

Chapter 2

Isolation and Cultivation of Human Scalp Interfollicular Epidermal Stem Cells

Longmei Zhao and Basil M. Hantash

Abstract

Skin regeneration is intricately controlled by epidermal stem cells. In human skin, the long-lived, slow-cycling, and highly proliferative stem cells are located in the basal layer of the interfollicular epidermis (IFE). The ability to isolate and culture human IFE stem cells (IFESCs) offers fascinating therapeutic potential for skin diseases as well as epithelial tissue engineering. Here we describe a straightforward strategy for generation of $\beta 1$ integrin⁺/CD24⁻ IFESCs from scalp with defined, serum-free, feeder-free medium and collagen I-coated culture plates. The use of defined media throughout isolation and cultivation allows for detailed investigation of the molecular events involved in ESC self-renewal and differentiation as well as provides a safe source for clinical use.

Key words Isolation, Cultivation, Epidermal stem cells, Human scalp, Serum free, Feeder free

1 Introduction

The epidermis forms the outer protective barrier of the body and regenerates throughout life. Skin regeneration is intricately controlled by epidermal stem cells (ESCs) (1), defined here as cells possessing the ability to self-renew—generation of daughter cells capable of undergoing single- or multi-lineage terminal differentiation (2). In human skin, the long-lived, slow-cycling, and highly proliferative stem cell compartment is located in the basal layer of the interfollicular epidermis (IFE) (3–5). Although evidence suggests that IFE, hair follicles, and sebaceous glands are all maintained by their own distinct stem cell compartments (6–10), IFE stem cells (IFESCs) possess the capacity to differentiate into all epidermal lineages. In addition, IFESCs participate in repair of skin after injury (6–9). The ability to isolate and culture human IFESCs offers fascinating therapeutic potential for skin diseases as well as epithelial tissue engineering.

Techniques to isolate and culture keratinocyte progenitor cells have been established, although limitations remain in many of

these procedures. In particular, many existing lines have been cultured using mouse embryonic fibroblasts (NIH 3T3) as the feeder layer and/or serum-sourced medium components. NIH 3T3 cells support keratinocyte progenitor cell growth from single cells into colonies while suppressing growth of human fibroblasts (11–13). However, continued use of feeders and serum containing culture medium will hinder the development of clinical applications by increasing the risk of transmitting animal viruses and immunogenic antigens as well as pose difficulty with quality control of these undefined components.

Keratinocyte progenitor cells rapidly adhere to collagen-coated dishes (12, 14). Low calcium medium appears to be effective for expanding proliferative keratinocytes while high calcium medium allows these cells to terminally differentiate (15). Cultivation of keratinocyte progenitor cells in serum-free medium led to enhanced expression of $\beta 1$ integrin, an ESC marker, relative to those cultured in serum containing medium (16). The method described here involves cultivation of IFESCs on collagen I-coated plates with defined serum-free medium containing low calcium in order to generate purified IFESCs from human scalp skin. This highly efficient process enables direct generation of keratinocyte progenitors from human skin tissue for potential use in research purposes and clinical applications.

2 Materials

2.1 Tissue

1. Human scalp samples were obtained from facelift procedures with Institutional Review Board approval.

2.2 Reagents and Medium

1. William's E serum-free medium (Life Technologies).
2. Progenitor cell-targeted (PCT) keratinocyte medium (Chemicon, CnT-07).
3. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen).
4. Phosphate buffered saline (PBS), without Ca^{2+} or Mg^{2+} , pH 7.4 (Invitrogen).
5. 0.25 and 0.05% trypsin-EDTA (Invitrogen).
6. Trypsin neutralizer solution (Invitrogen).
7. 0.4% trypan blue (Invitrogen).
8. Dispase (Invitrogen). Reconstituted in DMEM at 0.125 mg/ml. Aliquot could be stored at -20°C up to 6 months. If stored at 4°C , use within 2 weeks.
9. 10,000 U/ml penicillin and 10,000 mg/ml streptomycin (Invitrogen).
10. 12.5 $\mu\text{g}/\text{ml}$ fungazone (amphotericin B) (Invitrogen).

