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Ex Vivo Imaging and Genetic Manipulation of Mouse Hair Follicle Bulge Stem Cells

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Abstract

Stem cells that reside in the bulge of adult mouse hair follicles are a leading model of tissue stem cell research. Ex vivo culturing, molecular and cell biological characterizations, as well as genetic manipulation of fluorescence-activated cell sorting-isolated bulge stem cells offer a useful experimental pipeline to complement in vivo studies. Here we describe detailed methods for culturing, immunostaining, live cell imaging, and adenoviral infection of bulge stem cells for downstream applications such as in vitro clonal and in vivo patch assays.

Keywords Adenovirus, Bulge stem cells, Clonal assay, Hair follicle, Immunofluorescence, Live cell imaging, Patch assay

1 Introduction

The hair follicle is a skin appendage that undergoes dramatic remodeling during the postnatal hair cycle, which includes a growth phase (anagen), a destruction phase (catagen), and a resting phase (telogen) [1]. Fueling the hair cycle are the hair follicle stem cells (HFSCs) that reside in the bulge, a part of the outer root sheath that bulges out from the base of the non-cycling portion of the hair follicle, and their immediate progeny in the secondary hair germ (HG) [2, 3]. Transition from telogen into anagen occurs when the HG cells receive signals from the adjacent dermal papilla and begin to proliferate and differentiate into multiple cell types of the regenerative portion of the hair follicle, including the inner root sheath and the hair matrix that later produces the actual hair shaft [2]. The bulge HFSCs are generally quiescent but become proliferative during early anagen and differentiate into the outer root sheath that encases the inner parts of the growing hair follicle [2]. In catagen, most of the hair follicle cells that are located beneath the bulge undergo apoptosis, leaving a structure called strand which retracts up to the base of the bulge [1]. Some of the outer root sheath cells escape apoptosis and are incorporated into the base of the non-cycling portion of the hair follicle where they form a new

bulge and a new HG, which are HFSCs for the next hair cycle [2]. Understanding the function of HFSCs and the molecular regulation of their quiescence, activation, and differentiation represents an important direction in adult stem cell biology and may have implications on how we prevent and treat hair loss.

Various tools and techniques have been generated and/or applied to study HFSCs. With the knowledge of unique markers of the various hair follicle cell types, immunofluorescence can be used to examine the presence, absence, or fate alterations of cells in the context of various genetic perturbations. HFSCs can be identified by immunofluorescence using classical HFSC markers, such as CD34, or with pulse chase methods to identify HFSCs as label-retaining cells [2]. These analyses provide static snapshots of HFSCs, as skin must be frozen or fixed before sectioning and further analysis. More sophisticated imaging techniques now have abilities to track the migration, proliferation, and overall behavior of individual cells within the hair follicle in real time during various stages of regeneration [4, 5]. Coupled with fluorescent genetic labeling, these pioneering live imaging techniques uncover unprecedented information about stem cell dynamics in vivo. With flow cytometry and fluorescence-activated cell sorting (FACS), HFSCs can be quantified and isolated for downstream purposes such as gene expression analysis and in vitro assays [6, 7]. FACS-sorted HFSCs can be cultured and expanded on mitotically inactivated fibroblast feeders [6]. Cultured HFSCs can be functionally assessed for clonal growth potential and, with serial passaging, long-term self-renewal capability, as well as for regenerative capacity in host animals [6, 8, 9].

Here we describe methods for culturing and downstream analyses of sorted bulge HFSCs. Specifically, we expand on the existing clonal growth assay by incorporating live cell imaging to monitor the division events and movement tracks of individual cells within the growing bulge HFSC colonies. Furthermore, we provide protocols for growing bulge HFSCs on glass for immunofluorescence and for efficient adenovirus infection that minimally impacts their regenerative capacity in vivo.

2 Materials

2.1 J2-3T3 Fibroblast Culture, Mitotic Inactivation, and Feeder Layer Preparation

1. J2-3T3 fibroblasts.
2. F media (see [6] for detailed instructions).
3. Mitomycin C (Fisher Scientific, Cat. No. BP2531-2).
4. 0.22-μm filter (Millex, Cat. No. SLGV033RS).
5. 10-mL syringe (BD, Cat. No. 309604).
6. 1× PBS.
7. 15-mL conical vials.

