

Chapter 2

Functional Investigations of Keratinocyte Stem Cells and Progenitors at a Single-Cell Level Using Multiparallel Clonal Microcultures

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Abstract

The basal layer of human interfollicular epidermis is thought to contain a minor compartment of quiescent or slowly cycling epithelial stem cells. These primitive keratinocytes give rise to the progenitors, which are the proliferating keratinocytes and which can be defined as early to late progenitors, according to their differentiation status. Because of the intrinsic heterogeneity of the basal layer, the development of new methods suitable for functional analysis of basal keratinocytes directly isolated from skin samples is greatly needed. We describe here a new method that allows a rapid and multiparallel deposition of single keratinocytes into 96-well plates, using flow cytometry. The first step of the process allows the clonal analysis of the growth potential of freshly isolated epithelial cells in primary cultures. In a second step, various techniques of functional characterization can be performed on the progeny of the cloned cell, including the generation of reconstructed epidermis, colony assays, and secondary cloning. In a third step, a long-term characterization of the progeny of the cloned keratinocytes can be performed, either by successive subclonings or mass expansion cultures.

Key words: Keratinocytes, Single cell, Basal layer, Stem cells, Progenitors, Heterogeneity, Flow cytometry, Clonal cultures, Multiparallel, Epidermis reconstruction, Subcloning.

1. Introduction

Human epidermis comprises multiple layers of keratinocytes, which are in a continual process of cell replacement over a cycle of 28 days. The basal layer ensures the precisely regulated production of the differentiated keratinocytes which constitute the epidermal supra-basal layers. Stem cells represent a minor subpopulation of the basal keratinocytes, which possess the capacity for self-renewal and is

responsible for the long-term maintenance of epidermal integrity (1–4). Progenitors, also called transit amplifying cells, are the progeny of the stem cells and constitute the majority of the basal keratinocytes. They possess a limited proliferative capacity and are responsible for the short-term renewal of the epidermis. Keratinocyte progenitors are not a homogeneous population, but rather represent a gradient of differentiation, from primitive progenitors close to the stem cell up to more mature cells committed to differentiation and migration to the upper layers of epidermis. Because of this intrinsic heterogeneity of the basal layer, the development of new methods suitable for a functional analysis of basal keratinocytes at a single-cell level is an important challenge for both fundamental and applied research. An important requirement is that these methods can be used on cells freshly isolated from the skin tissue, in order to maintain cell characteristics close to their *in vivo* phenotype at the stage of microculture initiation. In addition, these methods should allow both short- and long-term assessment of the main keratinocyte properties, including epidermis reconstruction. Finally, they should be rapid and multiparallel, to permit analysis of large numbers of individual cells and thus large-scale screening assays.

We describe in this chapter a method which fulfills all these criteria. This method includes five experimental steps:

- (1) Extraction of epithelial cells from a tissue sample, for example, epidermal keratinocytes from normal or pathological human skin, completed or not by a selection of cell subpopulations with specific characteristics.
- (2) Initiation of parallel clonal microcultures by individual cell deposition into multiple separated culture wells, in conditions adapted for survival and growth. Single cell depositions are performed automatically, using flow cytometry.
- (3) Analysis of the primary growth capacity of individual cloned cells, using qualitative and quantitative parameters, including clone-forming efficiency and size of individual clones.
- (4) Epidermis regeneration from the progeny of the cloned cells. At the end of the primary culture, clones are individually detached from their culture substrate and then separately tested for their *in vitro* capacity to reconstruct epidermis.
- (5) Characterization of the long-term expansion of the individual cloned cells. This can be performed using a classical protocol, according to which mass cultures of single-cell origin are passaged continuously until their growth capacity has been exhausted. It can also be performed by serial subclonings.

This method enables the rapid plating of hundreds of clonal cultures, which opens the possibility for multi- and massively parallel functional screens. In addition, flow cytometry enables the single-cell deposition of keratinocytes with defined phenotypic criteria, which is not easily feasible with classical methods of micromanipulation. The principle of plating only one single keratinocyte per individual micro-well means that relatively low quantities of cells are needed, even if large cohorts of parallel microcultures are obtained. This characteristic of the proposed experimental system enables the future development of parallel screens using rare keratinocyte subpopulations, such as candidate stem or progenitor cells (5–8).