11. Recombinant human collagen I (Fibrogen, RhC1-003). Dissolve 100 mg of recombinant human collagen I in 2 ml of filter-sterilized 10 mM HCl. Aliquot could be stored at 4°C up to 6 months. When coating plates, further dilute to 25 mg/ml with sterile PBS and use 10 mg per 1 cm² dish area. Leave freshly coated plates for at least 1 h at room temperature. The coated plates could be stored at 4°C up to 2 weeks.
12. Fixation buffer: To make 8% (w/v) paraformaldehyde (PFA), dissolve 8 mg of PFA (Sigma) in 100 ml of PBS, heat the solution to 60°C and stir for at least 30 min, add a few drop of 10 M NaOH to completely dissolve PFA. Store at -20°C. Dilute to 4% (w/v) in PBS prior to use.
13. Blocking buffer: To make 10% (v/v) goat serum solution, add 1 ml of normal goat serum (Vector Laboratories) in 9 ml of PBS, store at 4°C.
14. Primary antibodies (recommended dilution): mouse anti- β 1 integrin monoclonal antibody (1:100, BD Biosciences), mouse anti-CD24 monoclonal antibody (1:100, BD Biosciences).
15. Preparation of ABC complex by adding 50 ml of avidin (Vector Laboratories, PK6102) and 50 ml of avidin-horseradish peroxidase (HRP) (Vector Laboratories, PK6102) in 10 ml of PBS containing 0.1% tween 20, mix immediately and allow to stand for 30 min ahead of application.
16. Prepare 3,3'-diaminobenzidine (DAB) solution by adding 30 ml of the DAB liquid chromogen solution (Sigma, D3939) to 1 ml of the DAB liquid buffer solution (Sigma, D3939). Use immediately.
17. Mayer's hematoxylin solution (1 \times , Sigma, MHS1).
18. Crystal mount aqueous mounting medium (Sigma, C0612).

3 Methods

The method described here enables production of high-purity ESCs from scalp skin. Initial cultures must be pure as fibroblast-like cells proliferate much faster than keratinocytes thereby dominating the latter in culture. IF ESCs immunostain positively for β 1 integrin (12) and negatively for CD24 (17) markers. Given use of defined and feeder-free culture media throughout isolation and cultivation, this system provides a unique means of investigating the molecular events involved in ESC self-renewal and differentiation.

3.1 Isolation of Human IF ESCs from Scalp Skin

The following procedures are performed in biosafety hoods. Investigators should be trained in the handling of human tissue and human pathogens before initiation of any studies.

1. Human scalp skin is placed in sterile William's E serum-free medium supplemented with penicillin (250 U/ml), streptomycin (250 mg/ml), and fungazone (1.25 mg/ml) immediately after surgery and stored at 4°C until use (see Note 1).
2. Rinse scalp skin several times in sterile PBS with constant agitation to remove clots.
3. Transfer scalp skin to a 100 mm petri dish and trim hairs with a sterile surgical scissor. While turning the dermal side upward, remove as much connective tissue (muscle and fat) as possible with a sterile scalpel and forceps and then cut trimmed scalp skin into pieces (~1 cm² in area).
4. Transfer small pieces of scalp skin into a 100 mm petri dish containing 10 ml of 0.125% dispase/DMEM and incubate for 16–18 h at 4°C (see Note 2).
5. Carefully transfer scalp skin to a 100-mm petri dish by turning the epidermal side upward. Gently scrape epidermis away from the dermis using a scalpel and Pasteur pipette (see Note 3).
6. Collect the epidermis into a sterile 50 ml conical tube containing 10 ml of prewarmed 0.25% trypsin-EDTA and trypsinize for 20–30 min at 37°C.
7. Gently shake the tube several times to release the trypsinized cells into solution (see Note 4).
8. Gently pipette cells with 2 ml of pipette and add an equal amount of trypsin neutralizer solution. Centrifuge at 800 rpm for 7 min.
9. Remove the supernatant by vacuum aspiration and resuspend the cells with 1 ml of prewarmed PCT keratinocyte medium.
10. Place 10 ml of cell aliquot into a 500-ml Eppendorf tube and add an equal volume of trypan blue solution, mix and leave at room temperature for 5 min. Determine the number of cells with a hemocytometer and eliminate dead cells from this count.
11. Dilute the cell suspension in an appropriate volume and plate the cells at 1×10^4 cell/cm² in collagen I-coated flasks with PCT medium containing 0.07 mM calcium (see Note 5) and incubate at 37°C in a humidified 5% CO₂ incubator (see Note 6).
12. Approximately 2 days later (see Note 7), remove the media and nonadherent cells and replace with fresh PCT medium containing 0.07 mM calcium. Change medium every other day.
13. After 10–14 days, tightly packed epithelial colonies should be observed (Fig. 1; see Note 8).