2.2 Plating and Culture of Primary HFSCs

1. Tissue culture dishes with mitotically inactivated J2-3T3 fibroblast layer (requires reagents from Sect. 2.1).
2. E media (see [6] for detailed instructions).
3. 1× PBS.
4. Versene:
   (a) 200 mL 10× PBS.
   (b) 0.4 g EDTA disodium salt (Sigma, Cat. No. E-6511).
   (c) Bring up to 2 L with deionized H_2O.
   (d) Autoclave.
   (e) 8 mL sterile 25% glucose solution in deionized H_2O.
5. 0.1% trypsin (Sigma, Cat. No. T4799).
6. 15-mL conical vials.

2.3 Clonal Assay

1. 6-well tissue culture plate (Falcon, Cat. No. 353046) or 60-mm gridded plates (Corning, Cat. No. 430166).
2. Tissue culture dishes with mitotically inactivated J2-3T3 fibroblast layer in F media.
3. E media.
4. 0.5% crystal violet (Sigma, Cat. No. HT90132-1L) in a 1:1 methanol/H_2O solution.

2.4 Live Cell Imaging

1. Keyence BZ-X700 microscope (or equivalent microscope capable of live cell imaging).
2. 6-well tissue culture plate (Falcon, Cat. No. 353046).
3. Mitotically inactive 3T3 fibroblast feeders.
4. F media.
5. E media.
6. Freshly sorted or passaged HFSCs.

2.5 Immunofluorescence

1. Collagen I solution at a concentration of 25 μg/mL in 0.02 M acetic acid (Sigma, Cat. No. C9791).
2. Glass coverslips (Fisher Scientific, Cat. No. 12-546).
3. Optional: 0.22-μm low protein-binding filter and 10-mL syringe.
4. F media.
5. E media.
6. 1× PBS.
7. 4% paraformaldehyde, made from powder (MP Biomedicals, Cat. No. 150146) in 1× PBS.
8. 1× PBS with 0.1% Triton X-100 (Sigma, Cat. No. T9284).

9. 20% normal goat serum (NGS)-gelatin solution (20 mL):
   (a) 4 mL NGS (Invitrogen, Cat. No. 16210-064).
   (b) 200 μL 10% Triton X-100 (Sigma, Cat. No. T9284).
   (c) 2 mL 10× PBS.
   (d) 200 μL 2% NaN_3 (Alfa Products, Cat. No. 50101).
   (e) 200 μL 5% Tween 20 (Fisher Scientific, Cat. No. EC500-018-3).
   (f) 200 μL 1% gelatin (Sigma, Cat. No. G-1890).
   (g) 13.2 mL distilled H_2O.
      - Mix and heat inactivate in 55 °C water bath for 30 min. Store at 4 °C for about 2 weeks.
10. 10% NGS-gelatin solution (10 mL):
   (a) 5 mL 20% NGS-gelatin solution (from step 9).
   (b) 50 μL 10% Triton X-100 (Sigma, Cat. No. T9284).
   (c) 500 μL 10× PBS.
   (d) 50 μL 2% NaN_3 (Alfa Products, Cat. No. 50101).
   (e) 50 μL 5% Tween 20 (Fisher Scientific, Cat. No. EC500-018-3).
   (f) 50 μL 1% gelatin (Sigma, Cat. No. G-1890).
   (g) 4.3 mL distilled H_2O.
11. 2% NGS-gelatin solution:
   (a) 1 mL 20% NGS-gelatin (from step 9).
   (b) 200 μL 10% Triton X-100 (Sigma, Cat. No. T9284).
   (c) 900 μL 10× PBS.
   (d) 200 μL 2% NaN_3 (Alfa Products, Cat. No. 50101).
   (e) 200 μL 5% Tween 20 (Fisher Scientific, Cat. No. EC500-018-3).
   (f) 200 μL 1% gelatin (Sigma, Cat. No. G-1890).
   (g) 7.75 mL distilled H_2O.
12. Primary antibodies of interest.
13. Appropriate secondary antibodies.
14. DAPI (Life, Cat. No. D1306).
15. Vectashield (Vector, Cat. No. H1000).

2.6 Adenoviral Infection of HFSCs

1. 6-well tissue culture dishes (Falcon, Cat. No. 353046) with mitotically inactivated J2-3T3 fibroblast layer.
2. 1× PBS.
3. E media.
4. Versene.

