Chapter 18

Isolation, Characterization, and Differentiation of Human Multipotent Dermal Stem Cells

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Abstract

Skin, as the body's largest organ, has been extensively used to study adult stem cells. Most previous skin-related studies have focused on stem cells isolated from hair follicles and from keratinocytes. Here we present a protocol to isolate multipotent neural crest stem-like dermis-derived stem cells (termed dermal stem cells or DSCs) from human neonatal foreskins. DSCs grow like neural spheres in human embryonic stem cell medium and gain the ability to self-renew and differentiate into several cell lineages including melanocytes, neuronal cells, Schwann cells, smooth muscle cells, adipocytes, and chondrocytes. These cells express neural crest stem cell markers (NGFRp75 and nestin) as well as an embryonic stem cell marker (OCT4).

Key words Skin, Stem cells, 3-D skin reconstruct, Cell differentiation

1 Introduction

Adult stem cells are well known for their potential therapeutic value and their accessibility from patient-derived samples to avoid ethical problems. Skin contains somatic stem cells that generate keratinocyte, melanocyte, and mesenchymal cell lineages. These somatic stem cells have traditionally been thought to be restricted in their differentiation and regeneration potential to the tissues in which they reside (1). The advantage of skin stem cell research is that skin is a readily accessible organ from which to obtain a biopsy. Hair follicle stem cells and keratinocyte stem cells from human epidermis have been well characterized. Human induced pluripotent stem (iPS) cells can be generated from human dermal fibroblasts following upregulation of different transcription factors, including Oct3/4, Sox2, c-Myc, Klf4, Lin28, and Nanog, using retroviral and/or lentiviral vectors (2–4). However, some issues of iPS cells have been recently observed such as low efficiency of the reprogramming process, high number of retroviral insertions, and tumorigenesis by reactivation of the c-Myc transgene (5).

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The dermis is a source of progenitor cells and stem cells for multiple lineages. The dermal papilla from whisker follicles has been reported to be of neural crest origin and to harbor skin-derived precursor cells (SKP) (6). We have developed a protocol to isolate dermal stem cells (DSCs) from human neonatal foreskins. These DSCs grow like three-dimensional (3-D) spheres when cultured in human embryonic stem cell medium HESCM4. DSCs differ from SKPs that are also derived from human foreskins (7). The neural crest marker NGFRp75, which has been used as a marker for isolating a pure or enriched population of neural crest stem cells (8), was expressed only at low or undetectable levels in SKPs, whereas it is highly expressed in DSCs. DSCs also express another neural crest marker nestin and stem cell marker OCT4 (9). NGFRp75 and OCT4 double-positive cells can be found in human foreskin dermis by immunofluorescence staining. DSCs show a self-renewal ability and can be differentiated into melanocytes, neuronal cells, smooth muscle cells, adipocytes, chondrocytes, and Schwann cells. We also demonstrated that DSCs can migrate to the epidermis, localize at the basement membrane, and become melanocytic marker positive cells when incorporated in 3-D skin reconstructs cultured together with dermal fibroblasts and epidermal keratinocytes. These data suggest that DSCs represent a potential reservoir for epidermal melanocytes in human skin (10, 11). We describe here the protocol for the isolation and differentiation of DSCs. This technique does not require the introduction of genetic materials, is robust and easily reproducible.

2 Materials

2.1 Medium and Solution Preparation

1. *Foreskin transporting medium*: Dulbecco's modification of Eagle's medium (DMEM; Cellgro #10-017-CM) supplemented with gentamycin (100 μg/mL; Cellgro #30-005-CR). After sterilization through a 0.2 μm filter, the medium is transferred into sterile containers in 20 mL aliquots and stored at 4°C for up to 1 month.
2. *Dispase solution* (*0.48%*): Dispase (grade II, 0.5 U/mg; Boehringer Mannheim #165859) 0.48 g is dissolved in 100 mL phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Cellgro #MT21-031-CM). Sterilize the enzyme solution through a 0.2 μm filter, aliquot into 5 mL/tubes, and store at −20°C for up to 3 months.
3. *Collagenase solution* (*1 mg/mL*): Collagenase type IV (Invitrogen #17104-019) 100 mg is dissolved in 100 mL DMEM to yield a final concentration of 1 mg/mL. Sterilize the enzyme solution through a 0.2 μm filter, aliquot into 5 mL/tubes, and store at −20°C for up to 3 months.

