Pour the ethanol out. Repeat the process.
4. Remove the entire dorsal skin using scissors and rinse in a 100 mL cup with 30–40 mL 1× DPBS and 2× gentamycin (100 mg/L).
5. Lay skin flat on a square Petri dish with the dermal side facing up; scrape off subcutaneous tissue with a scalpel until the remaining skin is nearly translucent. Keep scraped skin in the PBS until all pieces have been scraped (*see* **Note 2**).
6. Flatten skins on a fresh Petri dish, and cut the skin samples into ~1 × 1.5 cm strips. Transfer the skin pieces, epidermal side up, to a Petri dish containing 25 mL of 0.25% trypsin/saline and 2× gentamycin. Incubate at 37 °C for 70 min (*see* **Note 3**).
7. Transfer skin pieces from trypsin to a Petri dish with 10 mL SMEM containing 2× gentamycin and 5% FBS. Scrape off the epidermis with a sterile scalpel. Discard the dermis (*see* **Note 4**).
8. Pour the epidermis samples into a sterile 50 mL conical tube. Rinse the Petri dish with extra medium, and add the rinse medium into the conical tube containing scraped epidermis. Then add medium to 40 mL, and gently pipette the suspension up and down with a 10 mL pipette for 20 times (*see* **Note 5**).
9. Filter the suspension through a sterile 70 μm cell strainer into a 50 mL conical tube, and spin at 180 × *g* for 7 min at room temperature.
10. Resuspend the pellet in 10 mL SMEM medium and count the cell number.

3.2 FACS of Hair Follicle Stem Cells

1. Set up incubation of cells with antibodies
   (a) Compensation tubes (*see* **Note 6**):
   - Negative control: Aliquot 1×10^6 cells in 250 μL staining buffer (no stain).
   - FITC control: Aliquot 1×10^6 cells in 100 μL staining buffer. Add 20 μL CD49f-FITC antibody.

- PE control: Aliquot 1×10^6 cells in 100 μL staining buffer. Add 20 μL CD34-PE antibody.
- DAPI control: Aliquot 1×10^6 cells in 250 μL staining buffer. DAPI should be added immediately before FACS without additional incubation.

(b) FACS tubes:

Add CD49f-FITC and CD34-PE antibodies, 20 μL each in 100 μL staining buffer for every 1×10^6 cells (*see* **Note 7**).

2. Incubate for 30 min at room temperature.
3. Add 1× PBS to 1 mL and spin at $180 \times g$ for 5 min to remove supernatant.
4. Add 1–2 mL 1× PBS and suspend cells. Spin again to collect pellets.
5. Resuspend DAPI control samples in 250–500 μL staining buffer, and FACS samples in 1 mL staining buffer containing up to 10×10^6 cells.
6. Dilute DAPI 1:1000 in antibody-staining buffer. When all samples are ready for FACS, add 20 μL of the diluted DAPI into DAPI control tube (250–500 μL).
7. FACS is performed on a BD FACS Vantage with Diva Option, using a 100 μM nozzle, 15 PSI pressure and 30,000 kHz drop drive frequency (*see* **Note 8**).

3.3 Mouse Neonatal Dermal Cell Isolation for Reconstitution Assay

3.3.1 Medium Preparation

1. Chelex-treated fetal-calf serum
   (a) 500 mL fetal calf serum.
   (b) 25 g Chelex (Sigma, C7901-500G).

   Stir gently for 1 h on a magnetic platform at room temperature. Filter through 90 mm filter unit (Nalge Nunc, Rochester, NY, 162-0045), and then aliquot into 50 mL conical tubes. Store the treated serum at –20 °C. Thaw at 37 °C before using.
2. Complete EMEM Media:
   (a) $CaCl_2$-free Eagles minimal essential medium 500 mL (Sigma, M8167).
   (b) 50 mL Chelex-treated fetal calf serum.
   (c) 0.05 mmol/L $CaCl_2$ (25 μL of 1 M solution per 500 mL, Sigma, C-7902).
   (d) 2 mmol/L L-glutamine (Invitrogen, 25030-081, 5 mL per 500 mL).
   (e) 1× AB/AMY (Invitrogen 14190-144, 5 mL per 500 mL).
3. DNase I (40 mg/mL, 80 U/μL, 2.5 mL) stock solution
   (a) DNase I 100 mg (200,000 U) (Roche, Indianapolis, IN, 104159)
   (b) 40 mM Tris–Cl.