3.2 Subculture Human IFSCs

All solution and medium need be warmed to 37°C before use.

1. Aspirate off the culture medium in a T-75 flask with cells at nearly 80% confluency (see Note 9). Add 5 ml of prewarmed

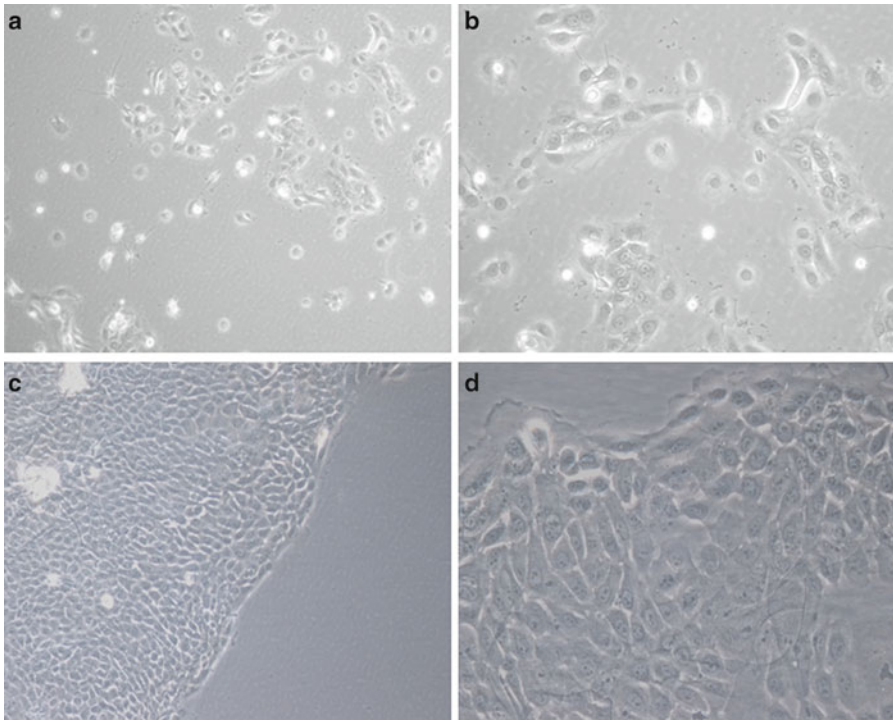


Fig. 1 (a, b) human IFESCs 3 days after plating. (c, d) Human IFESCs 10 days after plating. (a, c) 100× original magnification. (b, d) 200× original magnification

PBS, gently tilting the flask back and forth several times, then aspirate off PBS.

2. Add 3 ml 0.05% trypsin/EDTA solution per T-75 flask and ensure proper distribution of solution in the flask.
3. Incubate cells for 5–10 min at 37°C in a humidified incubator (see Note 10).
4. Gently tap the flask to dislodge the cells from the surface of the flask, and then add 7 ml of trypsin neutralizer solution per T-75 flask. Wash the flask by pipetting a few times over the cell attachment area (see Note 11).
5. Transfer detached cells to a sterile 15 ml conical tube.
6. Centrifuge cells at $180\times g$ (or about 1,000 rpm) for 7 min.
7. Aspirate supernatant without dislodging the pellet. Resuspend the cell pellet in an appropriate volume of culture medium and plate the cells at $1\times 10^4/\text{cm}^2$ in collagen I-coated flasks (see Note 12) with PCT medium containing 0.07 mM calcium (Fig. 2).

3.3 Immuno-cytochemistry

1. Plate the cells at 1×10^4 cell/ cm^2 in collagen I-coated chamber slides and culture at 37°C in a humidified 5% CO_2 incubator for a few days.

