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Epidermal Cells

Methods and Protocols

Second Edition

Edited by

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Preface

When the first edition of *Epidermal Cell Methods and Protocols* was published, it garnered a great deal of enthusiasm because it brought together into one convenient source a number of critically important protocols. Given its popularity, we decided it was time to extend the coverage by generating a second volume that includes protocols not included earlier and, in some cases, not yet available for the first volume.

I would like to take this opportunity to thank all the contributors for very graciously providing their protocols for this volume. Without them and their willingness to share protocol details, this new volume would not have materialized.

I would also like to thank Dr. John Walker, the Editor-in-Chief of the *Methods in Molecular Biology* series for his continued support.

Patrick Marton, the managing editor of the *Methods in Molecular Biology* series at Springer, also deserves thanks for always being available to answer my questions, for patiently listening to my suggestions, and for supporting this volume during its maturation stages. A very special thanks goes to David Casey for his invaluable help during the production stages of this volume.

Kursad Turksen

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Chapter 1

Isolation of Adult Mouse Stem Keratinocytes Using Magnetic Cell Sorting (MACS)

Corina Lorz, Carmen Segrelles, Marina Garín, and Jesús M. Paramio

Abstract

The functional and molecular characterization of mouse epidermal stem cells (SC) requires in many circumstances arduous technologies and elaboration of complicated transgenic mouse models. To avoid these difficulties, we have developed a simplified protocol to obtain primary keratinocytes from adult mouse skin followed by magnetic-coupled isolation of the CD34 positive epidermal SC population. This approach provides a reproducible and useful method to obtain large amounts of viable cells in a quick and affordable manner, which allows the analysis of pathologies or mutant mice with reduced epidermal SC, and for the molecular and genomic analysis of this population.

Key words: Keratinocyte, Skin stem cells, Isolation, Labeling, Magnetic cell sorting.

1. Introduction

Multipotent epidermal SC reside in a distinct localization within the mouse hair follicle, termed the bulge (1, 2). These cells have been extensively studied over the last few years; as a result specific cell surface markers have been found (3, 4) that allow their identification, isolation, and further analysis. Understanding the biology of epidermal SC is relevant to unraveling the processes of skin carcinogenesis, wound healing, and tissue regeneration. Here we present a successful method based on immune labeling of specific stem cell surface markers followed by magnetic cell sorting, to isolate multipotent epidermal SC for a variety of assays, including functional assays such as clonogenic cell assays and molecular biology studies (mRNA and miRNA profiles).

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2. Materials

2.1. Mice

Adult mice 7–9 weeks of age should be used. The harvesting procedure is optimal when the epidermis is in the telogen stage (quiescent period of the hair follicle cycle). Mice younger than 6 weeks or older than 10 weeks can be in anagen (growth period of the hair follicle cycle). At this stage of the cycle the epidermis is thick and tears easily during the keratinocyte harvest procedure; this may result in decreased yield and viability of the cells.

2.2. Reagents and Solutions

2.2.1. Isolation of Keratinocytes from Skin Samples

1. Lady's hair removing cream.
2. 70% ethanol in distilled water.
3. Sterile Dulbecco's phosphate-buffered saline, Ca^{2+} and Mg^{2+} free (DPBS, Sigma-Aldrich, St. Louis, MO; cat. no. D8537), with 5% antibiotic/antimycotic (Gibco, Invitrogen, Carlsbad, CA; cat. no. 15240-096).
4. Trypsin–EDTA solution (Sigma-Aldrich; cat. no. T3924).
5. Titriplex (ethylenediaminetetraacetic acid disodium salt, EDTA) 0.5 M, pH 8 (Merck KGaA, Darmstadt, Germany; cat. no. 1.08418).
6. DNAseI (Roche, Mannheim, Germany; cat. no. 13738000).
7. 0.4% Trypan blue solution (Sigma-Aldrich; cat. no. T8154).
8. Keratinocyte culture medium: minimum essential medium (EMEM) containing 4% fetal bovine serum (FBS) pretreated with Chelex 100 resin (*see* **Note 1**), supplemented with 0.2 mM $CaCl_2$ and 5% antibiotic/antimycotic (EMEM, Biowhittaker, Inc., Walkersville, MD; cat. no. BE06-1746; FBS, Biowhittaker; cat. no. DE14-801FH; Chelex 100 resin, Bio-Rad, Hercules, CA; cat. no. 142-2842; antibiotic/antimycotic, Gibco; cat. no. 15240-096).

Solutions 4, 5, 6, and 8 are 0.2 μm pore filtered and kept sterile.

2.2.2. Immunofluorescent and Magnetic Labeling

1. MACS/FACS buffer: PBS containing 5% heat-inactivated (56°C, 30 min) FBS and 0.09% (w/v) sodium azide, pH 7.4 (0.2 μm pore filtered).
2. Anti-mouse CD34, clone RAM 34 (eBioscience, San Diego, CA; cat. no. 13-0341).
3. Anti-rat FITC (Jackson ImmunoResearch Laboratories, West Grove, PA; cat. no. 712-095-153)
4. Rat control isotype (rat IgG2a,k, BD Pharmingen, San Diego, CA; cat. no. 553927).
5. Anti-FITC MicroBeads (Miltenyi Biotec, Auburn, CA; cat. no. 130-048-701).

2.3. Tools and Tubes

2.3.1. Isolation of Keratinocytes from Skin Samples

1. Electric veterinary clipper.
2. Sterile curved forceps and scissors.
3. 60-mm diameter sterile Petri dishes.
4. Number 10 round blade disposable sterile scalpels (Lamba, Intraven, Madrid, Spain; cat. no. 0010).
5. 40 μm cell strainer (BD Falcon, Franklin Lakes, NJ; cat. no. 352340).
6. 50-mL sterile polypropylene conical tubes (BD Falcon; cat. no. 352070).
7. Sterile stir bar and beaker (Erlenmeyer).
8. Sterile magnetic stirrer.
9. 4°C 50-mL tubes centrifuge.
10. Sterile laminar flow hood.

2.3.2. Immunofluorescent and Magnetic Labeling

1. 15-mL sterile polypropylene conical tubes (BD Falcon; cat. no. 352096).
2. 5-mL polypropylene round-bottom tubes (BD Falcon; cat. no. 352063).
3. 4°C centrifuge

2.3.3. Magnetic Cell Sorting (MACS)

1. LS column adapter (Miltenyi Biotec; cat. no. 130-090-544).
2. LS columns (Miltenyi Biotec; cat. no. 130-042-401).
3. Pre-separation filters (Miltenyi Biotec; cat. no. 130-041-407).
4. 14-mL polypropylene round-bottom collection tubes (BD Falcon; cat. no. 352059).

2.3.4. FACS Analysis

Fluorescence-activated cell sorting analysis of the samples can be done using a Beckman-Coulter Epics XL flow cytometer and EXPO32 ADC software (both from Beckman-Coulter, Fullerton, CA) or similar.

3. Methods

3.1. Isolation of Keratinocytes from Skin Samples

1. Sacrifice mice by CO_2 inhalation.
2. Remove the dorsal fur with an electric clipper and apply hair removing cream to the clipped area. Leave it on for 3 min approximately (*see* **Note 2**). Remove the cream, rinse with abundant distilled water, and then with 70% ethanol. The rest

of the procedure is done in a biosafety cabinet or a laminar flow hood to ensure sterility of the isolated keratinocytes for cell culture.