(c) 100 mM NaCl.

(d) 2 mM DTT (to make 10 mL 100 mM DTT stock solution, use 154 mg DTT (Sigma, 43815-1G), add distilled water to 10 mL).

(e) 1.15 mL distilled water (Invitrogen, 15230-147).

(f) 1.25 mL glycerol (Sigma, G5516) (Fig. 1).

3.3.2 Procedure

1. Sacrifice neonatal pups (day 0–2) according to the IACCU-approved protocol.
2. Remove heads, limbs, and tails from pups with sharp scissors.
3. Remove the skin from the muscle, rinse in DPBS with 1× AB/AMY three times.
4. Float isolated skins with epidermal side up in 2 mg/mL dispase/DPBS solution overnight at 4 °C in a 100 × 20 mm Petri dish. One dish can hold 20–30 mL dispase solution with ~20 pup skins (*see* **Note 9**).
5. Use sterilized forceps; peel off the epidermis from the dermis. Transfer dermis to a Petri dish containing complete EMEM to rinse off the dispase.
6. Transfer the dermis to a 50 mL conical tube, add 10 mL complete EMEM with 0.35% collagenase per ten pieces of dermis (*see* **Note 10**).
7. Use scissors to cut the dermis to very fine pieces (2–3 mm^2) until they can be easily pipetted up and down with a 10 mL pipette.
8. Incubate with moderate agitation in a 37 °C water bath for 25 min (*see* **Note 11**).
9. Add 20 μL of 20,000 U/mL DNase I/PBS solutions to each 10 mL collagenase digestion mixture. Incubate for another 5 min at 37 °C with agitation.
10. Dilute each 10 mL digestion mixture to 50 mL with complete EMEM, and pipet the mixture up and down for 20 times using a 10 mL pipet. Filter solution through 100 μm cell strainer.
11. Spin at 180 × g for 5 min at 4 °C. This is supernatant A and pellet A (Fig. 2).
12. Transfer supernatant A to a 50 mL tube; resuspend pellet A in 25 mL media and keep on ice.
13. Spin supernatant A at 520 × *g* at 4 °C for 5 min. This is supernatant B and pellet B (Fig. 2). Discard supernatant B, and resuspend pellet B in 25 mL media. Keep on ice.
14. Spin the resuspended pellet A mixture at 29 × *g* for 3 min at 4 °C. This is supernatant C and pellet C (Fig. 2). Transfer supernatant C to a new tube, and discard the pellet.