In this chapter, we describe in detail the practical procedures of clonal microcultures of keratinocytes from the interfollicular epidermis. However, the method can be applied to epithelial cells from other origins, such as stem cells from the hair follicle (9) or from the cornea (10). Because of its high discriminative efficiency, the method should have broad applications to characterize the potential of cell samples used for clinical protocols such as the grafts for burn patients (11) or therapy of genetic skin diseases (12).

2. Materials

2.1. Isolation of Keratinocytes and Fibroblasts

1. Dissection tools: autoclaved scissors, scalpel, and curved forceps (CML).
2. Sterile compress Nissan (10 × 10, 30G, 4-fold).
3. Sterile needles 18^{1/2}G (Becton Dickson; cat. no. 304622).
4. Corn remover (Sanodiane; cat. no. 0000678).
5. Cork plates.
6. Dermal Betadine 10% (Viatris).
7. Disinfectant (Anios; Amphospray; cat. no. 289047).
8. Phosphate-buffered saline without calcium and magnesium (PBS, Gibco; cat. no. 14190).
9. Trypsin (Gibco Life Technology; cat. no. 27250-018).
10. Antibiotics (Gibco; cat. no. 15140-122).
11. Dispase II (Roche; cat. no. 04942078001).
12. Collagenase II (PAA; cat. no. K11-240).
13. Trypan blue (Sigma; cat. no. T8154).
14. Sterile cell strainers 70 µm (BD Falcon; cat. no. 352350).
15. Filters 0.22 µm (Millipore; cat. no. 051246).
16. DMSO (Sigma; cat. no. D8418).

2.2. Solutions

1. Dermal Betadine diluted at 0.4% in PBS.
2. Trypsin: 0.25% in PBS with 2.5% penicillin–streptomycin, sterile filtered, kept at -20°C in 25 mL aliquots.
3. Dispase: 1 g in 10 mL PBS, stored as frozen aliquots of 200 μL . Before use, add 5 mL PBS to be at a final concentration of 4 mg/mL and filter through a 0.22 μm filter.
4. Collagenase: 100 mg in 1 mL PBS, stored as frozen aliquots of 150 μL . Before use, add 5 mL PBS to be at a final concentration of 3 mg/mL and filter through a 0.22 μm filter.

2.3. Flow Cytometry**2.3.1. Equipment**

1. MoFlo high-speed cell sorter (Cytomation/Coulter)
2. Excitation sources: 5 W water-cooled Argon Ion Laser (Coherent 70C) tuned to 488 nm, 7 W water-cooled Argon Ion Laser (Coherent 90C) tuned to MLUV lines and red laser diode.
3. Summit software for data analysis (MoFlo software).
4. Automated cell deposition unit (CyClone) allowing single-cell deposition for cloning purpose.

2.3.2. Reagents

1. Rat gamma globulins (Jackson Immuno Research; cat. no. 012-000-002).
2. Phycoerythrin-conjugated (PE) Rat anti-human $\alpha 6$ integrin CD49f (clone GoH₃; BD Pharmingen; cat. no. 555736) and corresponding isotype control (BD Pharmingen; cat. no. 555844).
3. PBS-2% BSA filtered through a 0.22 μm filter and stored as frozen aliquots at -20°C .
4. Bisbenzimidazole H33342 trihydrochloride Hoechst (Sigma; cat. no. B2261).

2.4. Keratinocyte Culture**2.4.1. Tissue Culture Labware**

1. 96-well cell culture plates, coated with type I collagen (BD BioCoat; cat. no. 356407).
2. Petri dishes 100 mm diameter, 58.1 cm^2 , coated with type I collagen (BioCoat, Becton-Dickinson; cat. no. 356450).

2.4.2. Components of Culture Medium and Working Concentrations

Keratinocytes are cultivated in a 3/1 mixture of DMEM 1 g/L D-glucose (Gibco; cat. No. 11880028) and Ham's F12 (Gibco; cat. No. 04195122 M), containing the following components:

1. Adenine hydrochloride (Sigma; cat. no. A9795): 180 μM .
2. Recombinant human epidermal growth factor (rhEGF) (Millipore; cat. no. GF001-500 μg): 10 ng/mL.
3. Hydrocortisone (Sigma; cat. no. H4881-100 mg): 0.4 $\mu\text{g}/\text{mL}$.
4. Apo-transferrin (Sigma; cat. no. T2252-100 mg): 5 $\mu\text{g}/\text{mL}$.
5. Tri-iodo thyronine (Sigma; cat. no. T2752-100 mg): 2 nM.