5. 0.1% trypsin (Sigma, Cat. No. T4799).
6. 15-mL conical vial.
7. IRES-GFP adenovirus (Vector Biolabs, Cat. No. 1761).

2.7 Patch Reconstitution Assay with Virally Infected HFSCs

1. Detailed lists of required reagents for the (1) isolation of neonatal dermal cells and (2) patch reconstitution assay can be found in [7].
2. Reagents from Sect. 2.6 are required for generating virally infected HFSCs.

3 Methods

3.1 J2-3T3 Fibroblast Culture, Mitotic Inactivation, and Feeder Layer Preparation

Culturing healthy fibroblasts and generation of a mitotically inactive feeder layer are a critical component of HFSC culture.

1. For J2-3T3 growth and propagation, see the detailed protocol in [6].
2. Allow cells to reach 100% confluency.
3. Prepare the mitomycin C solution.
   (a) Add 5 mL 1× PBS to 2 mg vial of mitomycin C and mix (0.4 mg/mL).
   (b) Filter sterilize with 0.22-μm filter.
4. Combine 12 mL F media with 240 μL of filter sterilized 0.4 mg/mL mitomycin C, and add to a confluent 100-mm plate of J2-3T3 fibroblasts (*see* **Note 1**).
5. Swirl gently to mix and incubate at 37 °C in incubator with 5% CO_2 for 2 h.
6. Aspirate media and rinse cells five times with 2 mL 1× PBS (*see* **Note 2**).
7. Add 2 mL 0.1% trypsin and incubate until cells start to lift off the plate (about 10 min).
8. Gently remove cells with P1000.
9. Add 8 mL F media to inactivate trypsin and transfer cell suspension to a 15-mL conical vial and centrifuge to pellet at 1000 RPM for 5 min.
10. Aspirate the supernatant and resuspend the cell pellet in 5 mL F media and count cells.
11. Seed appropriate number of mitomycin C-treated J2-3T3 fibroblasts in F media based on plate size. Use Table 1 for guide.
12. Swirl plate gently to evenly distribute feeders (*see* **Note 3**).

Table 1
Desired number of mitotically inactive J2-3T3 fibroblasts to plate depending on plate size

Plate type	J2-3T3 Fibroblast number
100 mm	1,000,000
60 mm	~360,000
35 mm	~170,000
6 Well	~170,000

13. Culture at 37 °C in incubator with 5% CO_2 for 2 days to allow fibroblasts to attach fully and spread to cover the entire dish before adding HFSCs (*see* **Note 4**).
14. Fibroblast feeder plates can be cultured for up to 1 week before adding HFSCs. F media should be changed every 3 days until usage.

3.2 Plating Freshly Isolated HFSCs

To plate HFSCs, a media switch from F media to E media must first occur.

1. Epidermal single cell suspension and sorting of HFSCs can be done using the detailed protocol in [6].
2. Prepare mitomycin C-treated J2-3T3 feeder layer by removing F media and then rinsing twice with 2 mL 1× PBS.
3. Add appropriate amount of E media to plate or well.
4. After HFSCs are obtained by FACS, count cells and add appropriate number of cells to each plate or well.
5. Swirl plate to mix.
6. Culture HFSCs at 35 °C in incubator with 5% CO_2 (*see* **Note 5**).
7. Replace E media every 3 days.
8. Colonies should become visible after ~7 days and then rapidly expand (*see* **Note 6**).

3.3 Passaging HFSCs

After ~2 weeks of culture and significant colony growth, HFSCs can be passaged onto a new mitomycin C-treated J2-3T3 fibroblast feeder layer.

1. Aspirate media. Add 2 mL versene and let sit for 2 min.
2. Vigorously pipette the versene with a P1000 to spray off all fibroblasts (*see* **Note 7**).
3. Aspirate versene, wash plate with 5 mL E media to remove residual fibroblasts, and then aspirate.

4. Add 2 mL 0.1% trypsin (no EDTA) and let incubate until cells start to lift up (about 10 min).
5. Add 8 mL E media to inactivate trypsin and spray to remove residual cells.
6. Put cells in a 15-mL conical vial and centrifuge to pellet at 1000 RPM for 5 min.
7. Aspirate media, resuspend pellet in 5 mL E media, and count cells.
8. Prepare mitomycin C-treated J2-3T3 feeder layer by removing F media and then rinsing twice with 2 mL 1× PBS.
9. Add appropriate amount of E media to plate or well.
10. Add appropriate number of HFSCs from step 7 onto mitomycin C-treated J2-3T3 feeder layer.
11. Culture HFSCs at 35 °C in incubator with 5% CO_2 (*see* **Note 5**).
12. Replace E media every 3 days.
13. Colonies should become visible after ~4 days and then rapidly expand (*see* **Note 8**).