3. Cut off area of dorsal skin without hair using forceps and scissors. Avoid under skin fat patches.
4. Rinse the skin in PBS containing antibiotic/antimycotic, place each skin with the dermis side facing down, and spread it out onto a 60-mm sterile dish. Add 5 mL of trypsin–EDTA solution pouring it carefully from the side of the dish so that the skin floats on the trypsin. Avoid trypsin contact with epidermis.
5. Incubate the dishes with the skins in a cell culture incubator (37°C) for 1 h followed by a 14 h (overnight) incubation at 4°C.
6. Rinse the skins with PBS containing antibiotic/antimycotic, place each skin with the dermis side facing down, and spread it out onto a 60-mm sterile dish. Holding the skin from the sides with forceps, scrape the surface (epidermis) using a scalpel, the blade of the scalpel must be kept perpendicular to the surface of the skin.
7. Collect the scraped epidermis using the surface of the scalpel blade in a 50-mL sterile Falcon containing 20 mL PBS (*see* **Note 3**) (**Fig. 1.1**).
8. Add 400 µL of 0.5 M EDTA (final EDTA=10 mM) and 100 µL of 1 mg/mL DNaseI (final DNaseI=5µg/mL) to the Falcon tube containing the scraped epidermis, cap the tube safely, and shake energetically up and down for a couple of times.

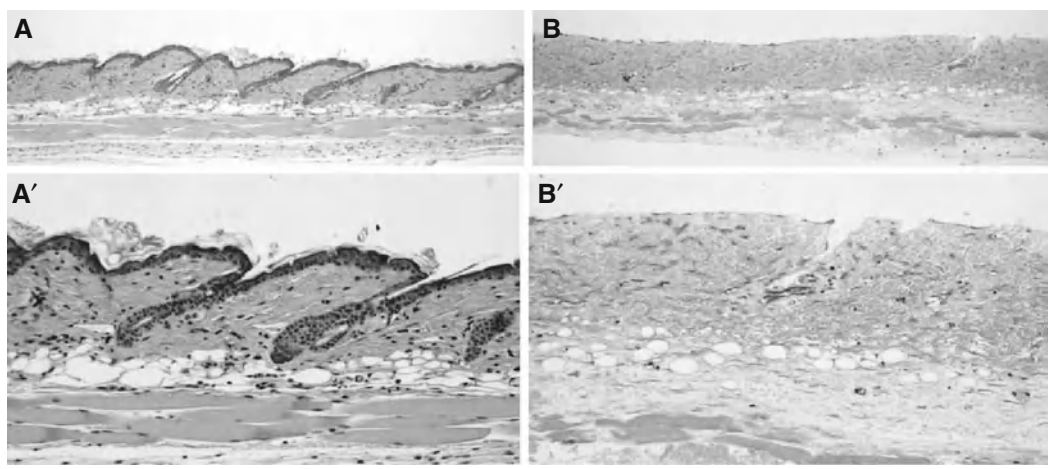

Fig. 1.1. H&E of paraffin-embedded sections from mouse dorsal skin samples. **A and A′,** skin sections from untreated mice. **B and B′,** skin sections from processed samples where the epidermal layer has been removed.Magnification: A and B, × 40; A′ and B′, × 100.

9. Transfer the contents of the Falcon to a sterile 50-mL Erlenmeyer containing a sterile stir bar of the appropriate size.
10. Place the Erlenmeyer on a magnetic stirrer and stir at medium strength for 30 min at room temperature.
11. Pipette energetically 15–20 times to disaggregate cell clusters. Pour the contents through a 40-μm cell strainer placed on a 50-mL Falcon tube. Add extra 5 mL of PBS to the Erlenmeyer to collect remainder epidermis and pour through the cell strainer.
12. Centrifuge tube at 190*g* (*see* **Note 4**) for 10 min at 4°C. Discard the supernatant and resuspend the cells in 5–10 mL of keratinocyte culture medium if they are to be used for colony formation assays or in MACS/FACS buffer if they are to be used for flow cytometry or magnetic cell sorting (*see* **Note 5**).
13. Cell yield can be evaluated by counting cells in a Neubauer or Malassez chamber. Viable keratinocytes are relatively small, round, and birefringent. Alternatively, 100 μL of the cell sample can be mixed with 100 μL 0.4% Trypan blue solution (*see* **Note 6**). Small round refringent cells exclude the dye and are scored as viable, while dark blue cells or large bright cells are non-viable. Expected cell yield ranges from 2 to 4 $\times$ 10^6 viable cells per mouse.

3.2. Immunofluorescent and Magnetic Labeling

For immunofluorescent labeling, keratinocytes should be resuspended in MACS/FACS buffer. The volume of buffer will depend on the amount of cells. The volumes and amounts described in this protocol are adapted to a cell number of 1.0–1.5 $\times$ 10^7 cells per sample. For other cell numbers, amounts and volumes should be recalculated accordingly. The following procedure can be done in 15-mL conical tubes or in 5-mL round-bottom FACS tubes. Cells must be kept cold at all times; incubations are performed on ice and centrifugations at 4°C.

1. Centrifuge tubes obtained in **Section** 3.1, **step 12** at 190*g* for 7 min at 4°C to pellet the cells. Discard the supernatant and resuspend the cells in 300 μL of MACS/FACS buffer.
2. *Important:* Put aside three aliquots (10–15 μL) in three 5-mL FACS tubes containing approximately 500,000 cells each; these will be the controls for the labeling procedure and will help to establish the cytometry settings (Tubes 1, 2, and 3: *see* **Section** 3.4.1, **Table 1.1**).
3. Continue with the tube of **step 1**. Add 30 μL of anti-CD34 and incubate for 30 min.
4. After the incubation wash the cells by adding 5 mL of MACS/FACS buffer to the tube and centrifuge at 190*g* for 7 min to pellet the cells. Discard the supernatant and resuspend the cells in 300 μL of MACS/FACS buffer.

Table 1.1.
Summary of the tubes and antibodies required for cell surface CD34 immunofluorescent and magnetic labeling.

Tube	*Sample*	*Volume of staining*	*Primary antibodies*	*FITC-labeled antibodies*	*MACS-labeled antibodies*
1	Unlabeled control	50 µL	none	none	none
2	FITC-labeled control	50 µL	none	5 µL anti-rat-FITC	5 µL anti-FITC MicroBeads
3	Isotype-labeled control	50 µL	5 µL control isotype	5 µL anti-rat-FITC	5 µL anti-FITC MicroBeads
4	Labeled sample	300 µL	30 µL anti-CD34	30 µL anti-rat-FITC	30 µL anti-FITC MicroBeads
5	CD34 negative selection	N/A			
6	CD34 positive selection	N/A			

5. Incubate for 30 min with 30 µL of anti-rat FITC antibody.
6. Wash cells by adding 5 mL of MACS/FACS buffer to the tube and centrifuge at 190*g* for 7 min to pellet the cells. Discard the supernatant and resuspend the cells in 300 µL of MACS/FACS buffer.
7. (*Optional*) Repeat washing step.
8. Incubate for 30 min with 30 µL of anti-FITC MicroBeads.
9. Wash the cells by adding 5 mL of MACS/FACS buffer to the tube and centrifuge at 190*g* for 7 min to pellet the cells. Discard the supernatant and resuspend the cells in 500 µL of MACS/FACS buffer.
10. Keep tube at 4°C. Before proceeding to the magnetic separation of the labeled sample, put aside an aliquot (15 µL) containing approximately 500,000 cells in a 5-mL FACS tube (Tube 4; *see* **Section** 3.4.1, **Table 1.1**).

3.3. Magnetic Cell Sorting (MACS)

Magnetic cell sorting is overall performed following the Miltenyi Biotec guidelines for its products. The procedure explained here is adapted for large-size (LS) columns.

1. Situate LS column into the appropriate MACS separator and place a pre-separation filter on top of the column.

2. Rinse the column by adding 3 mL of MACS/FACS buffer and discard the flow-through.
3. Apply labeled sample obtained in **Section** 3.2, **step 10** through the pre-separation filter onto the column, allow it to go through the column.
4. Rinse the tube from **Section** 3.2, **step 10** with an additional 500 μL of MACS/FACS buffer to collect remaining cells and repeat **step 3** of this section.
5. Wash the column by adding 3 mL of MACS/FACS buffer three times, allowing the buffer to go through the column each time.
6. Collect the effluent from **steps 3**, **4,** and **5** of this section in a round-bottom 13-mL tube; this is the unlabeled cell fraction. Place 600 μL of this sample in a 5-mL FACS tube for its analysis (Tube 5; *see* **Section** 3.4.1, **Table** 1.1).
7. Remove the column from the magnetic field of the MACS separator and place it on a clean 13-mL round-bottom collection tube.
8. Pipette 3 mL of MACS/FACS buffer in the column, remove the pre-separation filter, and apply firmly the plunge that is provided with the column, this will flush the labeled fraction retained in the column into the collection tube.
9. Place 400–500 μL of the sample collected in the previous **step 8** in a 5-mL FACS tube for its analysis (Tube 6; *see* **Section** 3.4.1, **Table 1.1**).