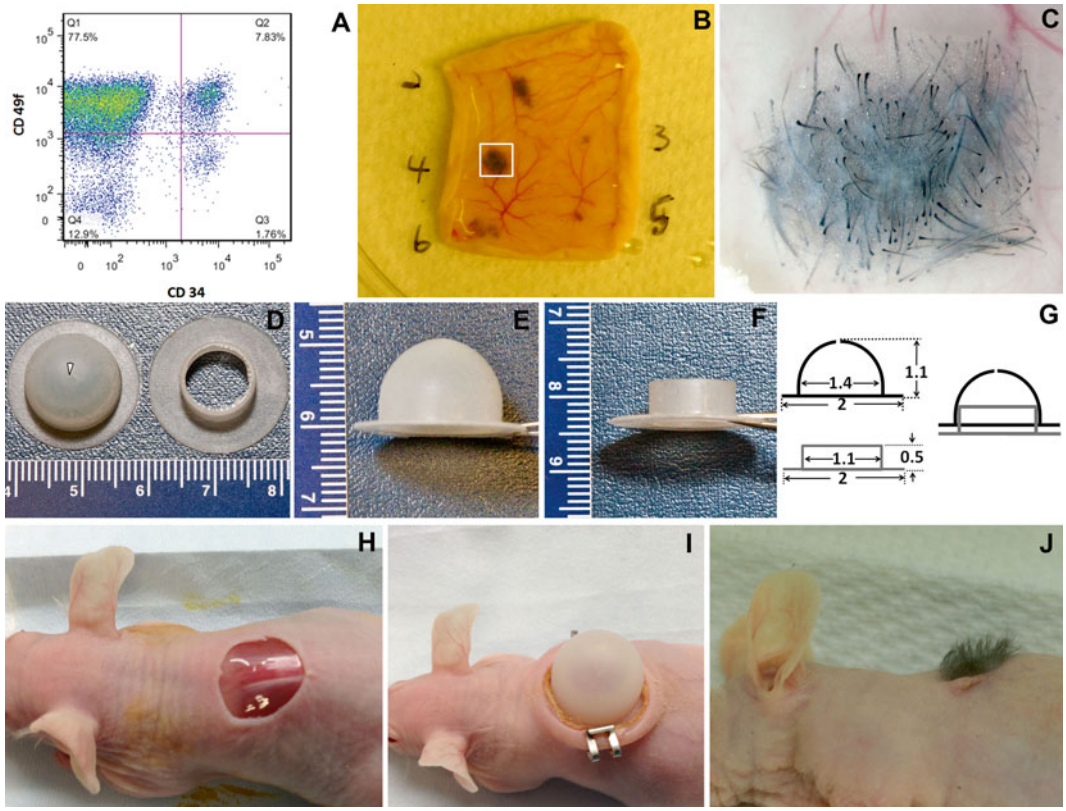

Fig. 1 FACS analysis and reconstitution assays for hair-follicle stem cells. (**a**) FACS analysis revealing the presence of bulge stem cells ($CD34^{+}CD49f^{+}$, approximately 8 % of the total input cells) among the keratinocytes isolated from the back skin of a 2-month-old mouse. (**b**) Patch-assay skin sample showing dermal view of skin harvested from a nude mouse which received multiple injections of a mixed suspension of dermal and epidermal cells. The *boxed area* shows pigmented de novo hair follicles reconstituted from injected cells. (**c**) An enlarged view of a representative injected site similar to the one in the *boxed area* in (**b**). As many as several hundred hair follicles with hair shafts can be seen at one injection site. (**d**–**g**) The dimensions of the silicone chamber used in the chamber assay. The chamber assembly consists of two parts, a dome and a base onto which the dome snaps. The rulers have metric units, with the smallest division representing 1 mm. (**d**) Top view of the dome (*left*) and the base (*right*). The *arrowhead* indicates the small hole on top of the dome, through which cell slurry is pipetted into the chamber. (**e**) Side view of the dome. (**f**) Side view of the base. (**g**) Schematic representations of the chamber, with the dimensions indicated in cm. The *right* illustrates the assembled chamber in use. The thickness of the chamber is uniformly 0.8 mm. (**h**–**j**) Chamber assays in nude mice. (**h**) Excision of the back skin on a nude mouse to prepare for the insertion of the chamber. (**i**) The inserted chamber is securely anchored to the skin with clips. (**j**) Hairs growing out of the site where the chamber was implanted

15. Spin resuspended pellet B and supernatant C at 180×*g* for 5 min at 4 °C. Aspirate supernatant. This is pellet D and E, respectively (Fig. 2). Resuspend both pellets in 10 mL complete EMEM each and combine in one tube.
16. Spin the combined suspension at 180×*g* for 5 min at 4 °C, and aspirate the supernatant. Resuspend the pellet in 20 mL complete EMEM. Spin the combined suspension at 180×*g* for 5 min at 4 °C and collect the pellet.