4. *Mouse embryonic fibroblast* (*MEF*) *derivation medium*: The medium contains 87% DMEM (Invitrogen #11965-092), 10% defined FBS (Invitrogen #16000-044; heat inactivate for 30 min at 57°C), 1% 200 mM L-glutamine (Invitrogen #21051-024), 1% nonessential amino acids 100× (Invitrogen #11140) and 1% penicillin-streptomycin 100×.
5. *MEF growth medium*: MEF derivation medium without penicillin–streptomycin.
6. *Human embryonic stem cell medium* (HES): 78% DMEM/F-12 (Invitrogen #11330-032), 20% Knockout-Serum Replacer (Invitrogen #10828-028), 1% 100 mM L-glutamine + β-mercaptoethanol (Invitrogen #21051-024—add 7 μL β-mercaptoethanol to 10 mL L-glutamine), 1% nonessential amino acids 100× (Invitrogen #11140), 4 ng/mL basic fibroblast growth factor (bFGF; Fitzgerald Industries #30R-AF015).
7. *Human embryonic stem cell medium 4* (HESCM4): 70% MEF-conditioned HES medium and 30% HES medium, sterilize through a 0.2 μm filter.
8. *L-Wnt3a cell growth medium and conditioning medium*: 90% DMEM (Cellgro #10-017-CM), add 10% FBS and 0.4 μg/mL G418 (Sigma #G-8168). Conditioning medium including 99% DMEM and 1% FBS.
9. *Differentiation medium:*
   (a) For melanocyte differentiation (100 mL): 50 mL Wnt3a conditioned medium, 30 mL DMEM-Low Glucose (Invitrogen #11885), 20 mL MCDB201 (Sigma #M6770), 1× ITS Liquid Medium Supplement (Sigma #I-3146), 1 mg/mL linoleic acid-BSA (LA-BSA; Sigma #L-9530), 10^{-4} M L-ascorbic acid (Sigma #A-4403), 100 ng/mL stem cell factor (SCF; Fitzgerald Industries, #RDI-307-255X), 0.05 μM dexamethasone (Sigma #D-2915), 20 pM Cholera toxin (Sigma #C-3012), 50 nM TPA (Sigma #P-1583), 4 ng/mL bFGF (Fitzgerald Industries #30R-AF015), 100 nM endothelin-3 (ET-3; American Peptide Co. #88-5-10).
   (b) For neuronal cell differentiation (100 mL): (1) 60 mL DMEM, 30 mL F12 (GIBCO #11765), 10 mL FBS, 40 ng/mL bFGF. (2) 60 mL DMEM, 30 mL F12, 10 mL FBS, 10 ng/mL nerve growth factor (Millipore #GF028), 10 ng/mL brain-derived neurotrophic factor (Peprotech #450-02-10), 10 ng/mL NT-3 (Stem Cell Technologies #02508).
   (c) For smooth muscle cell differentiation (100 mL): 90 mL DMEM-F12 (GIBCO m#11330), 10 mL FBS, 0.1 M nonessential amino acids solution and 60 pM transforming growth factor-β1 (TGF-β1; R&D Systems #240B).

(d) For adipocyte differentiation (100 mL): 90 mL low-glucose DMEM (Sigma #D6046), 10 mL horse serum (Invitrogen #26050-070), 1× ITS, 1 mg/mL LA-BSA, 1 μM hydrocortisone (Sigma #H4001), 60 μM indomethacin (Sigma #I7378), 0.5 mM isobutylmethylxanthine (Sigma #I5879).

(e) For chondrocyte differentiation (100 mL): 90 mL high-glucose DMEM, 10 mL FBS, 1× ITS, 1 mg/mL LA-BSA, 50 nM dexamethasone, 60 pM TGF-β1.

(f) For Schwann cell differentiation (100 mL): (1) 60 mL DMEM, 30 mL F12 (GIBCO #11765), 10 mL FBS. (2) Medium I plus 4 μM foskolin (Sigma #F3917).