3.4. FACS Analysis

3.4.1. Preparing the Controls and Samples for FACS Analysis

This is a summary of the control and sample tubes that must be prepared during the process of immunofluorescent and magnetic labeling and subsequent magnetic cell sorting. Flow cytometry analysis of these samples will ensure adequate labeling and enrichment of the CD34-positive fraction after magnetic separation (**Fig. 1.2**).

3.4.1.1. Unstained Control: Tube 1

1. Add 600 μL of MACS/FACS buffer to a 5-mL FACS tube containing approximately 500,000 unlabeled cells.
2. Keep tube at 4°C until used.

3.4.1.2. Control of Background Fluorescence: Tube 2

1. Add MACS/FACS buffer to a 5-mL FACS tube containing approximately 500,000 unlabeled cells to make a final volume of 50 μL.
2. Incubate for 30 min with 5 μL of anti-rat FITC antibody.
3. Wash cells by adding 5 mL of MACS/FACS buffer to the tube and centrifuge at 190*g* for 7 min to pellet the cells. Discard the supernatant and resuspend the cells in 50 μL of MACS/FACS buffer.
4. Incubate for 30 min with 5 μL of anti-FITC MicroBeads.

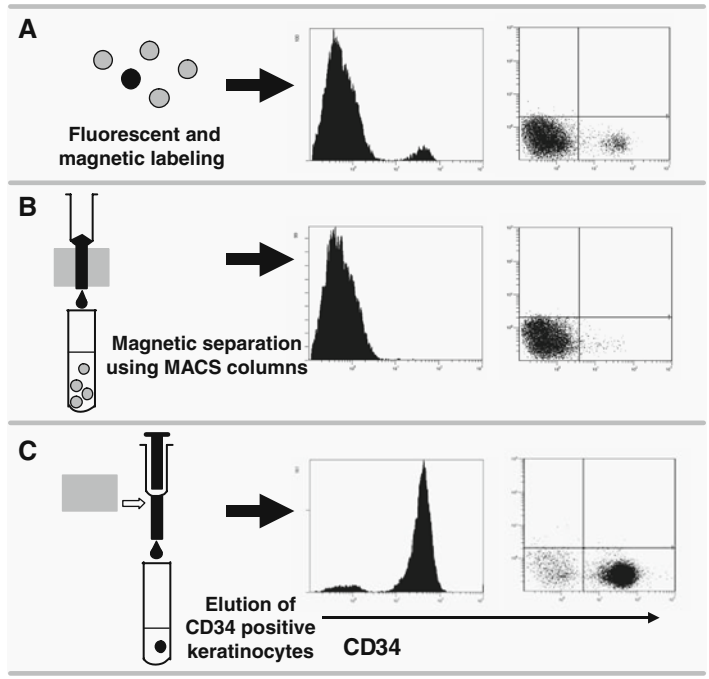

Fig. 1.2. Schematic of the process of cell labeling, magnetic separation (illustration in **A**, **B**, and **C**), and representative FACS plots for the assessment of CD34-positive keratinocytes in each fraction (represented by a solid circle in the illustration). **(A)** FACS plots showing CD34-FITC fluorescence signal of the labeled-presorted population (Tube 4). The smaller peak in the histogram plot and the events that fall in the lower left quadrant of the dot plot correspond to the CD34-positive population that represents about 8% of the total. **(B)** FACS plots corresponding to the MACS-sorted unlabeled cell fraction (Tube 5). **(C)** FACS plots corresponding to the MACS-sorted labeled cell fraction (Tube 6). Note the size of the peak in the histogram plot and the events fall largely in the lower left quadrant of the dot plot, both representing CD34-FITC-positive cells. CD34-positive cells represent an average of 80–90% of the MACS-sorted labeled cell fraction.

5. Wash the cells by adding 5 mL of MACS/FACS buffer to the tube and centrifuge at 190*g* for 7 min to pellet the cells. Discard the supernatant and resuspend the cells in 600 µL of MACS/FACS buffer.
6. Keep tube at 4°C until used.

3.4.1.3. Control Isotype: Tube 3

1. Add MACS/FACS buffer to a 5-mL FACS tube containing approximately 500,000 unlabeled cells to make a final volume of 50 µL.
2. Incubate for 30 min with 5 µL of rat-isotype control.
3. Wash cells by adding 5 mL of MACS/FACS buffer to the tube and centrifuge at 190*g* for 7 min to pellet the cells. Discard the supernatant and resuspend the cells in 50 µL of MACS/FACS buffer.

4. Incubate for 30 min with 5 μL of anti-rat FITC antibody.
5. Wash cells by adding 5 mL of MACS/FACS buffer to the tube and centrifuge at 190*g* for 7 min to pellet the cells. Discard the supernatant and resuspend the cells in 50 μL of MACS/FACS buffer.
6. Incubate for 30 min with 5 μL of anti-FITC MicroBeads.
7. Wash the cells by adding 5 mL of MACS/FACS buffer to the tube and centrifuge at 190*g* for 7 min to pellet the cells. Discard the supernatant and resuspend the cells in 600 μL of MACS/FACS buffer.
8. Keep tube at 4°C until used.

3.4.1.4. Presorted Population: Tube 4

1. Add MACS/FACS buffer to a 5-mL FACS tube containing approximately 500,000 labeled cells to make a final volume of 600 μL.
2. Keep tube at 4°C until used.

3.4.1.5. Unlabeled Cell Fraction: Tube 5

Collect 600 μL of the unlabeled cell fraction eluted from the column in a 5-mL FACS tube for its analysis.

3.4.1.6. Labeled Cell Fraction: Tube 6

Collect 400–500 μL of the labeled cell fraction eluted from the column in a 5-mL FACS tube for its analysis.

3.4.2. Overall Guidelines for FACS Analysis of the Samples

Flow cytometry analysis of the samples allows the quantification of CD34-positive cell enrichment (Tube 6) versus the depletion of this population in the negative unlabeled fraction (Tube 5) in the MACS-sorted cells. We used have a Beckman-Coulter Epics XL flow cytometer and Expo 32 ADC software to analyze the samples.

1. Cell debris are excluded from the analysis through gating in a forward versus side scatter linear scale plot and a minimum of 10,000 gated events were acquired.
2. Assessment of CD34-FITC labeling is performed by measuring FITC emission through the FL-1 channel. Histogram or dot logarithmic scale plots can be used to quantify the CD34-FITC-labeled population. If using dot plots
   a. unstained, secondary FITC-linked antibody and isotype-specific background fluorescence (Tubes 1, 2, and 3, respectively) fall in the lower left quadrant of the plot (**Fig. 1.2B**);
   b. fluorescence of CD34-FITC-labeled cells falls in the lower right quadrant of the plot. In our hands, CD34-positive cells represent an average of 80–90% of the labeled cell fraction (**Fig. 1.2C**).