6. Insulin (Sigma; cat. no. I5500-100 mg): 5 µg/mL.
7. L-glutamine (Gibco; cat. no. 25030-024): 2 mM.
8. Hyclone Fetal Bovine Serum (FBS); Research Grade EU Approved, FetalClone II, triple 0.1 µm sterile filtered: 10%.
9. Antibiotics (Gibco; cat. no. 15140-122): 100 U/mL penicillin and 100 µg/mL streptomycin.

2.5. Fibroblast Culture

1. DMEM: Dulbecco's modified Eagle's medium + GlutaMax-1; 4.5 g/L D-glucose, pyruvate (Gibco; cat. no. 31966).
2. Fetal bovine serum, Hyclone Fetal Bovine Serum Research Grade EU Approved, triple 0.1 µm sterile filtered: 10%.
3. Penicillin/streptomycin (Gibco; cat. no. 15140-122): 100 U/mL and 100 µg/mL.

2.6. Colony Assay

1. Ethanol (VWR; cat. no. 20821.206).
2. Eosin RAL 555 (RAL Reagents; cat. no. 361640).
3. Methylene blue RAL 555 (RAL Reagents; cat. no. 361650).

2.7. Equipment

Inverted fluorescence light microscope (AxioObserver D1, Zeiss, Zurich).

3. Methods

3.1. Keratinocyte Isolation

1. Collect human skin samples, for example from mammary reduction, after informed consent.
2. Place the skin dermal side up onto a sterile compress, stretch it with needles, and remove adipose tissue with a scalpel (*see Note 1*).
3. Decontaminate with Betadine 0.4% for 20 min and rinse three times in PBS. Decontaminate corn remover with Amphospray for 20 min.
4. Place the skin dermal side down onto a cork plate and stretch it with needles. The sterile corn remover is used to collect the upper part of the skin, containing the epidermis and the superficial dermis (approximately 1 cm wide and 2 mm thick).
5. Incubate tissue sample (epidermis + thin dermis) in 2.5 mg/mL trypsin for 15 hours at 4°C.
6. Separate epidermal sheets from the dermis by dissection with a pair of sterile curved forceps.
7. Neutralize trypsin with a solution of 80% DMEM and 20% FBS.

8. Transfer epidermal sheets in a 50-mL tube. Complete dissociation of the tissue is obtained by gentle pipetting at room temperature.
9. Filter samples through 70 μm cell strainers to eliminate residual aggregates and obtain single-cell suspensions of keratinocytes (*see Note 2*).
10. Determine cell number and viability by trypan blue exclusion.
11. Centrifuge at 4°C at 1000 rpm for 15 min. Eliminate the supernatant and resuspend keratinocytes in 1 mL of culture medium.

3.2. Fibroblast Isolation

1. Recover the dermal pieces after separating the epidermis from the dermis (*see Section 3.1.6, Methods*).
2. Place dermal pieces into a 50 mL tube and incubate in 4 mg/mL dispase and 3 mg/mL collagenase for 2 hours at 37°C to complete the digestion, vortexing every 20 min. Use 10 mL of dispase-collagenase solution per gram of tissue (*see Note 3*).
3. Quench the reaction by adding three volumes of 80% DMEM–20% FBS to the dermal cell suspension.
4. Filtrate through a 70 μm cell strainer, use one strainer for 20 mL of cell suspension.
5. Centrifuge at 1000 rpm for 15 min.
6. Count the cells and estimate cell viability with trypan blue exclusion.
7. Plate the dermal fibroblasts at 1500 cells/cm² per culture flasks in the fibroblast culture medium.
8. Freeze aliquots of fibroblasts at passage 2-4 in serum with 10% DMSO and store them as a master bank.
9. To prepare the feeder layer bank, amplify the fibroblasts of the master bank from passage 4 to 6 and expose them to a 60 Gy dose of γ rays.
10. Freeze aliquots of growth-arrested fibroblasts in serum with 10% DMSO and store them in liquid nitrogen until use as feeder layer.
11. For keratinocyte culture, feeder fibroblasts are thawed and seeded at 6000 cells/cm² in the culture labware the day before keratinocyte seeding.