(g) Skin reconstruct medium: Basic medium: 400 mL Keratinocyte-SFM (Invitrogen #10724), 2% dialyzed FCS (Gibco LTI #16440-034), 60 ng/mL bovine pituitary extract (BPE; Invitrogen #13028-014), 4.5 ng/mL bFGF, 100 nM ET3 (American Peptide Co. #88-5-10), 10 μg/mL SCF (Fitzgerald Industries #RDI-307-255X). (1) Add 1 ng/mL EGF (Invitrogen #10450-013) to 100 mL basic medium; (2) Add 0.2 ng/mL EGF (Invitrogen #10450-013) to 100 mL basic medium; (3) Add 2.4 mM $CaCl_2$ (Sigma; #C-7902) to 200 mL basic medium.

2.2 Additional Reagents and Equipment

0.25% trypsin/EDTA (Cellgro #25-053-CI).

Minimal essential medium with Eagle salts (10× EMEM) (Lonza #12-684F).

Fetal bovine serum (FBS) (Hyclone #SH-30071.02).

Sodium bicarbonate (Cambrex #17-613E).

Bovine tendon acid-extracted collagen I (Organogenesis #200/50).

10% buffered formalin (Surgipath #00600).

Forceps (Roboz, #RS5070).

Scissors (Roboz, #RS5840).

Iris scissors (Roboz, #RS5913).

Surgical blades (Feather, #2976).

Cell strainers 100, 70, and 40 μm (Becton Dickinson #08-771-19; #08-771-2; #08-771-1).

Shaker (Taitec, microincubator M-36).

CO_2 incubator (CO_2 at 5% (vol/vol); humidified, $T = 37°C$).

Cytospin 2 (Shandon).

Microscope slides (Fisher Scientific #12-550-15).

4-well chamber slides (Fisher #12-565-21).

Tissue culture 6-well trays with transwell (Organogenesis #9285).

Multi cassettes (Surgipath, #02293-BX).

TBS biopsy papers (Triangle Biomedical Sciences, #BP-B).

2.3 Animals, Cells, and Sources

1. CF-1 female mice (day 13–14 gestation) for MEF derivation (Jackson Laboratory).
2. L-Wnt-3A cells (ATCC, #CRL-2647).
3. Human fibroblasts (from neonatal human foreskins).
4. Human keratinocytes (from neonatal human foreskins).

3 Methods

3.1 Generation of Conditioned HES Medium from Mouse Embryonic Fibroblasts

1. Sacrifice a female CF-1 mouse (at day 13 or day 14 of pregnancy).
2. Sterilize the abdomen with 70% ethanol.
3. Pull up the skin using forceps, separate the hide from the peritoneum, and cut a nick in the skin with scissors.
4. Cut the peritoneum to expose the abdominal cavity. Separate the uterine horns from the abdominal cavity with a blunt-point forceps and scissors. Transfer the uterine horns to a 100 mm cell culture dish that contains 10 mL DPBS without Ca^{2+} and Mg^{2+}.
5. Wash the uterine horns with 10 mL DPBS without Ca^{2+} and Mg^{2+} three times.
6. Tease open the uterine walls using two fine-pointed forceps or a scissors to release the embryos into the culture dish.
7. Separate each embryo from its placenta and surrounding membranes.
8. Transfer the embryos to a new culture dish using forceps one by one and wash them three times using 10 mL DPBS without Ca^{2+} and Mg^{2+}.
9. Cut away brain and dark red organs from each embryo using a fine-tipped forceps, individually (see Note 1).
10. Wash the embryos three times with 10 mL DPBS without Ca^{2+} and Mg^{2+}, change to a new dish every time.
11. Remove the DPBS and add 2 mL 0.25% trypsin/EDTA solution to the washed embryos.
12. Mince each embryo finely using curved Iris scissors; add 5 mL 0.25% trypsin/EDTA solution.
13. Leave the dish on a shaker at 37°C for 20–30 min until individual cells are visible under the microscope.
14. Add 20 mL MEF derivation media to the plate after the incubation. Transfer the cells with media to a 50 mL tube.

