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Chapter 1

Interfollicular Epidermal Stem Cells: Boosting and Rescuing from Adult Skin

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Abstract

Epidermal stem cells isolation struggle remains, mainly due to the yet essential requirement of well-defined approaches and markers. The herein proposed methodology integrates an assemblage of strategies to accomplish the enrichment of the interfollicular epidermal stem cells multipotent fraction and their subsequent separation from the remaining primary human keratinocytes culture. Those include rapid adherence of freshly isolated human keratinocytes to collagen type IV through the β1-integrin ligand and Rho-Associated Protein Kinase Inhibitor Y- 27632 administration to the cultures, followed by an immunomagnetic separation to obtain populations based in the combined $CD49f^{bri}/CD71^{dim}$ expression. Flow cytometry is the supporting method to analyze the effect of the treatments over the expression rate of early epidermal markers keratins19/5/14 and in correlation to $CD49f^{bri}/CD71^{dim}$ subpopulations. The step-by-step methodology herein described indulges the boosting and consecutive purification and separation of interfollicular epidermal stem cells from human keratinocytes cultures.

Key words Epidermal stem cells, Collagen IV, Rock inhibitor, Immunomagnetic separation, Flow cytometry

1 Introduction

Human keratinocytes (hKC) have a limited lifespan in culture that constraints their proliferative capacity and consequently their clinical potential. The long-term function of the skin equivalents generated in a Regenerative Medicine context can be limited by the length of time needed to obtain epithelial sheets in vitro, during which patient is highly susceptible to infection, and also by extensive culture that may lead to terminal differentiation of the hKC to be grafted, thus compromising its success. Therefore, the use of epidermal stem cells (EpSCs) that play an important role in cellular regeneration, wound healing, and neoplasm formation (1) for this purpose enlarges the possibility of providing an alternative and clinically relevant active source of biological material.

Despite a wide effort among stem cells biologists community (2–6), EpSCs isolation difficulty remains, mainly due to the

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insufficiency of molecular markers that distinguish these cells from other proliferative cells within skin basal layer, highlighting the need for defining approaches and a panel of markers to obtain specific and well-characterized cell populations. P63 is abundantly expressed by holoclones and therefore recognized as being also present in EpSCs playing an important role in morphogenesis and in the expression pattern of the cultures (7). It has been also proposed that EpSCs exhibit a characteristic keratin profile that includes the typical K5 and K14 expression of the basal layer but not of K1/10 of the suprabasal layer cells. K19 appears also as an EpSCs-associated marker as it is expressed by cells present in the skin hair follicles bulge and in the deep epidermal rete ridges within thicker epidermis, being also expressed by a subpopulation of hKC in the human basal layer during proliferative lateral skin expansion (8).

Interestingly, the molecules related with cell-substratum adhesion are naturally meaningful as potential EpSCs markers, supported by the hypothesis that EpSCs require strong adherence to the basement membrane to maintain their stem cell characteristics or their position in the stem cell niche. (9). β 1-integrin was firstly identified in highly proliferating KC (holoclones) and was used to distinguish EpSCs and other basal cells (5). However, subsequent studies revealed that the majority of the cells of the basal layer in the human epidermis, EpSCs, and transient amplifying cells exhibit the expression of beta 1 integrin (10) and other putative markers such as the combination of CD49f (α6-integrin) and CD71 (transferrin receptor) (5). Human epidermal cells have been thus divided into three different subsets, $\alpha6^{bri}CD71^{dim}$, $\alpha6^{bri}CD71^{bri}$, and $\alpha6^{dim}$ expressing cells, the first being those with the highest proliferation rate and capability of long-term epidermal renewal (11), even at a limited dilution.

Despite the high importance of EpSCs, they constitute between 1% and 10% of the basal layer cells and, independently of the standardization of a characteristic panel of markers, boosting this population in culture through enrichment methods is a major demand. The involvement of Rho-Associated Protein Kinase (Rock) in tissue homeostasis, namely in the epidermis, is already recognized. Regardless of the unconsciousness of the exact timing of events, the key role that Rock plays in determining hKC fate was clearly demonstrated. By blocking Rock function, an inhibition of hKc terminal differentiation and an increase in cell proliferation was observed (12). It has also been shown that Rock inhibitor (Rocki) leads to an increased number of hKC in primary cultures that can survive and grow forming healthy colonies, thus suggesting its effect in boosting the cells exhibiting stem cell behavior (13) yet retaining the ability to differentiate and to form a stratified epithelium in adequate organotypic models (14). The herein proposed methodology describes an assemblage of strategies to accomplish enrichment and further purification of the EpSCs multipotent fraction present in

hKC primary cultures. The procedure combines the rapid adherence of primary hKC to β1-integrin ligand in collagen type IV and the administration of Rho-Associated Protein Kinase (Rock) Inhibitor Y- 27632 to the culture, together with subsequent immunomagnetic separation of subpopulations combining CD49f^{bri}/CD71^{dim} expression.