3.4 Clonal Assay

Clonal assays involve plating a low number of cells (1000 cells/ cm^2) such that each colony is generated by a single cell. There is flexibility in terms of what plate size to use. Generally use of a 6-well plate is recommended for ease of generating technical replicates. Gridded plates are useful for tracking the same colony over time and are also ideal for live cell imaging (Sect. 3.5).

3.4.1 Clonal Growth Assay

1. Generate mitomycin C-treated J2-3T3 fibroblast feeder layer as described in Sect. 3.1 using 6-well plates.
2. Prepare mitomycin C-treated J2-3T3 fibroblast feeder layer for HFSC as described in Sect. 3.2.
3. Add 1000 cells/cm^2 of HFSCs (~9,500 cells for 6-well plate) to appropriate volume of E media (2–3 mL for 6-well plate) in 15-mL conical vial, gently mix, and then add to well (*see* **Note 9**).
4. Swirl plate to mix.
5. Culture HFSCs at 35 °C in incubator with 5% CO_2 (*see* **Note 5**).
6. Replace E media every 3 days.
7. Culture HFSCs for 2 weeks.
8. After 2 weeks of culture, aspirate media. Add 2 mL versene and let sit for 2 min.
9. Vigorously pipette the versene with a P1000 to spray off all fibroblasts (*see* **Note 7**).
10. Aspirate versene, wash plate with 2 mL 1× PBS to remove residual fibroblasts, and then aspirate.

11. Add 2 mL 0.5% crystal violet staining solution in a 1:1 solution of water:methanol and incubate for 30 min.
12. Remove staining solution and then rinse with deionized water until water goes clear.
13. Allow plates to dry and then image and count/measure colonies.

3.4.2 Tracking Individual Colonies Using Gridded Plates

1. Prepare mitomycin C-treated J2-3T3 fibroblast feeder layer using 60-mm gridded plates.
2. Add 1000 cells/cm^2 of HFSCs (~21,000 cells per 60-mm gridded plate) to appropriate volume of E media (3 mL per 60-mm gridded plate) in 15-mL conical vial, gently mix, and then add to pre-prepared feeder plate (*see* **Note 9**).
3. Swirl plate to mix.
4. Culture HFSCs at 35 °C in incubator with 5% CO_2 (*see* **Note 5**).
5. Replace E media every 3 days.
6. After 7 days of culture, go through grids and look for colonies to track, and note colony locations.
7. Image individual colonies every 24 h (Fig. 1).
8. End-point clonal analysis can also be done by completing steps 8–13 in Sect. 3.4.1.