15. Add 3 mL to rinse the remaining tissue in the plate. Transfer to the 50 mL tube. Mix by pipetting a few times and wait for 1 min to allow the debris in the suspension to settle to the bottom of the tube.
16. Transfer the top 18 mL of the suspension containing the individualized cells into a new tube and spin down, resuspend the pellet in MEF derivation medium and divide into T75 flasks (three embryos/flask).
17. Spin down the remaining 12 mL with the debris, resuspend with MEF derivation medium and add into one T75 flask.
18. Bring the final volume to 20 mL/flask by adding additional MEF derivation medium.
19. Incubate the flasks in a humidified incubator at 5% CO_2 and 37°C for 2–3 days, until 80–90% confluent. At this time, the MEF are ready to be harvested and passaged.
20. MEF cells are split at 1:4 into 0.1% gelatin (Sigma #G1890) coated T75 flasks with MEF growth medium and grow to reach 80% confluence.
21. Aspirate the MEF growth medium. Add 20 mL HES medium to each flask and incubate 24 h.
22. Collect the conditioned medium, add 20 mL fresh HES medium, and incubate another 24 h. Collect the conditioned medium and mix with the day 1 conditioned medium, store at −70°C. This is the HES conditioned medium.

3.2 Generation of L-Wnt3a-Conditioned Medium

1. L-Wnt3a cells are cultured in L-Wnt3a growth medium.
2. Split 1:10 into 100 mm culture dishes when cells reach confluence, add 10 mL L-Wnt3a conditioning medium in each dish. Incubate cells 4 days at 37°C and 5% CO_2.
3. Collect the medium after 4 days and filter sterilize. This is Batch 1.
4. Culture the cells for another 3 days by adding 10 mL fresh L-Wnt3a conditioning medium.
5. Collect the medium and sterile filter. This is Batch 2. Discard the cells.
6. Mix Batch 1 and Batch 2 medium at a 1:1 ratio. This is the L-Wnt3a conditioned medium.

3.3 Dermal Stem Cell Culture

Day 1

1. Take out a foreskin from the transfer tube; rinse it with 70% ethanol for 1 min (see Note 2).
2. Transfer the foreskin to a sterile 100 mm culture dish, add 20 mL HBSS without Ca^{2+} and Mg^{2+}, wait for 2 min (see Note 3).

3. Open the foreskin ring with scissors and cut the foreskin into several pieces of approximately 5×5 mm^2 using a surgical scalpel blade (see Note 4).
4. Transfer the skin pieces into a 50 mL Falcon tube with 5 mL 0.48% dispase II, and incubate at 4°C overnight.

Day 2

1. Remove the Falcon tube containing the skin sample from 4°C and incubate it at 37°C for 5 min.
2. Pour the skin pieces with the dispase into a sterile 10 cm culture dish, transfer all skin pieces to a new dish using forceps.
3. Separate the epidermis from the dermis by holding the dermal part of each skin piece with one pair of forceps and gently remove the epidermal part with a surgical scalpel blade. Discard the epidermis. Repeat the procedure for each piece of skin.
4. Transfer all dermal pieces to a new dish and mince them as small as possible (see Note 5).
5. Collect the minced dermal pieces using a pipette and transfer to a 50 mL Falcon tube containing 2 mL 1 mg/mL collagenase IV. Incubate at room temperature for 24 h (see Note 6).

Day 3

1. Add 25 mL HBSS without Ca^{2+} and Mg^{2+} to the tube containing the dermis with collagenase IV. Mix well by pipetting up and down; serially filter through 100, 70, and 40 cell strainers (see Note 7).
2. Centrifuge the cell suspension at $200 \times g$ for 5 min.
3. Resuspend the cell pellet in 10 mL HESCM4 medium and seed the cells in two T25 flasks (see Note 8).
4. Put the flasks into an incubator with 5% CO_2 at 37°C.
5. After 48 h, aspirate 2.5 mL medium from the flask, and replace with 2.5 mL fresh HESCM4 medium. Change half the volume of the medium every 3–4 days.

3.4 Dermal Stem Cell Differentiation to Melanocytes, Neuronal Cells, Schwann Cells, Smooth Muscle Cells, Adipocytes, and Chondrocytes

1. Tissue culture-grade 4-well chamber slides (Becton Dickinson) are precoated with: 0.5 mL/well 10 ng/mL fibronectin (Advanced Biomatrix #5050; for melanocyte, chondrocyte, and adipocyte differentiation), 0.1% matrigel (BD Biosciences #354234; for neuronal and smooth muscle cell differentiation), and a mixture of 20 μg/mL laminin (BD Biosciences #354232) with 200 μg/mL poly-D-lysine (BD Biosciences #354210) (for Schwann cell differentiation) (see Note 9).
2. Collect dermal spheres and transfer into a 50 mL tube, spin down, remove the supernatant as much as possible.
3. Add 0.5 mL 1 mg/mL collagenase IV and 0.5 mL 0.25% trypsin/EDTA. Incubate at 37°C for 5 min.