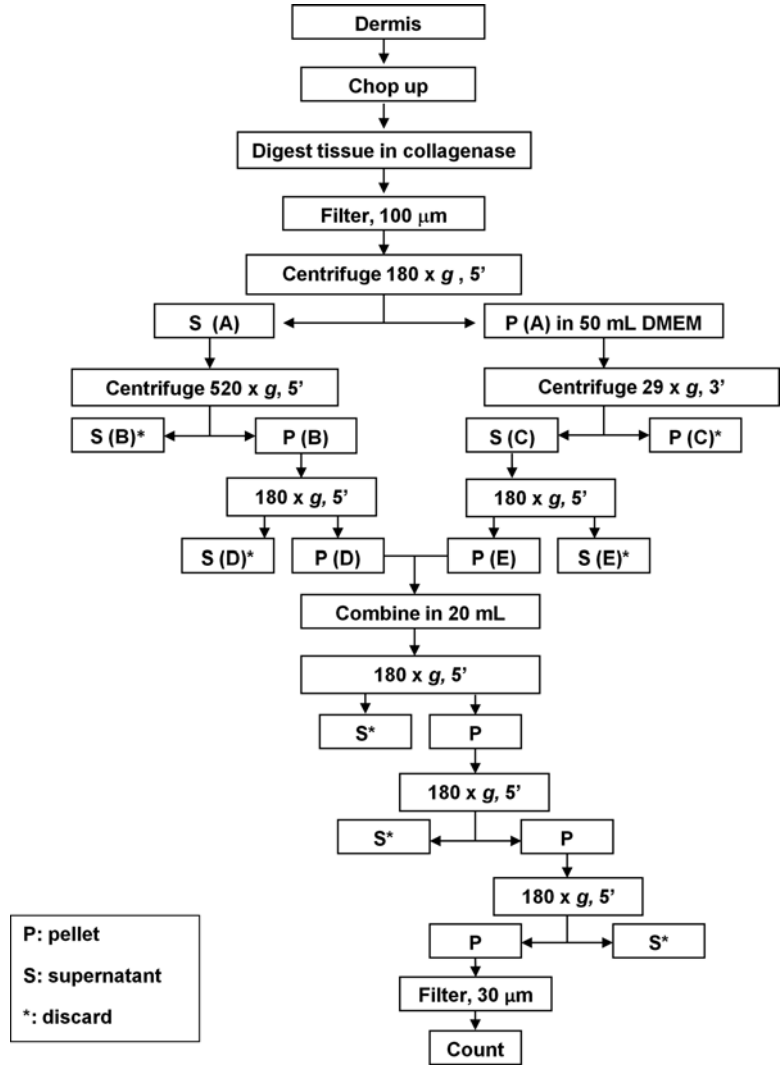

Fig. 2 Flow chart of mouse neonatal dermal-cell isolation. Details are described in the procedures for neonatal dermal cell isolation. S (A): supernatant A; P (A): pellet A; S (B): supernatant B; P (B): pellet B; S (C): supernatant C; P (C): pellet C; S (D): supernatant D; P (D): pellet D; S (E): supernatant E; P (E): pellet E. Asterisk denotes fractions to be discarded

17. Resuspend final pellet in 20 mL medium. Filter through a 40 μm cell strainer.
18. Count the cells. Store cells on ice until grafting (*see* **Note 12**).

3.4 Reconstitution Assays (See Note 13)

3.4.1 Patch Assay Procedure

1. For a single injection, mix 1×10^6 mouse neonatal dermal cells with 0.5×10^6 epidermal cells in 10 mL DMEM/F12 medium. Spin down the mixed cells at $350 \times g$ for 5 min at 4 °C (*see* **Note 14**).
2. Aspirate most of the supernatant, but leave approximately 70–80 μL of residual medium in the tube.

3. Transport the cell pellet on ice immediately to the animal facility.
4. Place nude mice in an induction container with 5% isoflurane and 1 L/min oxygen. Use the pedal (paw pinch) reflex to determine the depth of anesthesia.
5. Once mice are properly anesthetized, transfer them to a nose cone with 1% isoflurane and 1 L/min oxygen. Use the pedal (paw pinch) reflex again to determine the depth of anesthesia.
6. Disinfect the dorsal skin with iodine followed by alcohol swab. Tattoo the site of cell injection by piercing the mouse skin adjacent to the injection site once with a 25 G needle dipped in tattoo ink to mark the location of injection. Up to six injections can be performed per mouse.
7. Resuspend the cell pellet by pipetting. Draw the cells slowly into a 1 mL TB syringe with a 25 G needle to avoid shearing of the cells (*see* **Note 15**).
8. Insert the needle parallel to the skin, making sure the needle tip does not penetrate the whole dermis so that the cells, once injected, can remain in a confined space in the dermis. Inject the cell suspension into the dermis slowly, followed by gradual withdrawal of needle to avoid back flow of the fluid (*see* **Note 16**).
9. Place injected mice on their belly into pre-warmed cages lined with a paper towel. Use a heating pad on low setting to warm the cage. Moniter mice until they can get up by themselves when placed on their backs.
10. Sacrifice the mouse 2 weeks after cell injection. Harvest the skin and lay flat on a Petri dish. Visualize the hair follicles formed at each injection site from the dermal side of the skin under a dissecting scope (Fig. 1b and c).
11. Quantify the number of hair follicles formed in each injection before or after fixation (*see* **Note 17**).