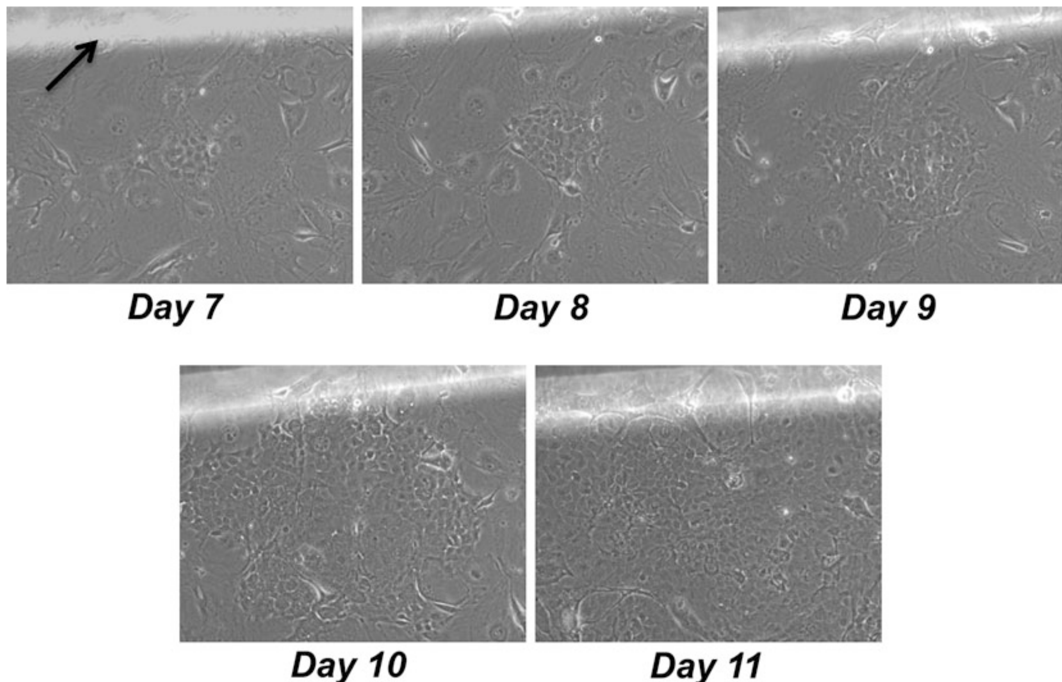

Fig. 1 Growth of a single HFSC colony over time. Note rapid expansion of colony size during the indicated time frame. Arrow indicates location of a grid on plate

3.5 Live Cell Imaging

Live cell imaging of HFSC colonies enables analysis of the migratory and proliferative behaviors of cells within the expanding colonies. We use the Keyence BZ-X700 live imaging system, which can utilize multiple tissue culture plate types.

1. Prepare cells for imaging by completing steps 1–6 in Sect. 3.4.2.
2. Consider starting live imaging at 7 days after plating of HFSCs as colonies should be visible at this time (*see* **Note 10**).
3. Adjust microscope setup to maintain temperature and CO_2 levels during live imaging.
4. Image each colony at 10× magnification every 15 min or less (*see* **Note 11**) for an 18-h duration.
5. Analysis of individual cells within colonies can be done using the "Manual Tracking" plugin in the ImageJ software from FIJI.
   (a) Carefully track individual cells through each image frame during the 18-h period to generate cell movement tracks with coordinates.
   (b) Set the original (X,Y) coordinate to the origin (0, 0), adjust to be in microns based on microscope's field of view, and then graph.
   (c) Consider calculating directionality and velocity of migration.
6. If desired, individual cell divisions can be counted by manually going through each frame.

3.6 Immunofluorescence

Immunofluorescence of HFSCs presents a challenge due to the technical complications of growing HFSCs on glass to allow for optimal colony growth, morphology, and imaging. We have assessed several commonly used glass-coating procedures, such as using poly-L-lysine, collagen I or IV, laminin, or fibronectin to coat glass coverslips before culturing, and found them all to yield less than ideal growth and morphology. Ultimately, co-culture using coverslips pre-seeded with a fibroblast feeder layer is the best at preserving HFSC viability and morphology for immunofluorescent imaging.

1. Generate working collagen solution (25 μg/mL in 0.02 M acetic acid) as per manufacturer's instructions. Filter through a low protein-binding filter to sterilize.
2. Cover surface of glass coverslips or glass chamber slides with working collagen solution for 1 h at room temperature in tissue culture hood.
3. Remove collagen and rinse three times with 1× PBS.
4. Coated coverslips/chamber slides can be store at 4 °C for up to a month if not immediately used.

5. Prepare mitomycin C-treated J2-3T3 fibroblast feeder layer as described in Sect. 3.2 using either chamber slides or glass coverslips. If using glass coverslips, place coverslips in 6-well plates, and add appropriate number of mitomycin C-treated J2-3T3 fibroblasts.
6. Add the needed volume of E media along with the desired number of HFSCs in 15-mL conical vial, gently mix, and then add to plate (*see* **Note 9** regarding media volume and cell density).
7. Culture HFSCs at 35 °C in incubator with 5% CO_2 (*see* **Note 5**).
8. Replace E media every 3 days.
9. Colonies become visible in approximately 7 days and fixation for immunofluorescence can be done when colonies reach the desired size.
10. Gently wash cells with 1× PBS, and then fix cells for 15 min in ice cold 4% paraformaldehyde.
11. Wash for 10 min with 1× PBS, and then twice in 1× PBS containing 0.1% Triton X-100 for 10 min each.
12. Block with 20% NGS-gelatin solution for 30 min at room temperature to overnight at 4 °C.
13. Incubate with primary antibody diluted in 10% (or lower, depending on the specific antibody used) NGS-gelatin solution overnight at 4 °C. Use of an anti-keratin 14 (K14) antibody helps to distinguish HFSC colonies from the fibroblast feeders (Fig. 2).