4. Notes

1. When culturing keratinocytes Ca^{2+} concentration must be kept low. Chelex resin is used to remove the Ca^{2+} from the FBS:
   a. Wash the Chelex 100 resin by stirring 180 g in 500 mL Milli-Q dH_2O for 5 min. Adjust pH to 7 with HCl. Allow the resin to settle and pour off the dH_2O.
   b. Repeat the previous step adjusting pH to 7 if necessary.
   c. Add 500 mL FBS to the resin and stir for 1 h at 4°C.
   d. Allow the resin to settle, collect and filter the FBS through a 0.22-μm filter to sterilize.
2. Use lady's hair removing cream, easily found at cosmetic shops. Follow the manufacturer's recommendations regarding time of application.
3. From the moment of their isolation cells are best maintained in ice (except when other condition is stated), this will increase the viability of the cells.
4. 190*g* is approximately 1,200 rpm in a 12-cm radius floating rotor centrifuge.
5. The initial volume of medium used to resuspend and count the cells can vary depending on the initial number of mice. Also, the medium will depend on the use of the keratinocytes. We recommend to use
   a. MACS/FACS buffer if they are to be labeled for magnetic cell sorting and/or analyzed by FACS.
   b. For mass culture or clonogenic cell assays we recommend following Wu and Morris protocol (5).
6. Mix equal volumes of cell sample and Trypan blue solution, pipet the mixture gently a couple of times and immediately place cells in a Neubauer or Malassez chamber for counting. Viable cells exclude trypan solution; however, after a time dye will also enter and positively stain viable cells.

Acknowledgments

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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 suprabasal layers. Stem cells represent a minor subpopulation of the basal keratinocytes, which possess the capacity for self-renewal and is

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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 microwell 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 α6 integrin CD49f (clone GoH_3; 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. Bisbenzimide 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 µg/mL.
4. Apo-transferrin (Sigma; cat. no. T2252-100 mg): 5 µg/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 steptomycin.

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/cm^2 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/cm^2 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 α6 (*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/cm^2) 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% CO_2.

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 ($6000/cm^2$) in the keratinocyte growth medium.
2. Plate the keratinocytes at the low density of 5 keratinocytes/cm^2 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/cm^2 (*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 μm 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 ½ 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: $\mathrm{PD} = (\log N/N_0)/\log 2$, 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: $\mathrm{PD} = (\log N/N_0)/\log 2$.

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Chapter 3

Growth and Stratification of Epithelial Cells in Minimal Culture Conditions

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Abstract

Biological risk management is required in modern tissue engineering. Particular attention should be paid to the culture medium and the scaffold used. In this perspective, it is important to define minimal culture conditions which allow proper growth and differentiation of epithelial cells in vitro. We propose a simple experimental system which permits the generation of three-dimensional epidermal constructs using a collagen layer as a scaffold mimicking the entire dermal tissue and without the need of any feeder layer. Although showing significant differences compared to natural epidermis, these epidermal constructs appear useful to study keratinocyte differentiation and epidermis histogenesis.

Key words: Epidermal construct, Type I collagen, Human keratinocytes, Epithelial histogenesis, Tissue engineering, Biological risk management, Immunohistochemistry, Flow cytometry, Confocal immunofluorescence, Ultrastructural analysis.

1. Introduction

Normal histogenesis requires a network of complex interactions between different cell types and the environment where these cells live. Advances in biotechnology, including the generation of bioreactors (1), and in knowledge about molecular signaling and stem cell biology (2–6) have permitted significant successes in tissue engineering. Epithelial cells, in particular keratinocytes, have been extensively studied with regard to their capability of growing in vitro to produce epithelial tissues, including epidermis. According to classical experimental studies of tissue recombination, it has been shown that an interplay between epithelial cells and connective tissue is required in epithelial histogenesis (7–10).

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Complex in vitro systems of keratinocytes and fibroblasts have led to the generation of the first engineered organ ever made, the skin (11–17).

In these systems, putative epidermal cells grow, differentiate, and stratify under induction of connective tissue, which includes cells, extracellular matrices, growth factors, and other chemical stimuli. The precise role of a "feeder layer" made of lethally irradiated murine fibroblasts, that is used in the generation of such skin-like constructs in vitro, is still unclear.

Several studies have demonstrated that connective tissue-derived extracellular matrices are necessary to induce epidermal cell stratification in vitro. Commonly used matrices include collagens and glycosaminoglycans, such as hyaluronic acid (15, 18, 19). Moreover, it has been shown that fibroblasts in the dermal equivalent of artificial skin are responsible for de novo synthesis of matrices that have not been added to the culture (20). Conversely, epidermal cells have been shown to deposit appropriate matrices in the basement membrane at the dermal–epidermal interface, such as type IV collagen and laminin (21), thus reconstructing the dermo-epidermal junction.

A major problem in designing the strategy to engineer a tissue or a complete organ is that artificial products would be preferably suitable for clinical application in the human (reviewed by *22*). In this view, any hazardous molecule or unknown contaminant should be excluded not only in the final product but also in each step along with the engineering pathway, in particular in the culture medium. Indeed, viruses, prions, or exogenous antigens may be potentially included in the engineered construct, thus generating a hazardous or harmful situation in case of transplant in the human (23). In other words, safety should be considered a prerequisite in modern tissue engineering.

Keeping this as our goal, we decided to establish minimal experimental conditions that would allow the generation of epidermal constructs in vitro. In a series of experiments, we found that type I collagen alone was able to promote and sustain the growth and differentiation of human keratinocytes, thus leading to the generation of a model of artificial epidermis, without the need of any feeder layer (24, 25).

The generation of these epidermal constructs included three experimental stages, i.e., the isolation of keratinocyte stem cells (or other cells provided by stemness properties), the growth of epithelial colonies on the collagen substrate, and finally the generation of three-dimensional epidermal constructs.

Morphological, immunochemical, and cytokinetic features of epithelial colonies grown on the collagen layer were typical of keratinocytes and comparable to those reported for keratinocytes grown on a feeder layer (**Figs. 3.1 and 3.2**). The stratification of keratinocytes generated three-dimensional synthetic constructs

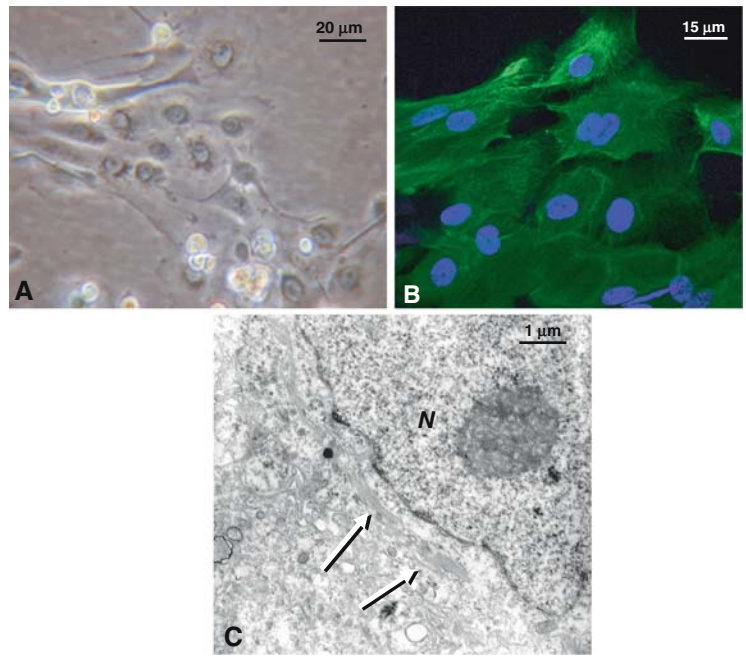

Fig. 3.1. Human keratinocytes seeded on collagen-coated coverslips. **(A)** Seven days after isolation, small cell clusters form large mosaics of polygonal and squamous cells (scale bar: 20 μm). **(B)** All cells show an intense cytoplasmic staining for cytokeratin (green fluorescence). Nuclei were stained in blue with Hoechst 33258 (bar: 15 μm). (C) Three to four weeks after isolation, ultrastructural analysis demonstrates abundant tonofilaments within the cytoplasm (arrows) of keratinocytes grown and differentiated in a three-dimensional construct (N: nucleus; scale bar: 1 μm).