4. Pipette up and down for 1 min, then add 9 mL soybean trypsin inhibitor. Spin down. Resuspend the sphere cell pellet in the various differentiation media and seed the cells in coated chamber slides (see Note 10).
5. For neuronal and Schwann cell differentiation, use medium I during the first week and switch to medium II during the second week.
6. Incubate for 2–3 weeks, replace 1/2 fresh medium twice a week. Cells are ready to fix for staining using differentiation markers.

3.5 DSCs Differentiation to Epidermal Melanocytes in Three-Dimensional Skin Reconstruct Culture

1. Coat the transwell (Organogenesis) with the collagen mixture: 0.59 mL 10× minimal essential medium (EMEM), 50 μL 200 mM L-glutamine, 0.6 mL FBS, 120 μL 7.5% sodium bicarbonate, 4.6 mL bovine collagen I and mix well. Add 1 mL of the mixture into one transwell of tissue culture trays.
2. Collect dermal spheres by centrifugation and resuspend 6,600 dermal spheres in 0.75 mL HESCM4 medium (see Note 11).
3. Trypsinize fibroblasts and collect cells by centrifugation and resuspend 0.45×10^6 cells in 0.75 mL skin reconstruct medium I.
4. Mix the following reagents in a 50 mL tube: 1.65 mL 10× MEM, 150 μL 200 mM L-glutamine, 1.85 mL FBS, 350 μL 7.5% sodium bicarbonate, 14 mL bovine collagen I, 0.75 mL dermal spheres from step 2, 0.75 mL fibroblasts suspension from step 3 and mix well. Add 3 mL to each coated transwell. Incubate for 45 min at 37°C in a 5% CO_2 tissue culture incubator. Add skin reconstruct medium I (2 mL inside and 10 mL outside of the transwell). Incubate for 4 days.
5. Harvest human keratinocytes, resuspend 3×10^6 cells in 600 μL skin reconstruct medium I.
6. Remove the skin reconstruct tray from the incubator, aspirate medium from both inside and outside of transwells.
7. Add skin reconstruct medium I (1.5 mL inside and 10 mL outside of insert). Drop 100 μL keratinocyte suspension to each inside transwell. Incubate for 2 days.
8. Remove skin reconstruct medium I from both inside and outside of transwells. Add skin reconstruct medium II (2 mL inside and 10 mL outside). Incubate for another 2 days.
9. Aspirate skin reconstruct medium II both inside and outside of transwells, add 7.5 mL skin reconstruct medium III to only the outside of the transwells (see Note 12). Change medium III every other day until day 18.
10. Harvest the skin reconstruct at day 18: Remove the transwell from the tray with forceps. Cut out the reconstruct (including

the polycarbonate filter) by tracing a circle close to the edge with a scalpel blade. Place the reconstruct in a histology cassette (Surgipath #02275-BX) between two black TBS biopsy papers (Triangle Biomedical Sciences, #BP-B) and soak the whole cassette in 10% formalin (Fisher Healthcare #245-685) for 4–6 h. Then place the cassette in 70% for paraffin embedding.

3.6 Immunostaining

For spheres:

1. Cytospin the spheres on a microscope slide using a Cytospin 2 (Shandon) (see Note 13).
2. Dry 1 h at room temperature.
3. Fix with acetone for 10 min at 4°C.
4. Wash three times with 1× PBS without Ca^{2+} and Mg^{2+}.
5. Block 30 min with 3% BSA (MP Biomedicals #810034).
6. Remove blocking solution and add primary antibodies diluted in 1× PBS without Ca^{2+} and Mg^{2+}. Incubate overnight at 4°C.
7. Remove the primary antibody solution and wash three times with 1× PBS without Ca^{2+} and Mg^{2+}.
8. Incubate slides in secondary antibodies for 45 min at room temperature in the dark.
9. Wash twice with 1× PBS without Ca^{2+} and Mg^{2+}.
10. Mount with Vectashield mounting medium for fluorescence with DAPI (Vector #H-1200) and coverslip (Fig. 1).