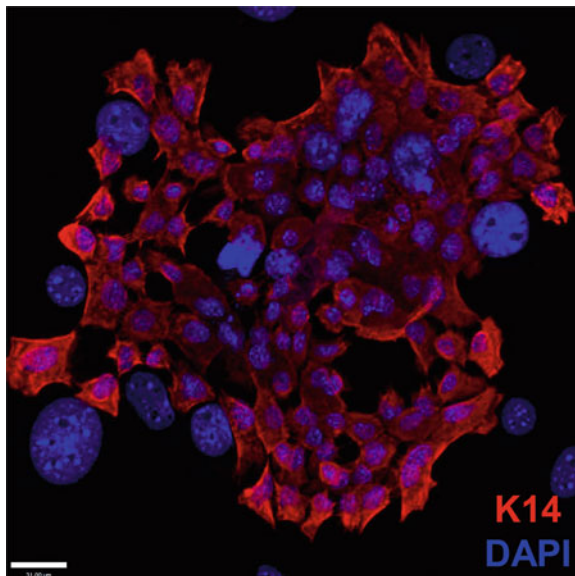

Fig. 2 Immunofluorescent staining for K14 expression in cultured HFSCs. DAPI stains the nuclei

14. Wash three times with 1× PBS for 10 min at room temperature, and then wash with 2% NGS-gelatin solution for 15 min at room temperature.
15. Incubate with secondary antibody diluted in 2% NGS-gelatin solution for 1 h at room temperature in the dark.
16. Wash three times with 1× PBS containing 0.1% Triton X-100 for 10 min at room temperature in the dark.
17. Stain with DAPI at a final concentration of 2 μg/mL in 1× PBS for 10–20 min in the dark.
18. Wash three times with 1× PBS for 5 min each.
19. Mount slides with Vectashield and seal edges with nail polish.
20. Image colonies using epifluorescence or confocal microscopy. Note that the HFSCs often push the fibroblast feeders away as the colonies expand, resulting in a single layer of cells. However, the HFSC colonies may begin to stratify if cultured for too long.

3.7 Adenoviral Infection of HFSCs

Viral infection of HFSCs enables manipulation of gene expression and functional characterization. We found it necessary to temporarily remove the mitomycin C-treated J2-3T3 fibroblast feeders in order to maximize the viral infection efficiency of HFSCs. In our experiments, we utilized commercially available adenoviruses that express GFP so infection efficiency can be estimated based on GFP expression (Fig. 3).

1. Prepare mitomycin C-treated J2-3T3 fibroblast feeder layer as described in Sect. 3.2.
2. Add 1000 cells/cm^2 of HFSCs (~9500 cells for 6-well plate) to appropriate volume of E media (2–3 mL for 6-well plate) in 15-mL conical vial, gently mix, and then add to well (*see* **Note 9**).
3. Swirl plate to mix.
4. Culture HFSCs at 35 °C in incubator with 5% CO_2 (*see* **Note 5**).
5. Replace E media every 3 days.
6. Culture HFSCs for 2 weeks so there are enough cells to infect.
7. Harvest HFSCs in a single well by following steps 1–7 in Sect. 3.3, and use for counting the number of cells (*see* **Note 12**).
8. To the remaining wells, add 2 mL versene and let sit for 2 min.
9. Vigorously pipette the versene solution with a P1000 to spray off all fibroblasts (*see* **Note** 7).
10. Aspirate versene, wash plate with 2 mL E media to remove residual fibroblasts, and then aspirate.
11. Mix appropriate amount of viruses based on cell count in step 7 to achieve multiplicity of infection (MOI) of 50 to 1 mL E media in a 1.5-mL Eppendorf tube and then carefully add to a well.