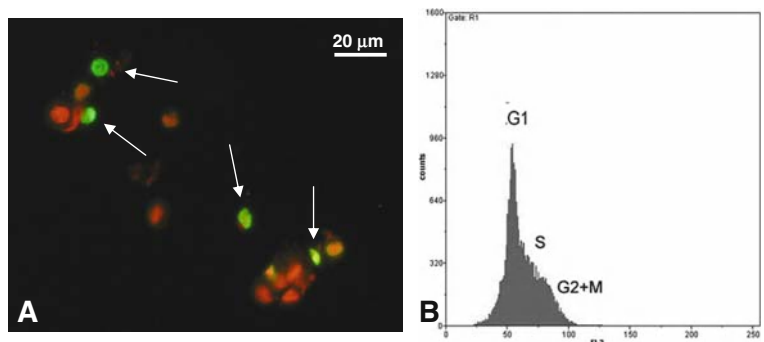

Fig. 3.2. Immunofluorescence and flow cytometry analyses of keratinocyte proliferation. **(A)** Nuclear immunolocalization of bromodeoxyuridine (green fluorescence, arrows) in keratinocytes seeded on collagen slips, whereas nuclei were stained with propidium iodide (red fluorescence, scale bar: 20 μm). **(B)** The histogram shows the distribution in different phases of the cell cycle (G0/G1, S, G2, and M) of epithelial cells after 3 days in culture medium, as revealed by DNA flow cytometry.

displaying a tissue architecture comparable to natural epidermis. Basal, intermediate, and keratinized cell layers were clearly recognizable in the constructs. Nevertheless, the number of cell layers was lower compared to natural skin, and electron microscopical analysis revealed that the overall organization of these layers was poor compared to natural epidermis, including the formation of junctional complexes, basement membrane, and keratinization. The lack of complete epithelial–mesenchymal interactions which occur during normal skin histogenesis may account for such an incomplete maturation of epidermal constructs. Immunohistochemical analysis revealed that molecular markers of cell differentiation pathway were expressed according to a normal spatial and temporal pattern. Accordingly, immature cells were integrin-positive, thus showing their attachment to the collagen matrix, and displayed proper markers of basal keratinocytes (p63 and laminin). An example result is shown in **Fig. 3.3**. Differentiated epithelial cells expressed specific markers of keratinocyte terminal differentiation, including involucrin and filaggrin. Also cytokeratin pattern expression was normal throughout different cell layers.

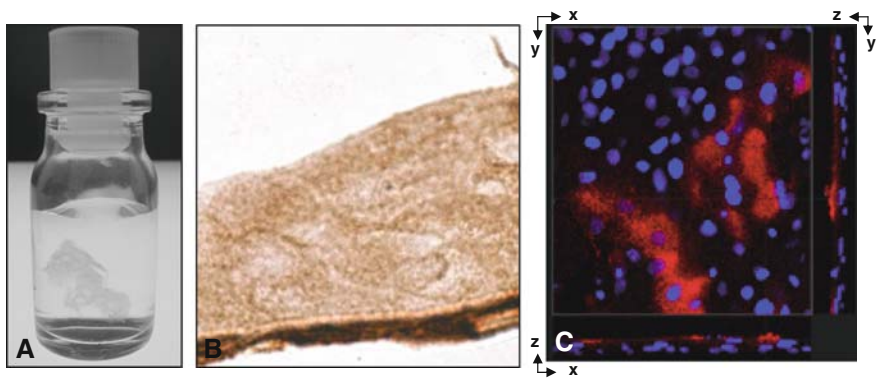

Fig. 3.3. Three-dimensional epithelial construct. **(A)** The epidermal construct is macroscopically visible as a three-dimensional opalescent sheet. **(B)** Immunostaining of the construct revealed that epithelial cells were positive for several proliferation and differentiation markers. For example, all basal cells displayed a cytoplasmic immunoreactivity for laminin (brown staining, scale bar: 10 μm). **(C)** Epidermal constructs can be observed using laser scanning confocal microscopy. Cells were stained with antibodies against proteins expressed at different stages of keratinocyte differentiation. The image was obtained by acquiring fluorescence signals at 0.3 μm intervals (section thickness 10 μm). Example of this analysis is shown in (C), where basal cells show a cytoplasmic immunostaining for laminin (red fluorescence). Nuclear DNA was stained with Hoechst 33258 (blue fluorescence). *x–y* cross-section shows the cytoplasmic expression of laminin in the cells of the lower layer alone. An orthogonal projection of two cross-sectional cuts *x–z* and *y–z* is also shown (upper side of the projections is the lower basal layer of the epidermal construct).

Although unexpected, our results suggest that simplified experimental conditions may be sufficient to produce epithelial tissues in vitro. Each protocol in tissue engineering should be

checked to define exactly which steps are essential to generate the designed tissue and which step may be hazardous and should be modified, according to a risk management approach.

2. Materials

2.1. Cell Culture

1. Epidermal keratinocytes isolated by human skin biopsies obtained from clinically healthy skin from different body areas from donors of different age
2. Protease Type IX (Sigma-Aldrich, St. Louis, MO; cat. No. P-6141)
3. Trypsin 1X type III 0.05% with ethylenediaminetetraacetic 0.01% (Eurobio, Les Ulis, Cedex B, France; cat. no. 492015)
4. Collagen type I from calf skin (Sigma-Aldrich, St. Louis, MO; cat. no. C9791)
5. Culture flasks and coverslips previously coated with bovine collagen type I (Sigma): 10 mg of calf skin in 2 ml of 0.1 M acetic acid and 8 ml of sterile water
6. Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO; cat. no. D5671)
7. Ham's F12 Medium (Sigma-Aldrich, St. Louis, MO; cat. no. N4888)
8. Fetal bovine serum (BioWest, Nuaille, France; cat. no. S1810)
9. Hydrocortisone (Sigma-Aldrich, St. Louis, MO; cat. no. H-0135)
10. Insulin (Sigma-Aldrich, St. Louis, MO; cat. no. I-0516)
11. Apo-transferrin, bovine (Sigma-Aldrich, St. Louis, MO; cat. no. T1428)
12. 3,3′,5′-Triiodo-L-Thyronine free acid T3 (Sigma-Aldrich, St. Louis, MO; cat. no. T0281)
13. L-Glutamine 200 mM stock solution (Eurobio, Les Ulis, Cedex B, France; cat. no. 010129)
14. Sodium pyruvate 100 mM (Sigma-Aldrich, St. Louis, MO; cat. no. S8636)
15. Penicillin 10,000 Ul/ml + Streptomycin 10,000 Ul/ml (Eurobio, Les Ulis, Cedex B, France; cat. no. CABPES01-0U)
16. Epidermal growth factor (EGF) 10 ng/ml (human recombinant, Sigma-Aldrich, St. Louis, MO; cat. no. E-9644)

2.2. Reagents for CEC Medium Preparation

2.2.1. Collagen Type I Solution

1. Dissolve 10 mg of calf skin collagen type I in 2 ml of 0.1 M acetic acid
2. Add 8 ml of sterile water
3. Shake the solution to dissolve all substances

2.2.2. Insulin Solution

To prepare insulin solution at 5 mg/ml concentration, dissolve 500 mg insulin powder in 100 ml HCl 0.005 N (0.5 ml in 100 ml distilled H_2O). Filter solution with 0.2 μm filter.

2.2.3. Apo-transferrin/ 3,3′,5′ -Triiodo-L-Thyronine T3 Mixture

1. Sol. A: dissolve 500 mg Apo-transferrin in 60 ml PBS.
2. Sol. B: dissolve 13.6 mg T3 in minimum volume NaOH 0.02 N (2 ml NaOH 1 N in 98 ml PBS) and add distilled H_2O until 100 ml final volume.
3. Apo-transferrin 5 mg/ml/T3 1.36 μg/ml mixture: mix 60 ml Sol. A with 1 ml Sol. B and add PBS until 100 ml. Filter solution with 0.2 μm filter.

2.2.4. Hydrocortisone Solution

1. Dissolve 25 mg hydrocortisone powder in 5 ml ethanol 95%.
2. Withdraw 4 ml of this solution and add DMEM medium until 100 ml final volume to have a stock solution of 0.2 mg/ml concentration.
3. Filter solution with 0.2 μm filter.

2.2.5. Epidermal Growth Factor

Dissolve 0.1 mg EGF powder in 10 ml distilled H_2O to have a stock solution of 0.02 mg/500 μl concentration (*see* **Note 1**).