2 Materials

2.1 Labware (see Note 1)

Petri dishes (Greiner Bio One, Cat. No. 391-2080).

Forceps (RSG, Cat. No. 311.105).

Surgical scissors (RSG, Cat. No. 101.130).

Cell culture flasks (75 cm^2, 150 cm^2) (BD Falcon, Cat. No. 353136, 353028).

6-well culture plates (BD Falcon, Cat. No. 353224).

15 mL Falcon tubes (BD Falcon, Cat. No. 352097).

50 mL Falcon tubes (BD Falcon, Cat. No. 352070).

Flow cytometry tubes (BD Falcon, Cat. No. 352052).

Pipettes (Corning Science Products, Cat. No. 4489).

Cell strainers of 100 μm pore size (BD Falcon, Cat. No. 352360).

Eppendorf tubes 1.5 mL (Laborspirit, Cat. No. 200400P).

DynaMag™-2 magnet (Invitrogen, Cat. No. 123-21D).

0.22 μm pore membrane filters (Sarsted, Cat. No. 83.1823.101).

2.2 Reagents

Phosphate buffer saline (PBS) (Sigma, Cat. No. P4417).

Distilled water (diH_2O).

Antibiotic/antimycotic solution (Gibco, Cat. No. 15240062).

Dispase (BD Biosciences, Cat. No. 354235).

Trypsin–EDTA (Gibco, Cat. No. 25300-062).

Keratinocyte Serum Free Medium (KSFM) Kit with l-Glutamine, EGF, and BPE (Gibco, Cat. No. 17005-075).

Y-27632 dihydrochloride monohydrate (Sigma, Cat. No. Y0503).

Acetic acid (vWR, Cat. No. 20104.334).

Human placenta collagen type IV (Sigma, Cat. No. C5533).

Bovine serum albumin (BSA) (Sigma, Cat. No. A2153).

Dynabeads M-450 Epoxy (Life Technologies, Cat. No. 14011).

Sodium phosphate (Sigma, S0876).

Permeabilization buffer (10×) (eBioScience, Cat No. 00-8333).

CD49f-APC antibody (eBioscience, Cat. No. 17-0495-82).

CD71-PE antibody (BD Biosciences, Cat. No. 555537).

Cytokeratin 19-AF488 antibody (ExBio, Cat. No. A4-120-C100).

Cytokeratin 14-FITC antibody (AbD Serotec, MCA890F).

Keratin 5 antibody (Covance, Cat. No. PRB-160P).

Alexa Fluor 488 Goat anti-Rabbit (Invitrogen, Cat. No. A-11008).

Formaldehyde (vWR, Cat. No. ALFA33314K2).

Sodium azide (Sigma, Cat. No. 13412).

2.3 Reagents Setup

1. *Dispase stock solution (25 U/mL)*: dilute dispase, 1:2 in PBS (see Note 2).
2. *Collagen IV stock solution (1 mg/mL)*: Add 5 mL of 0.25% acetic acid and let to dissolve overnight at 4°C (see Notes 2 and 3).
3. *Rocki stock solution (1 mM):* Reconstitute 1 mg of Y-27632 dihydrochloride monohydrate in 2.96 mL of diH_2O.
4. *Dynabeads buffer 1*: prepare a buffer of 0.1 M Sodium phosphate in diH_2O and adjust pH to 7.4–8.0 (see Note 4)
5. *Dynabeads buffer 2*: make a 0.1% BSA solution in PBS and adjust the pH to 7.4.
6. Coating of immunomagnetic beads with CD71 and CD49f antibodies (see Note 5)
   6.1 Transfer 10 μL of dynabeads to an eppendorf tube.
   6.2 Place the tube in a magnet for a minute and discard the supernatant. Remove the tube from the magnet.
   6.3 Resuspend the beads in 50 μL of Dynabeads buffer 1 and add 4 μL of CD71 or 2 μL of CD49f antibody.
   6.4 Incubate for 16–24 h at room temperature with gentle tilting and rotation.
   6.5 Repeat step 6.2 and resuspend beads in Dynabeads buffer 2.
7. *Permeabilization buffer*: Dilute permeabilization buffer (10×) in diH_2O to obtain a 1× working solution, store at 4°C.
8. *Labeling buffer*: Prepare a 3% BSA solution in PBS.
9. *Acquisition buffer*: Make a 1% formaldehyde and 0.1% Sodium azide solution in PBS, filter (0.22 μm pore membrane), and store at RT.
10. *Antibiotic/antimycotic solution*: Make a 1% antibiotic solution in PBS
11. *Dispase working solution (2.5 U/mL)*: Dilute 1:10 of stock dispase solution in 1% solution of antibiotic/antimycotic in PBS
12. *Rocki working solution (10 μM)*: Dilute Rocki stock solution (1 mM) 1:100 in KSFM, in order to have KSFM supplemented with 10 μM Rocki.