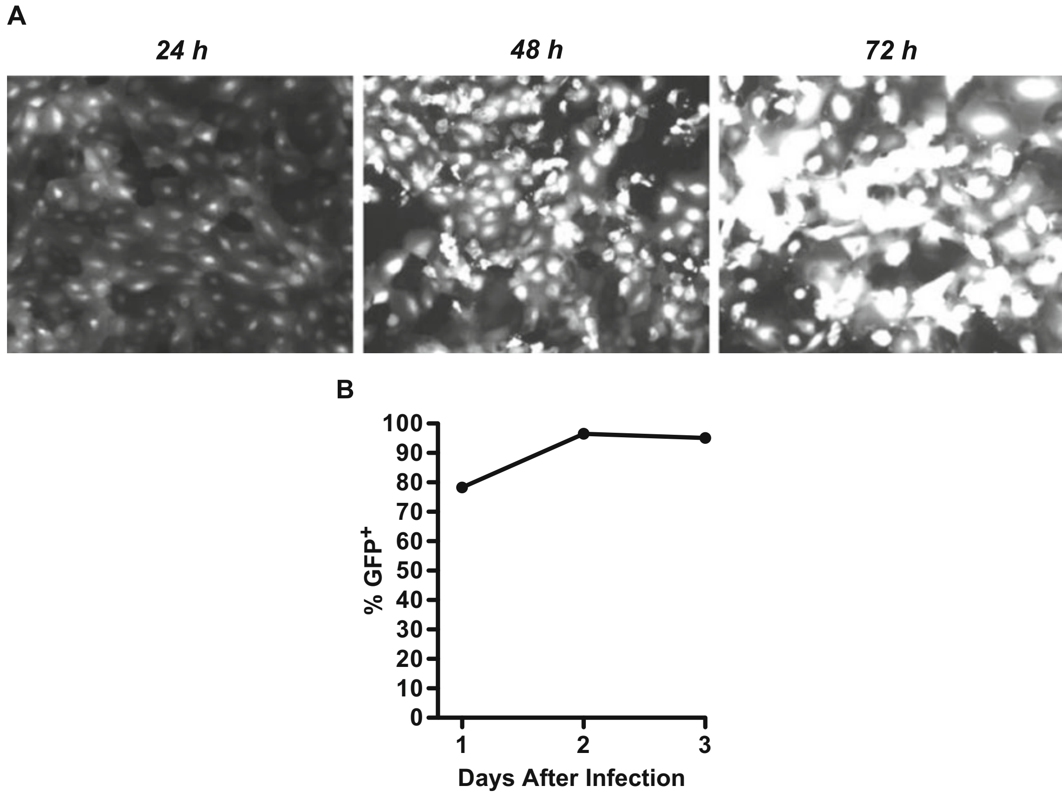

Fig. 3 GFP expression as a measure for efficiency of adenovirus infection in HFSCs. GFP protein was visualized by (**a**) fluorescence microscopy and (**b**) flow cytometry

12. Incubate cells with viruses overnight at 35 °C in incubator with 5% CO_2.
13. The next day, check cells under the microscope for GFP expression (Fig. 3).
14. Prepare mitomycin C-treated J2-3T3 fibroblast feeder layer as described in Sect. 3.2.
15. Remove the virus-containing media from HFSCs and then briefly rinse with 2 mL 1× PBS.
16. Add 1 mL 0.1% trypsin (no EDTA) and let incubate until cells start to lift up (about 10 min).
17. Add 4 mL E media to inactivate trypsin and spray to remove residual cells.
18. Put cells in a 15-mL conical vial and centrifuge at 1000 RPM for 5 min to pellet cells.
19. Aspirate media, resuspend pellet in 2 mL E media, and count cells.

20. Add 1000 cells/cm^2 of HFSCs (~9,500 cells for 6-well plate) to appropriate volume of E media (2–3 mL for 6-well plate) in 15-mL conical vial, gently mix, and then add to well (*see* **Notes 9** and **13**).
21. Culture HFSCs at 35 °C in incubator with 5% CO_2 (*see* **Note 5**).
22. Replace E media every 3 days.
23. Colonies should begin to be visible after ~4 days and then rapidly expand (*see* **Note 8**).
24. Proceed with downstream applications.

3.8 Patch Reconstitution Assay with Virally Infected HFSCs

The regenerative capacity of virally infected HFSCs can be assessed using a well-established "patch" assay [7], where HFSCs can be combined with newborn dermal cells and then subcutaneously injected into the backs of immunocompromised Nu/J mice. After 2 weeks, hair follicles form at the site of injection (Fig. 4).