2.3. CEC+ Medium Preparation

1. Mix DMEM medium and Ham's F12 medium in the ratio of 3:1.
2. Add 10% fetal bovine serum; 1 mg/ml L-Glutamine 200 mM; 0.11 mg/ml 100 mM sodium pyruvate; 5 μg/ml Apo-transferrin/1.36 ng/ml T3 mixture; 5 μg/ml insulin; 0.4 μg/ml hydrocortisone; 1% penicillin/streptomycin 10,000 Ul/ml; 10 ng/ml EGF.
3. Filter the limpid red solution.
4. Conserve CEC+ medium at +4°C (*see* **Note 2**).

2.4. Tissue and Antigen Investigation

1. Human skin biopsies obtained from clinically healthy skin from different body areas from donors of different age
2. Monoclonal antibody to pan-cytokeratin, clone LU5 (BMA, Augst, Switzerland; cat. no. T-1302)
3. Monoclonal antibody to vimentin, clone V9 (Biogenex, San Ramon, CA, USA; cat. no. 074 M)
4. Monoclonal antibody to filaggrin, clone FLG01 (Oncogene, USA; cat. no. CP71)

5. Monoclonal antibody to p63, clone 4A4 (Oncogene, USA; cat. no. OP132)
6. Monoclonal antibody to involucrin, clone SY5 (Sigma-Aldrich, USA; cat. no. I-9018)
7. Monoclonal antibody to laminin, clone LAM-89 (Sigma-Aldrich, USA; cat. no. L8271)
8. Monoclonal antibody to integrin β1 subunit, clone JB1 (Biogenex, San Ramon, CA, USA; cat. no. 298 M)
9. Monoclonal antibody to integrin α2β1, clone BHA2.1 (Chemicon, USA; cat. no. MAB1998)
10. Monoclonal antibody to 5-bromo-2′-deoxyuridine, clone BU-1 (Amersham Biosci, UK; cat. no. RPN202)

2.5. Immuno cytochemistry and Immunohistochemistry

1. Phosphate-buffered normal saline PBS (Sigma-Aldrich, St. Louis, MO; cat. no. P4417)
2. Tween 20 (Sigma-Aldrich, St. Louis, MO; cat. no. P-1379)
3. Bovine serum albumin, BSA (Sigma-Aldrich, St. Louis, MO; cat. no. A9647)
4. Hoechst 33258 dye (Sigma-Aldrich, St. Louis, MO; cat. no. B-2883)
5. Mowiol 40-88 (Sigma-Aldrich, St. Louis, MO; cat. no. 32,459-O)
6. Alexa 594 phalloidin anti-mouse antibody (Molecular Probes, Eugene, Oregon, USA; cat. no. A-12381
7. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Sigma-Aldrich, St. Louis, MO; cat. no. F-5387)
8. Paraformaldehyde powder (BDH, UK; cat. no. 29447)
9. Paraffin wax, pastillated, congealing point 56°C (BDH, England; cat. no. 361077E)
10. Pepsin A, from porcine stomach mucosa (Sigma-Aldrich, Germany; cat. no. P7125)
11. Normal goat serum (NGS; Sigma-Aldrich, Germany; cat. no. G9023)
12. Harris' hematoxylin and eosin G (BDH, England; cat. no. 351945S)
13. Supersensitive link-label IHC detection system (Streptavidin-biotinylated peroxidase complexes; Super Sensitive kit, BioGenex; cat. no. QP900-9L)
14. 3,3′-Diaminobenzidine tetrahydrochloride, DAB (liquid DAB BioGenex; cat. no. HK153-5 K)
15. Phosphate-buffered normal saline (PBS) 0.1 M, pH 7.4: Sol. A: 5.52 g potassium dihydrogen phosphate (NaH_2PO_4 H_2O). Dissolve in 200 ml water. Sol. B: 28.48 g disodium

hydrogen phosphate (Na_2HPO_4 $2H_2O$). Dissolve in 800 ml water. To use, mix Sol. A with Sol. B (1:4) to obtain 0.2 M buffer. Dilute 1:1 with water and check pH for a 0.1 M buffer.

16. 0.05 M Tris-buffered normal saline (TBS), pH 7.4: 6.07 g Tris-(hydroxymethyl)methylamine (Tris base), 8.7 g sodium chloride. Dissolve in 900 ml deionized water. Add concentrated hydrochloride acid HCl (12–14 ml) until pH reaches 7.4 and make up to 1 l with water.
17. Phosphate-buffered normal saline + Tween20 + Albumin (PTA, pH 7.4): 1 PBS pastillated 0.2 M (Sigma) in 200 ml water + 0.02% Tween20 + 1% bovine serum albumin.
18. 0.05 M Tris–HCl, pH 7.4 (substrate buffer): 6.61 g Tris–HCl, 0.97 g tris base. Dissolve in 900 ml deionized water. Adjusted pH with 1 N HCl and make up to 1 l with water.
19. Paraformaldehyde buffered 4% at pH 7.4 within PBS 0.1 M: dissolve 8 g paraformaldehyde powder in 100 ml water with stirring. Heat to 55°C and add 1 N NaOH, one drop at a time, until the solution becomes clear. Cool at room temperature and add 100 ml PBS 0.2 M, pH 7.4, to reach a final concentration of 0.1 M.
20. 0.01 M Citrate buffer, pH 6.0: 1.9 g citric acid. Dissolve in 900 ml water, then add 2 M NaOH to adjust pH 6.0 make up to 1 l with water. The buffer can be kept at room temperature for several days.
21. Diluent of primary antibody and link antibody: 0.1 g BSA, 0.01 g sodium azide (NaN_3). Dissolve in 10 ml TBS, pH 7.4.
22. DAB solution: dissolve 1 mg DAB in 2 ml substrate buffer to obtain a final DAB concentration of 0.05%. Add 10 µl hydrogen peroxide (H_2O_2) 3%, just before use (final concentration 0.01–0.02%). Make fresh solution each time and use within 10 min (*see* **Note 3**).
23. DPX mounting medium (EMS, Rome, Italy; cat. no. 13512).

2.6. Transmission Electron Microscopy Analysis

1. Glutaraldehyde stock solution 25% (BDH, England; cat. no. 360802F).
2. Paraformaldehyde buffered 4% at pH 7.4 within PBS 0.1 M: dissolve 8 g paraformaldehyde powder (BDH, UK; cat. no. 29447) in 100 ml water with stirring. Heat to 55°C and add 1 N NaOH, one drop at a time, until the solution becomes clear. Cool at room temperature and add 100 ml PBS 0.2 M, pH 7.4, to reach a final concentration of 0.1 M.
3. Sodium cacodylate buffer 0.1 M, pH 7.4 (BDH, England; cat. no. 301183 V).
4. Osmium tetroxide (EMS, Washington, PA 19034, USA; cat. no. 19110).