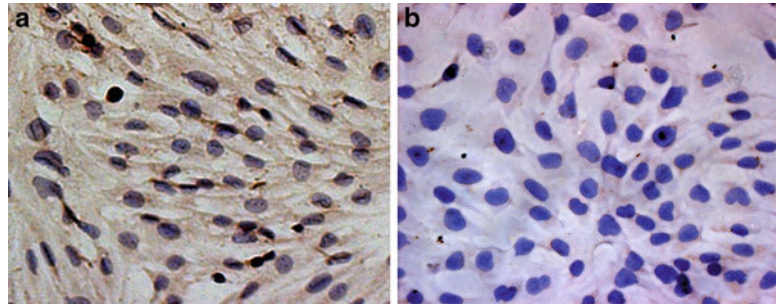


Fig. 2 Immunostaining of primary passage 1 IFESCs cultured for 14 days. (a), More than 90% of cells are positive for $\beta 1$ integrin staining. (b) IFESCs are negative for CD24 staining

2. Aspirate the culture medium from each well and gently rinse the cells twice with PBS at room temperature (see Note 13).
3. Fix cells by incubating them in 4% (v/v) PFA in PBS for 20 min at room temperature.
4. Rinse cells three times with PBS (see Note 14).
5. Incubate cells with 1:100 diluted H_2O_2 (30%) for 10 min at room temperature (see Note 15).
6. Rinse cells three times with PBS.
7. Block cells with 10% normal goat serum for 30 min at room temperature.
8. Incubate cells with primary antibodies in fresh blocking buffer at the appropriate dilution (see Note 16) overnight at 4°C or for 2 h at room temperature.
9. Wash cells three times with PBS.
10. Incubate cells with biotinylated secondary antibodies (see Note 17) at a dilution of 1:200 in fresh blocking buffer for 30–60 min at room temperature.
11. Wash cells three times with PBS.
12. Incubate cells with the ABC complex solution for 30 min at room temperature.
13. Drop off the ABC complex solution and cover cells with DAB reagent solution.
14. Monitor carefully under light microscopy during the reaction to prevent overdevelopment and high background (see Note 18).
15. Stop the reaction when the color is satisfactory by dropping off the DAB reagent solution and covering cells with PBS.
16. Rinse cells three times with PBS.
17. Incubate cells for 5 min with Mayer's hematoxylin solution.
18. Rinse cells three times with PBS.
19. Mount slide with mounting medium.

4 Notes

1. For maximum viability, skin tissue should be processed as soon as possible after surgery. However, when necessary tissue can be kept at 4°C for a few days without significant loss of viability. The yield of stem cells per cm³ skin is affected by the age and health of donor. Younger donors generate more stem cells than older ones.
2. Use enough dispase medium to completely cover tissue. To obtain optimal digestion, tissue should be evenly distributed in dispase medium.
3. The epidermis should peel away quite easily. Take care not to take dermis with the epidermis. This is a critical step to obtain pure populations of epidermal cells. If the epidermis does not peel easily, the tissue should be incubated for up to several hours at 37°C or placed in fresh dispase solution and incubated for an additional 24 h at 4°C. The pieces of peeled epidermis should be kept in calcium- and magnesium-free Hanks' balanced salt solution during this procedure.
4. If the epidermis is not completely digested, keep the tube at room temperature for several minutes to allow the epidermal pieces to float to the surface while cellular debris settles to the bottom of the tube. Using sterile forceps, remove the undigested epidermis into a new 50 ml sterile conical tube. To obtain maximum keratinocyte yield, undigested pieces of epidermis could be incubated in prewarmed trypsin solution. The process may be repeated up to three times. Cells harvested in this way are pooled and counted.
5. PCT medium is defined, serum-free, feeder-free media for superior isolation and growth of progenitor cells. Keratinocyte progenitor cells are selected by using low calcium conditions and avoiding use of fetal calf serum in the culture medium.
6. Ensure proper distribution of cells by quickly moving the flask back and forth several times. Avoid opening the incubator door after plating.
7. There may not be many adherent cells at 24 h. Wait for at least 2 days to allow cells to settle down to the bottom of the dish.
8. At this time point, cells may require daily feeding.
9. Split cells when the culture reaches 60–80% confluency. Avoid splitting cells at more than 80% confluency as cells tend to undergo differentiation when crowded in culture.
10. Incubate the flasks at room temperature until cells have become completely round which usually takes approximately 10–15 min.
11. Sometimes fibroblast-like cells are observed contaminating the culture. To eliminate these contaminants, use two-step enzyme