3.4.2 Chamber Assay

1. For each single assay, mix 10×10^6 mouse neonatal dermal cells with 5×10^6 epidermal cells in DMEM/F12 medium. Spin down the mixed cells at $350 \times g$ for 5 min.
2. Aspirate most of the supernatant, but leave approximately 200 μL of residual medium in the tube.
3. Transport the cell pellet on ice immediately to animal facility.
4. Administer bubrenorphine (0.5 mg/kg) by subcutaneous injection 30 min before the procedure and 6–8 h post surgery.
5. Place mice in an induction container with 5% isoflurane and 1 L/min oxygen. Use the pedal (paw pinch) reflex to determine the depth of anesthesia.

6. Once mice are anesthetized, transfer them to a nose cone with 1% isoflurane and 1 L/min oxygen. Use the pedal (paw pinch) reflex again to determine the depth of anesthesia. If the mouse responds to pedal pressure, increase isoflurane (e.g. by 1–2%) until the mouse does not respond to a pedal pinch. Monitor respiration and heartbeat continuously during surgery.
7. Place eye ointment on each eye of the mouse to prevent drying out. Disinfcet the dorsal skin with iodine followed by alcohol swab.
8. Using curved scissors, cut a piece of full thickness skin (about 1 cm diameter) from the back of the mouse by lifting it with forceps (Fig. 2h).
9. Moisturize the area where the chamber dome will be grafted with PBS. Pull the skin by forceps to create a slit-like opening and insert the dome flange under the skin. Securely anchor the chamber to the skin using wound clips (Fig. 2i, *see* also **Note 18**).
10. Apply cell slurry through the hole on top of the grafting dome (Fig. 2d).
11. After cell implantation, place mice on their belly into prewarmed cages lined with a paper towel. Use a heating pad on low setting to warm the cage. Apply additional eye ointment as necessary. Monitor mice until they are awake.
12. Remove domes after 7 days. The grafted area heals within 3 weeks. Euthanize animals and collect skins 30 days post-grafting or later as needed to fit study purpose (*see* **Note 19**).

4 Notes

1. We routinely use 6- to 8-week C57BL female mice for isolation of bulge stem cells, since hair follicles are in the telogen phase at this stage and can be easily scrapped off the skin surface after trypsin treatment. Each female mouse will yield ~25×10^6 total epidermal cells prior to sorting, while the yield from a male mouse is ~usually 20–30% lower.
2. Use a new scalpel for each mouse skin, and keep scalpel holder and forceps in 70% ethanol between samples.
3. We found that when younger mice (such as 42–45 days) are used, the trypsin digestion time can be reduced by 5–10 min. On the other hand, when older mice are used, the trypsin digestion time may need to be increased by 5–10 min. However, we do not normally recommend more than 70 min of trypsin treatment as prolonged trypsin digestion would increase cell death. The trypsin treatment can also be replaced

by overnight (less than 16 h) dispase (2 mg/mL/1× DPBS) treatment at 4 °C. However, in our experience the trypsin treatment usually gives a higher yield than the overnight dispase treatment.