5. Epoxy embedding medium, Accelerator (Fluka, Seelze, Germany; cat. no. 45348).
6. Epoxy embedding medium, hardener DDSA (Fluka, Seelze, Germany; cat. no. 45346).
7. Epoxy embedding medium, hardener NMA (Fluka, Seelze, Germany; cat. no. 45347).
8. Epoxy embedding medium (Fluka, Seelze, Germany; cat. no. 45345).
9. Collidine (2,4,6-Trimethylpyridine, Merk, Darmstadt, Germany; cat. no. 0250).
10. Uranyl acetate EM (TAAB, Aldermaston; cat. no. UOO6).
11. Lead citrate (EMS, Rome, Italy; cat. no. 17800).
12. Sodium cacodilate buffer: dissolve 21.4 g $Na(CH_3)_2AsO_2$ $3H_2O$ in 250 ml distilled H_2O (Sol. A). Mix 50 ml Sol. A with 8 ml HCl 0.2 M until it reaches pH 7.2. Add distilled H_2O until 100 ml final volume.
13. Diluted Karnovsky solution (KD): Mix 65 ml 4% buffered paraformaldehyde with 50 ml sodium cacodilate buffer. Add 25 ml 25% glutaraldehyde solution and 15 ml 5% $CaCl_2$. Add distilled H_2O until 250 ml final volume and pH 7.35 (*see* **Note 4**).
14. Collidine buffer: mix 2.67 ml collidine $C_8H_{11}N$ with 60–70 ml distilled H_2O. Add 9 ml HCl 1 N (pH 7.4). Add distilled H_2O until 100 ml final volume.
15. Osmium tetroxide solution: dissolve 1 g osmium OsO_4 in 25 ml distilled H_2O and conserve at +4°C. To use the solution, add 25 ml collidine buffer and 25 ml distilled H_2O.
16. Uranyl acetate solution: saturated uranyl acetate solution in distilled H_2O.
17. Reynold's lead citrate solution: dissolve in 30 ml distilled H_2O 1.33 g $Pb(NO_3)_2$ and 1.76 g sodium citrate $C_6H_5Na_3O_7$ $2H_2O$. Add 8 ml NaOH 1 N until it reaches a clear solution. Keep in the dark and conserve at room temperature.
18. Epon mix: Sol. A: mix 62 ml Epon with 100 ml DDSA and conserve at +4°C. Sol. B: mix 100 ml Epon with 89 ml NMA and conserve at +4°C. To use, mix Sol. A with Sol. B 1:1 and add 0.2 ml accelerator for each 10 ml resin.

2.7. Cytometric Analysis

1. Hydrochloric acid 37% (Carlo Erba, Rodano, Italy; cat. no. 403871)
2. Sodium tetraborate (Merck; cat. no. 6306)
3. Bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO; cat. no. A9647)

4. Propidium iodide (5 μg/ml; Calbiochem, CA; cat. no. 537059)
5. RNase type I 1A, free DNase (50 Kunitz U/ml; Sigma-Aldrich, St. Louis, MO; cat. no. R7397)
6. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich, St. Louis, MO; cat. no. F-5387)
7. Rinse buffers and antibodies diluent (PTA) or phosphate-buffered normal saline + Tween20 + albumin (PTA) pH 7.4: 1 PBS pastillated 0.2 M (Sigma) in 200 ml water + 0.02% Tween20 + 1% bovine serum albumin. Make fresh solution each time

2.8. Instruments

1. Light microscope (Axiophot, Zeiss, Germany).
2. Zeiss Axiophot fluorescence microscope (Carl Zeiss, Oberkochen, Germany).
3. Leica TCS SP2 confocal microscope (Heidelberg, Germany).
4. Zeiss EM10 electron microscope (Carl Zeiss, Oberkochen, Germany).
5. Partec PAS II flow cytometer (Partec, Munster, Germany).
6. Whirlpool microwave oven (model MT232, 750 W).

3. Methods

3.1. Cell Culture Procedures: Isolate Epidermal Keratinocytes from Human Skin Biopsies

1. Cut human skin biopsies into small fragments (~2 × 2 cm).
2. Digest with 2.5 mg/ml protease type IX at 4°C for 24 h to separate the epidermis from dermal layer, according to Häkkinen et al. (26).
3. Peel the epidermal sheet from the underlying dermis and trypsinize for 10 min at room temperature, to dissociated single keratinocytes.
4. Centrifuge the isolated cells at 200*g* for 10 min at 15°C and re-suspend gently the pellet of keratinocytes in the CEC growth medium with low Ca^{2+} concentration (27).
5. Seed the keratinocytes in culture flask (25 cm^2) or on coverslip (12 mm Ø), previously coated with bovine collagen type I, at a final seeding cell density of approximately 10–20 × 10^6 viable cells per well and 2–3 × 10^5 per coverslip.
6. Incubate cell cultures at 37°C in 5% carbon dioxide with CEC+ medium.
7. Change the medium every 2 days, adding 10 ng/ml EGF.

3.2. BrdU Incorporation

1. Incubate living keratinocytes for 30 min with 5 ml containing 30 μM BrdU to label S phase cells.
2. Wash with 0.1 M PBS and proceed with the harvesting and fixation with 70% ice ethanol.

3.3. Immuno cytochemistry for Stemness and Differentiation Antigens

This section describes the immunocytochemical method used to detect expression of typical stemness and differentiation proteins in epidermal cells grown on collagenated-coverslips in vitro.

1. Fix cells seeded on coverslips with 4% buffered paraformaldehyde for 15 min; then, rinse well with PBS for 3 min.
2. Post-fix in 70% ethanol for 30 min and wash in PBS for 5 min twice.
3. Dip the coverslips within a PTA solution for 20 min at room temperature, to reduce non-specific background staining.
4. Incubate the cells seeded on coverslips with primary antibody (at specific dilution in PTA buffer, *see* **Note 5**) at room temperature for 1 h.
5. Rinse well with PTA solution for 10 min twice.
6. Incubate with secondary antibody conjugated with fluorochrome (at dilution 1:100 in PTA) at room temperature for 30 min, then wash in PTA for 10 min three times.
7. Wash again with PBS for 5 min twice.
8. Incubate with Hoechst 33258 solution (concentrated 0.5 μg/ml PBS) to counter stain nuclear DNA.
9. Wash with PBS for 10 min three times.
10. Mount with Mowiol solution (*see* **Note 6**).

3.4. Immuno histochemistry for Cell Differentiation and Proliferation Antigens

This section describes the immunohistochemical method used to detect expression of typical proliferation and differentiation proteins in natural skin and three-dimensional epidermal constructs sections.

3.4.1. Sample Preparation

1. Detach gently (using a scalpel) three-dimensional epidermal constructs from the flask.
2. Wash the specimens with PBS 0.1 M and fix with the fixative solution pH 7.4 (4% buffered paraformaldehyde) for 6 h.
3. Wash in TBS three times for 15 min and start to dehydrate through graded alcohols: 70% ethanol for 2 h; 85% ethanol for 2 h; 100% ethanol for 2 h; xylene 10 min.
4. Embed samples in liquid paraffin.
5. Cut paraffin sections (5–10 μm). Float on warm (not hot) water and pick up on poly-L-lysine coated slides. Dry for several hours at 37°C.

6. Remove paraffin in two changes of xylene and take through graded alcohol to water.
7. Block endogenous peroxidase: treat with 0.3% H_2O_2 in water for 30 min (a shorter time may be adequate).
8. Place slides in TBS and proceed with immunostaining according to indirect immunoperoxidase protocol ((28, 29), *see* **Note 7**).

3.4.2. Staining Procedure for Paraffin Sections with Monoclonal Antibody to BrdU

1. Dip the section within a solution of 0.5% pepsin in 0.01 M HCl for 30 min at 37°C (*see* **Note 8**).
2. Wash well with tap water for 5 min, then wash in TBS for 5 min.
3. In the humidified box treat with NGS 1:20 in TBS for 30 min to reduce non-specific background staining; then remove excess serum from sections.
4. Incubate the section with primary antibody (at dilution 1:500 in specific buffer) at 4°C overnight in a humidified chamber, then rinse well with TBS for 10 min three times.
5. Incubate with link antibody (biotinylated anti-immunoglobulins), diluted 1:50 in buffer, at room temperature for 20 min, and then rinse well with TBS for 10 min three times.
6. Incubate with peroxidase-labeled streptavidin (at dilution 1:50 in specific diluent) at room temperature for 20 min, then rinse well with TBS for 10 min three times.
7. Add enough DAB solution to cover completely the sections and incubate for 3–5 min.
8. Wash with TBS for 10 min and then counterstain the section with hematoxylin.
9. Wash slides with tap water for 10 min and dehydrate the sections through graded alcohol and xylene.
10. Mount the sections in permanent DPX mounting medium.