For monolayer differentiated cells:

1. Fix the differentiated cells in chamber slides with 4% paraformaldehyde for 20 min.
2. Wash slides three times with 1× PBS.
3. Permeabilize cells using 0.5% Triton X-100 (Sigma #T9284) for 5 min.
4. Repeat steps 4–10 for sphere staining.
5. To detect differentiated adipocytes, fix the differentiated cells with 4% paraformaldehyde, and cover them with Oil Red O solution for 16 min. After washing slides with isopropanol, stain nuclei with hematoxylin solution.

For paraffin embedded skin reconstructs:

1. Deparaffinize in xylene twice for 10 min.
2. Redehydrate in 100, 100, 95, 70, and 50% ethanol for 2 min.
3. Wash three times with 1× PBS without Ca^{2+} and Mg^{2+}.
4. Antigen retrieve with trypsin.
5. Repeat steps 4–10 for monolayer cells (Fig. 2).

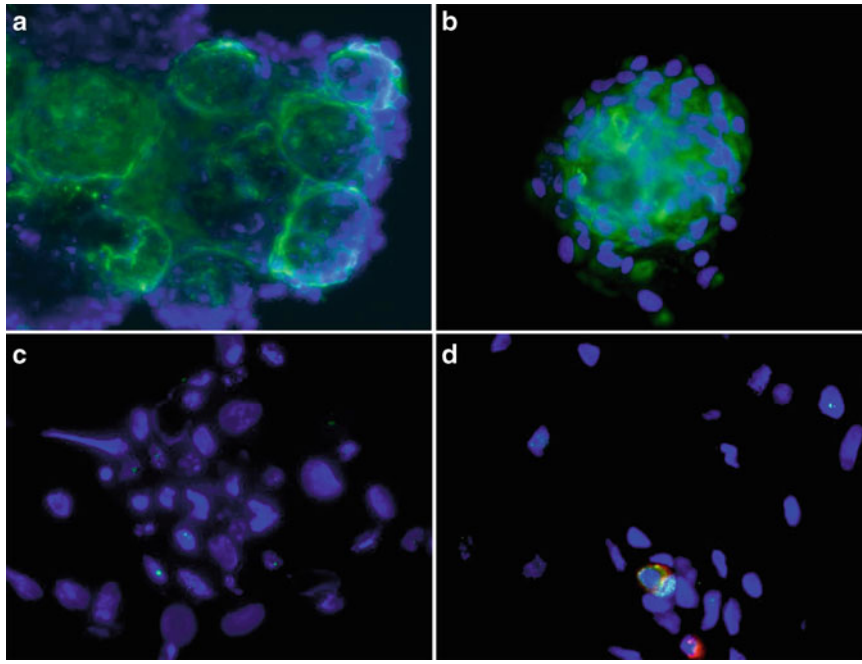

Fig. 1 Dermal spheres express stem cell markers. (**a**) Multiple dermal spheres are positive for the neural stem cell marker NGFRp75 (*green*). (**b**) A dermal sphere expressing nestin (*green*). (**c**) Embryonic stem cell marker OCT4 (*green*) expression is typically localized to nuclei. (**d**) Paraffin embedded foreskin stained with antibodies to NGFRp75 (*red*) and OCT4 (*green*). Nuclei stained with DAPI (*blue*)

4 Notes

1. Remove as much blood and organ tissue as possible to avoid contamination with other cells and to keep the pure MEF population.
2. Foreskins from the hospital are not always sterile. Use 70% ethanol to clean each foreskin to avoid contamination.
3. The purpose for this step is to completely wash out the ethanol.
4. Small size for easy enzymatic digestion to separate the epidermis from the dermis.
5. The smaller the skin is minced, the more single cells will be recovered after digestion.
6. Shake several times during this incubation for better digestion.
7. Pipet 60–80 times for better release of single cells from tissue clumps and filter with a cell strainer to remove clumps of unbroken cells and connective tissue.
8. Each T25 flask contains 3×10^6 to 4×10^6 cells in 5 mL HESCM4 medium.