4. To maximize the yield of epidermal cells, hold one corner of the skin with forceps, scrape in the direction away from the forceps. Then hold the skin at another corner, and repeat the scraping. Repeat this procedure for the remaining two corners.
5. The pipetting time can be optimized based on the force and speed of the pipettor. The goal is to fully dissociate the tissues without causing damage to the cells.
6. Cell number for compensation samples can be reduced to as few as 0.25×10^6 if not enough cells were obtained.
7. Antibody titer optimization: We found the BD Pharmingen antibodies listed here work well for the FACS of bulge stem cells. The antibody concentrations listed are based on the manufacturer's recommendation. We found that using 1/5 to 1/10 of the recommended concentration also worked well. However, the concentrations of antibodies used need to be optimized by each user when they first perform this experiment.
8. FACS parameter optimization: Cells are collected using the following filters: CD49f FITC 530/30-A, CD34 PE 585/42-A, and DAPI 450/20-A with UV excitation. Sorting parameters are set at: 15 psi, a 100 μm nozzle, and 30,000 kHz drop drive frequency. This configuration permits a trigger rate of approximately 6000 cell/s with abort rates of less than 20%. Purity check on the $CD34^+CD49f^+$ population ranges, typically around 95–98%. Data are analyzed using DIVA software. When 6- to 8-week telogen skin is used for cell isolation, the percentage of the $CD34^+CD49f^+$ population is usually between 5% and 10% of the total cells (*see* Fig. 1a).
9. Dispase treatment overnight at 4 °C can be shortened to 2 h by incubation at 37 °C, if it is necessary to speed up the isolation process.
10. Collagenase I solution: Weigh 35 mg collagenase I, dissolve in 10 mL complete EMEM medium, filter by pushing through a syringe attached to a 0.22 μm filter unit. Use 10 mL solution for every ten pup dermis.
11. Invert the tube and resuspend the tissue pieces in the collagenase solution thoroughly 4–5 times during incubation can help increase the yield of isolated cells.
12. Dermal cells are best used fresh after isolation for reconstitution assay. Dermal cells can be frozen in complete EMEM containing 5% DMSO for future use. However, we found that

previously-frozen dermal cells yield approximately 50% less hair follicles than freshly-isolated dermal cells in reconstitution assays.

13. Both reconstitution assays, the patch assay, and the silicone chamber dome grafting surgery should be carried out with strict adherence to institutional guidelines for minimizing distress in experimental animals. Surgery instruments should be sterilized before use.
14. As a positive control for the constitution experiment, 1×10^6 freshly-isolated mouse neonatal dermal cells, combined with 1×10^6 freshly-isolated mouse neonatal epidermal cells per injection, will yield roughly 200–300 hair follicles. We found that the ratio of dermal to epidermal cells, ranging 5:1 to 1:5, while maintaining the total number of injected cells, had little effect on hair-forming efficiency in the reconstitution assay. When sorted cells are used, the number of hair follicles formed may vary due to the purity of the cells and the level of cell damage resulting from sorting. The number of cells used per injection will need to be adjusted and optimized by individual researchers, based on the available number of sorted cells and hair-forming potential of the cells used.
15. Withdraw the plunger prior to loading the cell suspension in the syringe, so that an air space forms between the plunger and the cell suspension. Then carefully inject the cell suspension only, while avoiding injecting the air into the skin. This is to prevent losing cells to the dead space of the needle hub during the injection.
16. Given the indispensible roles of the reciprocal interactions between dermal and epidermal cells during hair morphogenesis, it is important to maintain the target cells in close proximity following injection. To achieve this, the injected cells should be placed above the panniculus carnosus layer of the skin. Therefore, it is critical to maintain the needle-entry angle in parallel to the skin surface and make sure that the needle does not penetrate the full thickness of the skin during the injection. This is to ensure that the cell mixture stays in a confined space in the skin, without being diluted through subcutaneous diffusion.
17. One advantage of the patch assay is that it is a semi-quantitative assay. The number of hair follicles formed by various cell populations in different injections can be counted under a dissecting scope once the skin is harvested. The number of hair follicles formed per injection can be manipulated by injecting less or more cells per test in order to make the quantification manageable.
18. Currently, the silicone chamber is not available from any commercial source as far as we know. However, the chambers can

be custom-made by individual researchers. To assist interested researchers, the chamber and its dimensions are shown in Fig. 1d–j.

19. If the number of hair follicles formed in a chamber assay needs to be quantified, the harvested fresh skin can be submerged in 2 mg/mL dispase solution at 4 °C overnight. The epidermis can be peeled off from the dermis, and the dermis can be fixed with 4% paraformaldehyde/PBS solution at room temperature for 1 h, followed by chromogenic staining for alkaline phosphatase activity in the dermal papilla (DP). The number of stained DPs can be counted under a dissection microscope or in digital images of the stained skin using ImageJ software, which can be downloaded from NIH website.

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