3.3. Single Keratinocyte Plating by Flow Cytometry

3.3.1. Keratinocyte Labeling

1. Keratinocytes from the basal layer of epidermis are sorted according to their high level of expression of integrin $\alpha 6$ (*see Note 4*).
2. Resuspend keratinocyte samples in PBS-2% BSA.
3. Block unspecific antibody fixation with Rat γ globulins.

4. Add the PE-conjugated antibody (anti-CD49f-PE) and incubate at 4°C for 30 minutes.
5. Wash the cell suspension twice in PBS-2% BSA.

3.3.2. Flow Cytometry Instrument Settings and Controls

1. Set up the MoFlo using a low sheath pressure (20 psi) and a 100 µm nozzle (*see Note 5*).
2. Wash and rinse the fluidic system to obtain sterile conditions.
3. Adjust the cell sorter for standard FITC and PE detection, using 488 nm laser excitation and 530/30 and 580/30 nm band pass fluorescence filters, respectively.

Use the ratio area/peak on PE fluorescence for doublet discrimination.

4. Set up the MoFlo for sorting. Use the single-cell mode and choose one-half drop sort droplet envelope (*see Note 6*).
5. Set up the single-cell deposition unit. Select the 96-well plate mode (*see Note 7*). Check that the drop is well centered into each well and choose 1 cell per well. Exclude the outside rows for deposition.
6. Use standard flow cytometric gating on FSC and SSC parameters to exclude cellular debris and large differentiated keratinocytes.
7. Gating on PE fluorescence intensity is performed to select the population of interest.
8. Use the ratio area/peak on PE fluorescence for doublet discrimination.

Run the sorting at a low flow rate of 500–1000 cells per second.

3.3.3. Quality Control of Single-Cell Plating

To qualify the cell deposition procedure, single-cell plating is performed in conditions enabling easy observation and counting of individual cells in the 96-well plates.

1. Prepare three 96-well plates filled with PBS containing 10 µg/mL Hoechst 33342 (*see Note 8*).
2. Incubate the plates after cloning for 30 minutes at 37°C.
3. Search for the presence of a single keratinocyte in each well, under the UV light of an inverted fluorescence microscope.
4. Determine the deposition efficiency for the current experiment (*see Note 9*).
5. Once the quality of deposition has been verified, run the sorting for the experiment.

3.3.4. Deposition of Keratinocytes in Primary Clonal Microcultures

1. The day before cloning, prepare the 96-well plates with feeder fibroblasts (6000/cm²) in the keratinocyte growth medium.

2. Deposit a single keratinocyte per well using the Cyclone unit of the MoFlo.
3. Place the culture plates immediately after cloning at 37°C in an incubator with a fully humidified atmosphere containing 5% CO₂.

3.4. Primary Clonal Growth

1. Renew medium three times a week until the end of the primary culture.
2. Note the wells with proliferating clones to select them and obtain the clone-forming efficiency.
3. Trypsinize each clone 2 weeks after cloning and count cell numbers to quantify the potential of growth in terms of cell output (total cell number obtained) (*see Note 10*) and number of population doublings (PD) (*see Note 11*).
4. Depending on the number of cells obtained at the end of the primary culture, different functional assays can be performed on the progeny of selected clones at this stage, including colony assays, epidermis reconstruction, and long-term culturing.

3.5. Colony Assays

1. The day before the assay, prepare the 96-well plates with feeder fibroblasts (6000/cm²) in the keratinocyte growth medium.
2. Plate the keratinocytes at the low density of 5 keratinocytes/cm² in 100 mm Petri dishes coated with type I collagen (*see Note 12*).
3. Twelve days later, rinse twice in PBS and fix the cultures with 70% ethanol; stain successively with eosin and blue RAL 555 (RAL Reagents), and rinse with water.
4. Count colony numbers according to their size.

3.6. Epidermis Reconstruction

The potential of the progeny of cloned keratinocytes to regenerate epidermis can be analyzed at the end of the primary culture using a model of tissue reconstruction on dead de-epidermized human dermis (DED), as described (13, 7, 8).