selection. After washing with PBS, incubate cells with 0.05% trypsin/EDTA at room temperature for 5–10 min, then collect the solution and discard. Incubate cells with fresh 0.05% trypsin/EDTA at room temperature for another 5–10 min and collect cells into a 15-ml sterile conical tube and spin down. If only a few keratinocyte colonies are present in culture, place 10 mm outer diameter borosilicate glass cloning cylinders over regions containing a high percentage of cells with an epithelial morphology and harvest cells from one or more cylinders by trypsinization.

12. A variety of coating matrices may be used to subculture ESCs including collagen I and collagen IV. Precoated culture plates/flasks are also available commercially.
13. Do not let cells dry out during immunostaining.
14. Cells can be stored in 0.02% (w/v) sodium azide in PBS at 4°C for several days after this step.
15. Blocking of endogenous peroxidase can be performed after primary antibody incubation.
16. The researcher should test serial concentration of primary antibody to determine the optimal concentration.
17. Be sure that the correct isotype specific secondary antibody for each primary antibody is used.
18. This process should not take longer than 2 min.

Acknowledgments

The protocols described here were developed under the support of a generous gift from the Minnesota Jewish Foundation (2006) and an ASDS Cutting Edge Grant (BMH). The authors thank Shu Jiang, M.D., Ph.D. for her technical assistance.

References

1. Quesenberry P, Levitt L (1979) Hematopoietic stem cells (second of three parts). *N Engl J Med* 301:819–823
2. Lajtha LG (1979) Stem cell concepts. *Differentiation* 14:23–34
3. Ghazizadeh S, Taichman LB (2005) Organization of stem cells and their progeny in human epidermis. *J Invest Dermatol* 124:367–372
4. Lavker RM, Sun TT (1982) Heterogeneity in epidermal basal keratinocytes: morphological and functional correlations. *Science* 215:1239–1241
5. Lavker RM, Sun TT (2000) Epidermal stem cells: properties, markers, and location. *Proc Natl Acad Sci USA* 97:13473–13475
6. Levy V, Lindon C, Zheng Y et al (2007) Epidermal stem cells arise from the hair follicle after wounding. *FASEB J* 21:1358–1366
7. Ghazizadeh S, Taichman LB (2001) Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. *EMBO J* 20:1215–1222
8. Ito M, Liu Y, Yang Z et al (2005) Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat Med* 11:1351–1354
9. Levy V, Lindon C, Harfe BD et al (2005) Distinct stem cell populations regenerate the follicle and interfollicular epidermis. *Dev Cell* 9:855–861

10. Owens DM, Watt FM (2003) Contribution of stem cells and differentiated cells to epidermal tumours. *Nat Rev Cancer* 3:444–451
11. Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–343
12. Jones PH, Watt FM (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell* 3: 713–724
13. Roh C, Roche M, Guo Z et al (2008) Multipotentiality of a new immortalized epithelial stem cell line derived from human hair follicles. *In Vitro Cell Dev Biol Anim* 44:236–244
14. Jones PH, Harper S, Watt FM (1995) Stem cell patterning and fate in human epidermis. *Cell* 80:83–93
15. Ji L, Allen-Hoffmann BL, de Pablo JJ et al (2006) Generation and differentiation of human embryonic stem cell-derived keratinocyte precursors. *Tissue Eng* 12:665–679
16. Lorenz K, Rupf T, Salvetter J et al (2009) Enrichment of human beta 1 bri/alpha 6 bri/CD71 dim keratinocytes after culture in defined media. *Cells Tissues Organs* 189:382–390
17. Jiang S, Zhao L, Purandare B et al (2010) Differential expression of stem cell markers in human follicular bulge and interfollicular epidermal compartments. *Histochem Cell Biol* 133:455–465



<http://www.springer.com/978-1-62703-329-9>

Skin Stem Cells

Methods and Protocols

Turksen, K. (Ed.)

2013, XII, 321 p., Hardcover

ISBN: 978-1-62703-329-9

A product of Humana Press