1. Generate virally infected HFSCs by following steps in Sect. 3.7. Cells should have been growing for 2 weeks and ready for use once step 2 below is completed.
2. Isolate newborn primary dermal cells using the protocol from [7].
3. Harvest HFSCs by following steps 1–7 in Sect. 3.3.
4. Combine 100,000 infected HFSCs with 500,000 newborn dermal cells in 1.5-mL Eppendorf tube and centrifuge at 1000 RPM for 5 min to pellet the cells.
5. As dermal-only negative control, add 500,000 newborn dermal cells to 1.5-mL Eppendorf tubes, and centrifuge to pellet at 1000 RPM for 5 min (*see* **Note 14**).
6. Very carefully remove the media from the vials as to not dislodge the cell pellets.

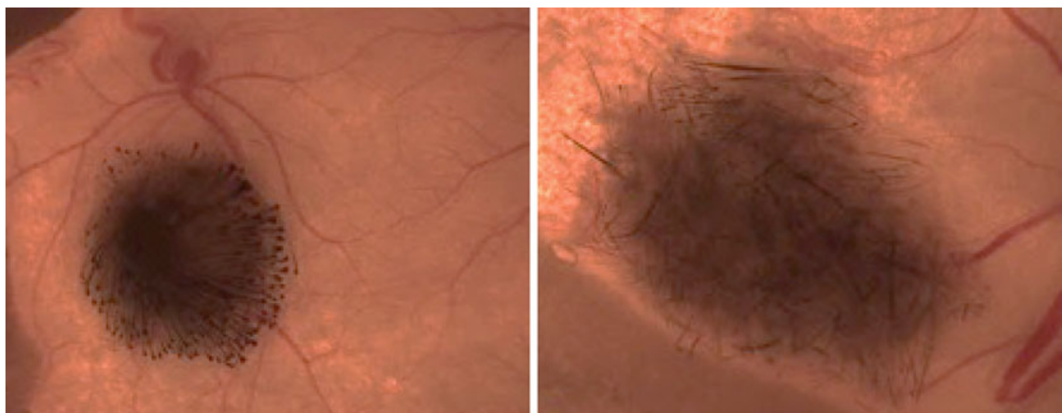

Fig. 4 Result of a "patch" assay using newborn keratinocytes (left) and infected HFSCs (right)

7. Add 50 μL of E media to each pellet and then place the cells on ice and bring to the appropriate mouse procedure room.
8. Follow approved mouse protocols to sedate Nu/J mice, and sterilize the outer skin surface surrounding the desired injection site.
9. Carefully resuspend the cells in each 1.5-mL Eppendorf tube, and then draw cell suspension into syringe with a 25G needle.
10. Carefully inject the cell suspensions into the backs of the Nu/J mice (see [7] for detailed injection instructions).
11. Return mice to their cages and follow approved post-operation procedures.
12. Sacrifice mice 2 weeks later to examine hair follicles at each of the injection sites.

4 Notes

1. It is critical to not use over-confluent J2-3T3 fibroblasts.
2. If not used immediately, add 10 mL F media to mitomycin C-treated J2-3T3 fibroblasts. Cells can be kept in the incubator for a week before use.
3. Even distribution is critical for optimal HFSC growth.
4. It generally takes ~2 days for mitomycin C-treated J2-3T3 fibroblasts to attach and spread over the entire dish. It is not recommended that HFSCs be added until the tissue culture plastic is fully covered.
5. After initial seeding of HFSCs, do not move plate within the first 72 h to allow the cells to attach to the feeders.
6. HFSCs grow as colonies. Colonies derived from HFSCs isolated from p49 mice (when their hair follicles are in telogen) become visible within a week and will begin to expand very rapidly after coming out of quiescence.
7. Feeders are easily removed and HFSCs will remain attached. HFSCs will begin to detach if treated for extended amount of time.
8. Passaged HFSCs form colonies faster than freshly sorted HFSCs.
9. Adding cells to the full volume of media in a conical vial followed by mixing facilitates even distribution of HFSCs in the well.
10. It is recommended that imaging be performed between 7 and 9 days. Generally, beyond 10 days after plating, the HFSC colonies become too large for an entire colony to be captured in a single field of view.

11. Imaging every 15 min or less is recommended to capture the dynamic cellular changes, as HFSCs expand fairly quickly at this time.
12. An extra well is used for counting cells before infection to calculate how much viruses to add to achieve an ideal MOI.
13. This plating density is good for a clonal assay. Different plating density may be desired per specific downstream assay.
14. A dermal-only control is necessary to gauge how much the dermal cells might be contaminated with newborn epidermal cells, which are regeneration-competent.

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