3 Methods

3.1 Isolation of Human Keratinocytes from Adult Skin

3.1.1 Processing Human Skin

1. Remove the exceeding fat tissue from the dermis with scissors and scalpel.
2. Wash the skin samples with *antibiotic/antimycotic solution* (±20 s).
3. Cut skin into 0.5 cm^2 pieces.

3.1.2 Epidermal–Dermal Separation by Dispase

Incubate skin pieces in dispase working solution (2.5 U/mL) overnight at 4°C in a 250 mL flask.

After incubation place the skin samples on a Petri dish and peel off epidermis from dermis using two pairs of forceps.

3.1.3 Isolation of Human Keratinocytes: Digestion of Epidermis with Trypsin

1. Place epidermis (dermal side up) in a new Petri dish.
2. Add 0.05% trypsin–EDTA.
3. Incubate the samples at 37°C for 5–7 min.
4. Add an equal amount of KSFM.
5. Scrape of cells carefully with a cell scraper.
6. Pipette rigorous up and down several times.
7. Poor cell suspension trough a 100 μm pore size cell strainer into a 50 mL Falcon tube.
8. Wash with PBS, passing the liquid through the 100 μm pore size cell strainer.
9. Centrifuge for 5 min at 290 × *g*.
10. Wash pellet with 5 mL of PBS.
11. Poor cells trough a 100 μm pore size cell strainer into a 50 mL Falcon tube.
12. Centrifuge for 4 min at 290 × *g*.
13. Resuspend cell pellet (hKC) in KSFM.

3.2 EpSCs Enrichment Strategies (see Note 6)

3.2.1 Rapid Adherence to Collagen IV

1. Coat tissue culture surface with collagen IV, by incubating 5 μg/cm^2 at 37°C, for at least 1 h (see Note 3).
2. Wash with PBS.
3. Plate 2×10^4 hKC/cm^2 in KSFM.
4. Change medium every 2–3 days and keep the culture until 80% confluent.

3.2.2 Rock Inhibitor

1. Plate 2×10^4 cells/cm^2 in Rocki working solution (10 μM).
2. Change medium every 2–3 days; keep them in culture until 80% confluent.

3.2.3 Combined Approach

1. Perform steps 1 and 2 described in section 3.2.1.
2. Proceed as described in section 3.2.2.

3.3 Intermediate Analysis

An intermediate analysis of a fraction of the obtained cells after each treatment should be performed in order to assess the effect in the increased fraction of interest—EpSCs fraction. Therefore in this section a flow cytometry protocol using a combination of CD49f-APC and CD71-PE markers is described.

1. Harvest the adherent cells cultured under the described conditions with trypsin–EDTA.
2. Transfer cells to a 15 mL falcon tube and add labeling buffer up to 10 mL.
3. Centrifuge cell suspension at $200\times g$ for 5 min.
4. Count cells using a hemocytometer.
5. Discard supernatant and resuspend cell pellet to a concentration of 0.5–10^6 cells/mL in fresh labeling buffer.
6. Add 100 μL of cell suspension to each flow cytometry tube (see Note 7).
7. Add 4 μL of CD71-PE and 2 μL CD49f-APC antibodies; reserve one tube per condition without antibody as control.
8. Incubate 30 min at room temperature.
9. Wash by adding 2 mL of PBS per tube and centrifuge at $250\times g$ for 3 min.
10. Resuspend cell pellets in 500 μL of acquisition buffer.
11. Acquire data in flow cytometer.
12. Analyze simultaneous expression of CD71 and CD49f (Fig. 1).