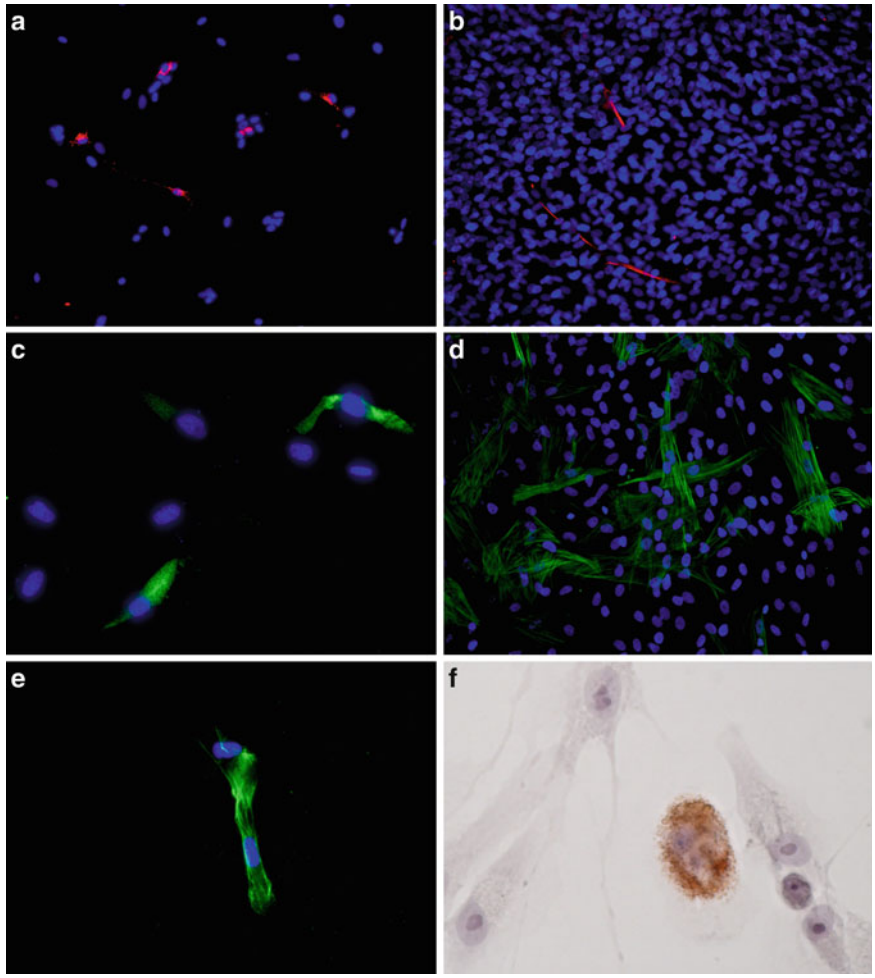

Fig. 2 DSCs can be differentiated into several cell lineages. Dermal spheres cultured in differentiation medium for 2 weeks and then stained with lineage specific antibodies. (**a**) Melanocytic differentiation is performed in melanocyte differentiation medium. Immunofluorescent staining show pigmentation marker HMB45-positive cells (*red*). (**b**) Neural differentiated cells become immunoreactive to β3 tubulin (*red*). (**c**) CNPase is used to detect differentiated Schwann cells (*green*). (**d**) Differentiated smooth muscle cells are positive for smooth muscle actin (*green*). (**e**) Differentiated chondrocytes express collagen II (*green*). (**f**) Oil-red-O staining detects a differentiated adipocyte (*red*). Nuclei stained with DAPI (*blue*, (**a**)–(**e**)) or hematoxylin (**f**)

9. Add 0.5 mL coating solution (fibronectin, or Matrigel or a mixture of laminin with poly-D-lysine), incubate at 37°C overnight before use.
10. Spheres cannot be completely dissociated. Seed single cells and spheres together in chamber slides. Aspirate coating solution from the chamber slides before seeding cells.
11. The dermal spheres are easily detached by tapping the flasks since most spheres have a slightly lower adherence to the

culture flasks. Mix well, pipet 100 μL to one well of a 96-well plate, settle for a few minutes and count using a microscope.

12. Air-lift the epidermis: Add skin reconstruct medium III only outside of the insert to expose the epidermis in air. This step induces keratinocyte differentiation to form a thick epidermis.
13. Collect spheres with medium in a 50 mL tube, wait 5 min to let the spheres settle down to the bottom of the tube, discard the top 2/3 medium which contains single dead cells. Spin down the tube with spheres and wash the sphere pellet with DPBS without Ca^{2+} and Mg^{2+}. Resuspend spheres in 1 mL DPBS without Ca^{2+} and Mg^{2+}. Drop 200 μL of the sphere suspension to each cytospin chamber with a microscope slide and spin at 800 rpm for 3 min.

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