1. Seed keratinocytes onto DED (2 × 2 cm) in a ring diameter of 0.5 cm.
2. Keep the cultures immersed for 1 week in a medium of the same composition as that used for bi-dimensional growth of keratinocytes.
3. Raise the cultures to the air–liquid interface and carry on for 2 weeks in the absence of transferrin, tri-iodo thyronine, and adenine.
4. Perform histological examination of the reconstructed epidermis after hematoxylin–eosin–safran (HES) staining of tissue sections.

3.7. Long-Term Clonal Growth

The long-term growth capacity of individual primary clones can be assessed using two different methods.

3.7.1. Mass Expansion Cultures

1. Plate the progeny of selected primary clones obtained 2 weeks after cloning in 100 mm diameter Petri dishes coated with type I collagen.
2. Use the same culture medium and feeder layer for the long-term growth as those used for the primary clonal microcultures.
3. Trypsinize the keratinocytes 1 week later, count the cells, and replate at a density of 1000 cells/cm² (*see Note 13*).
4. Serially cultivate these mass cultures until keratinocyte growth capacity has been exhausted.

3.7.2. Serial Subclonings

1. Trypsinize selected clones and resuspend keratinocytes in PBS-2% BSA.
2. Perform iterative clonal cultures using the same procedures as those described in **Section 3.3**, except that keratinocytes are used directly, without sorting on the integrin $\alpha 6$ marker.
3. At each cloning, the number of cells in each clone is determined until keratinocyte growth capacity has been exhausted.

For the two different methods, the growth potential of a selected clone is determined by the cumulative cell output (total cell number obtained) and the total number of population doublings (TPD) (*see Note 14*), from the initiation of the clonal culture up to senescence.

4. Notes

1. This step is hazardous, disposable scalpels are recommended.
2. Filtration of keratinocytes through a 70 μ m cell strainer must be performed on large volumes in order to avoid cell loss. Use one strainer for 20 mL of cell suspension.
3. Dissolve dispase and collagenase in PBS and filter just prior use.
4. Different keratinocyte populations can be analyzed at the single level by the clonal microculture process, from the whole epidermal population to populations fractionated by cytometry after antibody labelling, for example, to isolate stem cells and progenitors on specific markers.

5. Keratinocytes are fragile cells, susceptible to mechanical stress. This unusual low pressure and the choice of a large 100 μm nozzle permit to slightly increase both the viability of the sorted cells and the efficiency of cell deposition in wells.
6. Single-cell mode provides a specific number of sorted cells and a purity more than 99%. The sort droplet envelope may be chosen between $\frac{1}{2}$ and five. This mode enhances the purity in selecting the events well centered in the sorted drop.
7. The Cyclone unit allows single-cell deposition in a variety of culture dish formats, from a simple slide to multi-well plates (24, 96, 384, and 1536 well trays).
8. Beware of possible mutagenic effects of DNA dyes, wear gloves, and avoid aerosol formation at all times.
9. Hoechst labelling of sorted keratinocytes is used to estimate the number of wells where no cell or one single keratinocyte has been deposited. We found that the deposition efficiency was high, routinely reaching 70–80%.
10. A profile of the distribution of the clones according to their size is thus obtained. Integrin $\alpha 6$ sorting allowed isolation of the various keratinocyte populations of the basal layer. The profile obtained with this sorting revealed the high heterogeneity in growth potential of these basal keratinocytes.
11. The number of population doublings is calculated using the following formula: $\text{PD} = (\log N / N_0) / \log 2$, where N_0 represents the number of plated cells and N the total number of cells obtained at the end of the primary culture. We found that the more proliferative clones performed around 15 population doublings in 2 weeks.
12. The standard colony assay can be performed at any time during cultures, from the first replating at the end of the primary clonal microculture to the end of the serial mass cultures. The number of clones is counted according to their size. This assay allows quantifying the number of clonogenic cells present within the progeny of the keratinocyte clones.
13. The cell densities recommended for optimal expansion of keratinocytes in mass expansion cultures ranged from 500 to 1000 keratinocytes/ cm^2 .
14. The total number of population doublings (TPD) is the sum of the population doublings from the primary culture to the end of the culture, calculated at each passage using the following formula: $\text{PD} = (\log N / N_0) / \log 2$.

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