3.4 $CD71^-/CD49f^+$ Rescuing: Immunomagnetic Selection

3.4.1 Depletion of $CD71^+$ Cells

1. Wash CD71 coated beads by placing the tubes in a magnet for 1 min, discarding the supernatant and adding 1 mL of Dynabeads buffer 2, twice.
2. Incubate the cells harvested on Subheading 3.3, **step 1** and resuspended in Dynabeads buffer 2 with the washed CD71 beads for 30 min at 2–8°C with gentle tilting and rotation.
3. Place the tubes in a magnet for 2 min.
4. Transfer the supernatant containing the unbound cells to a fresh 15 mL Falcon tube ($CD71^-$ cell fraction).
5. Count cells using a hemocytometer.
6. Plate 2×10^4 cells/cm^2 in new tissue culture vessels/flasks with the correspondent treatments (described in Subheading 3.2).
7. Culture cells until 80% confluent by changing medium 2–3 days.

3.4.2 Positive Selection of $CD49f^+$ Cells Among $CD71^-$ Population

1. Harvest $CD71^-$ cells with trypsin–EDTA and resuspend them in Dynabeads buffer 2.
2. Wash CD49f coated beads by placing the tubes in a magnet for 1 min, discarding the supernatant and adding 1 mL of Dynabeads buffer 2, twice.

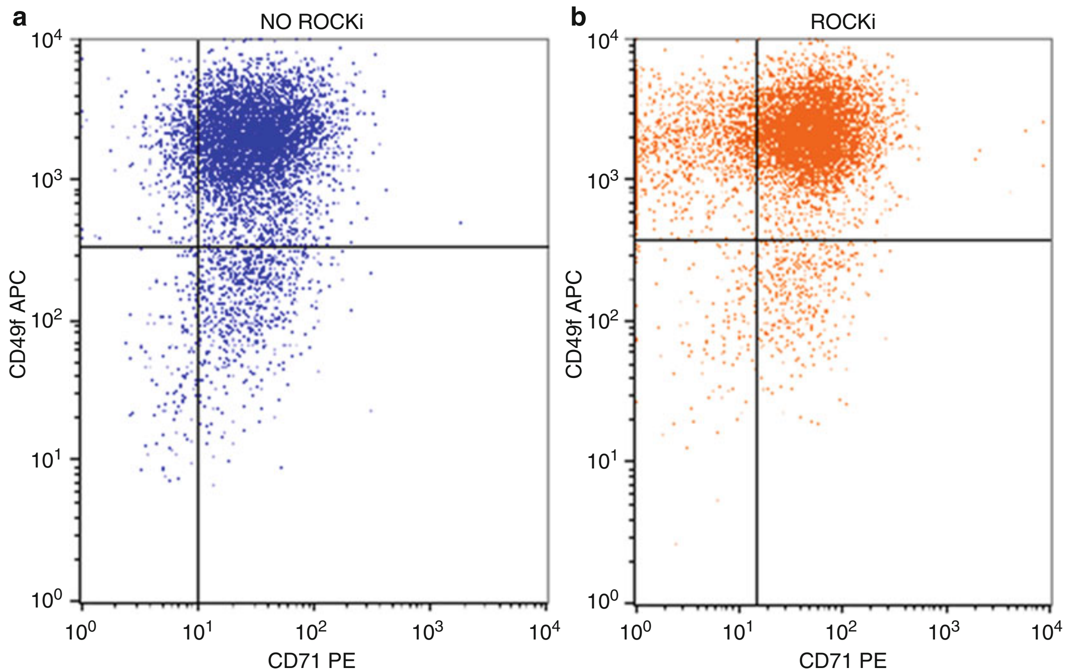

Fig. 1 Dot plots of CD49f/CD71 staining on human keratinocytes isolated from the same human sample and cultured without any treatment (**a**) and after EpSCs enrichment with Rocki (**b**), showing an increase of the population of interest by the differential expression of $CD49f^{bri}/CD71^{dim}$ (6.20% in **a** and 10.37% in **b**)

3. Incubate $CD71^-$ cells for 20 min at 2–8°C with gentle tilting and rotation.
4. Place the tubes in a magnet for 2 min.
5. Discard the supernatant and gently wash the bead-bounded cells, four times, by adding 1 mL of Dynabeads buffer 2.
6. Place the tubes in the magnet for 1 min and discard the supernatant.
7. Resuspend the cells in fresh KSFM.
8. Plate 2×10^4 cells/cm^2 in new tissue culture vessels/flasks with the correspondent treatments (described in Subheading 3.2) for further cell expansion and analysis.

3.5 Analysis

The analysis of the expression of the early epidermal markers on the obtained cell fraction by flow cytometry is advisable to validate the success of the employed strategies. Thus, this section comprises the protocol for identifying the expression of the intracellular markers using Cytokeratin 19-AF488 and Cytokeratin 14-FITC, and Keratin 5, respectively by direct and indirect staining.

3.5.1 Direct Staining

Follow the protocol from Subheading 3.3, steps 1–11, using 5 μL of Cytokeratin 19-AF488 and 4 μL of Cytokeratin 14-FITC in separate tubes.

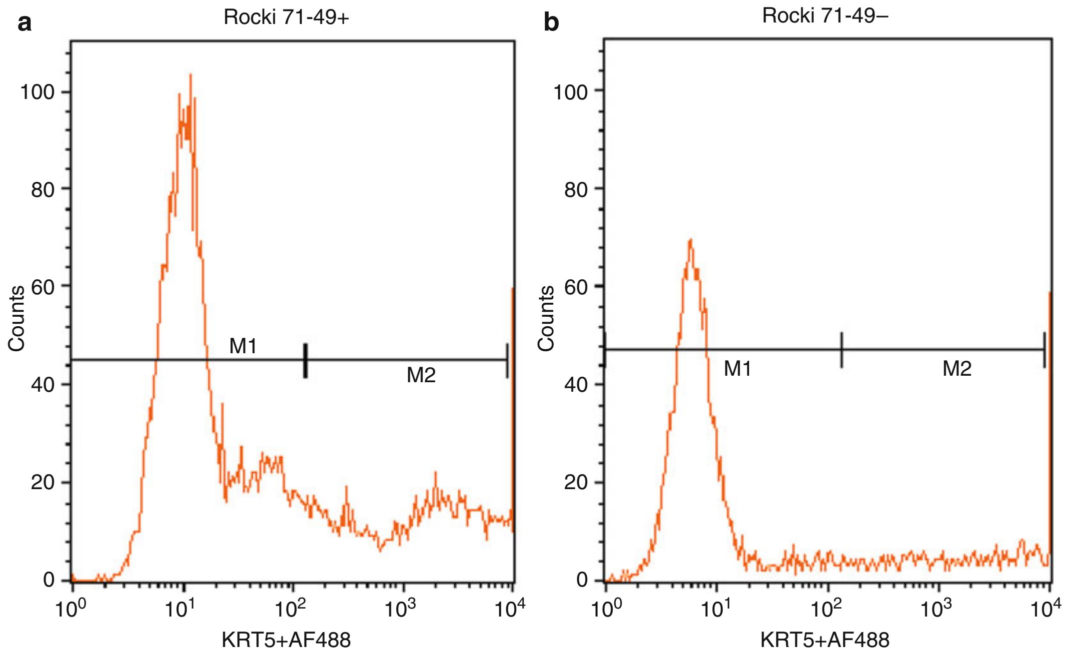

Fig. 2 Expression fluorescence profile of Keratin 5 (early epidermal marker) in both CD71-/α6+ cells (25.45%) (**a**) and CD71-/α6- (17.42%) (**b**), showing the higher expression of K5 in the fraction of interest, thus illustrating its early differentiation stage

3.5.2 Indirect Staining

1. Follow the protocol from Subheadings 3.3, steps 1–6.
2. Incubate cells with 200 μL of permeabilization buffer for 10 min at RT.
3. Centrifuge for 5 min at 250 × *g* and remove supernatant.
4. Resuspend cells in 200 μL of keratin 5 antibody diluted 1:500 in labeling buffer.
5. Incubate for 1 h at room temperature.
6. Wash by adding 2 mL of PBS, centrifuge at 250 × *g* for 5 min, and remove supernatant.
7. Resuspend cells in 200 μL of Alexa-fluor-labeled secondary antibody diluted 1:500 in labeling buffer.
8. Incubate 45 min at 4°C protected from light.
9. Wash by adding 2 mL of PBS, centrifuge at 250 × *g* for 5 min, and remove supernatant.
10. Resuspend cell pellets in 500 μL of acquisition buffer.
11. Acquire data in flow cytometer (Fig. 2).

4 Notes

1. All the labware has to be sterilized prior use.
2. It is recommended to make aliquots that should be kept at –20°C, avoiding repeated freeze-thawing.
3. Coating with collagen IV can be previously performed incubating solution overnight at 4°C, without drying.
4. Alternatively, a solution of 0.1 M sodium borate sulfate in diH_2O, pH 7.4–8, can be used as buffer 1.
5. This protocol is adapted from the section "coupling of ligands to dynabeads" of the Dynabeads m-450 Epoxy manufacturer's instructions.
6. Cells cultured, in these different treatments, present distinct adherence and proliferation rates. No specific treatment, in which cells are cultured in noncoated plates and in KSFM, should be performed as a control. hKC cultured in Rocki and No treatment take almost 1 week to first adhere.
7. The remaining cells should be used as described in Subheading 3.4 and kept in culture for comparison purposes in the end of the experiment.

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