3.4.3. Staining Procedure for Paraffin Sections with Antibodies to Pan-CK, Laminin, and $\alpha 2\beta 1$ Integrin

1. Pre-treat the paraffin sections with protease (0.5% pepsin in 0.01 M hydrochloric acid or 0.1% trypsin in distilled water, pH 7.6) for 10–30 min at 37°C.
2. Wash two to three times with TBS for 5 min.
3. In the humidified box treat with NGS 1:20 in TBS for 30 min to reduce non-specific background staining.
4. Remove excess serum sections and incubate with antibody at 4°C overnight. Mouse monoclonal antibodies to Pan-cytokeratin, diluted 1:400; Laminin, diluted 1:2000; α2β1 Integrin, diluted 1:50 (*see* **Note 9**).
5. Rinse well with TBS for 10 min three times.

6. Incubate with link antibody (biotinylated anti-immunoglobulins), diluted 1:50 in buffer at room temperature for 20 min.
7. Wash in TBS buffer three times for 10 min.
8. Incubate with horseradish streptavidin-peroxidase (diluted 1:50) at room temperature for 20 min, and then wash in TBS buffer for 10 min three times.
9. Add enough DAB solution to cover completely the sections and incubate for 3–5 min.
10. Wash with TBS for 10 min and counterstain the section with hematoxylin.
11. Rinse slides with tap water for 10 min.
12. Dehydrate the sections through graded alcohol and xylene.
13. Mount the sections with permanent medium.

3.4.4. Staining Procedure for Paraffin Sections with Antibodies to Filaggrin, p63, and Integrin β1 Subunit

1. Place the slides in a plastic slide carrier and immerse them completely in 0.01 M citrate buffer.
2. Microwave a 750 W for 5 min (the buffer must boil) for antigen retrieval, according to the antibody supplier's protocol (30).
3. Let the buffer cool down until it is possible to remove slides without danger of buffer salts precipitating upon the sections as they dry. Then, wash in TBS buffer for 5 min twice.
4. In the humidified box, treat with NGS 1:20 in TBS for 30 min to reduce non-specific background staining; then, remove excess serum from sections.
5. Incubate with antibody at 4°C overnight. Mouse monoclonal antibodies to Filaggrin antigen, diluted 1:200 in specific buffer; p63 antigen, diluted 1:1000; Integrin β1 subunit, diluted 1:100.
6. Rinse well with TBS for 10 min three times.
7. Incubate with link antibody (biotinylated anti-immunoglobulins), diluted 1:50 in buffer at room temperature for 20 min.
8. Wash in TBS buffer three times for 10 min.
9. Incubate with horseradish streptavidin-peroxidase (diluted 1:50) at room temperature for 20 min, and then wash in TBS buffer for 10 min three times.
10. Add enough DAB solution to cover completely the sections and incubate for 3–5 min.
11. Wash with TBS for 10 min and counterstain the section with hematoxylin.

12. Rinse slides with tap water for 10 min.
13. Dehydrate the sections through graded alcohol and xylene.
14. Mount the sections with permanent medium.

3.4.5. Staining Procedure for Paraffin Sections with Antibodies to Vimentin and Involucrin

1. In the humidified box, treat with NGS 1:20 in TBS for 30 min to reduce non-specific background staining; then, remove excess serum from sections.
2. Incubate with antibody at 4°C overnight. Mouse monoclonal antibodies to Vimentin, diluted 1:400 in specific buffer; Involucrin, diluted 1:200.
3. Rinse well with TBS for 10 min three times.
4. Incubate with link antibody (biotinylated anti-immunoglobulins), diluted 1:50 in buffer at room temperature for 20 min.
5. Wash in TBS buffer three times for 10 min.
6. Incubate with horseradish streptavidin-peroxidase (diluted 1:50) at room temperature for 20 min, and then wash in TBS buffer for 10 min three times.
7. Add enough DAB solution to cover completely the sections and incubate for 3–5 min.
8. Wash with TBS for 10 min and counterstain the section with hematoxylin.
9. Rinse slides with tap water for 10 min.
10. Dehydrate the sections through graded alcohol and xylene.
11. Mount the sections with permanent medium.

3.5. Staining Procedure for Ultrastructural Analysis

3.5.1. TEM Processing

1. Primary fixation for ultrastructural analysis: dip the section within the diluted Karnovsky's solution at +4°C for 4 h.
2. Wash well with cacodylate buffer 0.2 M (pH 7.4) for 15 min three times.
3. Post-fixation: dip the sections within the osmium tetroxide solution at +4°C for 1 h.
4. Dehydrate through a series of ethanols and propylene oxide: 25% ,50%, 70%, 96% ethanol (each for 15 min at +4°C), 100% ethanol (three times for 15 min at +20°C), and propylene oxide (three changes, each of 15 min) (*see* **Note 10**).

3.5.2. Resin Embedding Protocol for Ultrastructural Analysis (Epon Mix)

1. Resin infiltration: 2:1 mix of propylene oxide:resin for 1 h
2. 1:1 mix of propylene oxide:resin for 1 h
3. 1:2 mix of propylene oxide:resin for 1 h
4. 100% resin overnight
5. Change in to fresh resin for 1 h
6. Fresh resin in embedding moulds at +70°C for 48 h (*see* **Note 11**)

3.5.3. Cutting Ultrathin Sections

1. Cut with ultramicrotome in thick sections of 80–90 nm
2. Put the sections on nickel grids of 200 mesh
3. Stain with aqueous uranyl acetate for 20–30 min +20°C in dark (*see* **Note 12**)
4. Wash in distilled H_2O for 10 min
5. Dip in lead citrate solution for 10 min
6. Wash in distilled H_2O for 10 min
7. Dry with air

3.6. Staining Procedure for Cytometric Analysis

1. Trypsinize the epidermal cells grown for different days on flasks coated with collagen type I.
2. Centrifuge cell suspension at 400*g* for 3 min.
3. Fix the pellet with 70% ethanol at –20°C for 10 min and conserve at –20°C.

3.6.1. Propidium Iodide DNA Staining

1. Centrifuge fixed epidermal cells at 400*g* for 3 min.
2. Wash pellet with PBS for 10 min and then centrifuge at 400*g* for 3 min.
3. Stain pellet with a solution of 50 µg/ml propidium iodide, 0.1% Nonidet-P-40, and type 1A RNase (50 Kunit U/ml) in PBS for 30 min at room temperature.
4. Conserve samples at 4°C for about 24 h.
5. Before flow analysis, filter cells to remove aggregates.

3.6.2. Biparametric Analysis by Flow Cytometry of BrdU vs Propidium Iodide

1. To detect BrdU, centrifuge the fixed cell suspension at 400*g* for 3 min.
2. Wash pellet with PBS for 5 min and then centrifuge at 400*g* for 3 min.
3. Treat the pellet with 1 ml HCl 2 N for 30 min at room temperature to denature the DNA.
4. Neutralize with 1 ml sodium tetraborate 0.1 M for 15 min, then centrifuge for 3 min at 400*g* at room temperature.
5. Wash with PBS for 10 min, centrifuge and incubate pellet with PTA for 30 min to reduce non-specific background staining.
6. Centrifuge for 3 min at 400*g* at room temperature; then, incubate the pellet with BrdU-antibody diluted 1:100 in PTA for 1 h at room temperature. Shake each tube containing samples every 10 min to avoid sedimentation.
7. Add 1 ml PTA in each tube and centrifuge for 3 min at 400*g* at room temperature; then remove supernatant and rinse pellet well with PTA for 10 min twice.

8. Incubate cells with secondary antibody FITC-conjugate goat anti-mouse, diluted 1:100 in PTA, for 30 min and shake each tube with samples every 10 min to avoid sedimentation.
9. Add 1 ml PTA in each tube and centrifuge for 3 min at 400*g* at room temperature; then, remove supernatant and rinse the pellet well with PTA for 10 min twice.
10. Centrifuge for 3 min at 400*g* and incubate pellet with a solution of 5 μg/ml propidium iodide, 0.1% Nonidet-P-40, and type 1A RNase (50 Kunitz U/ml) in PBS for 30 min at room temperature to stain DNA.
11. Store samples at 4°C for about 24 h.
12. Before flow analysis, filter cells to remove aggregates using filter.

3.7. Quantitative Evaluation of Flow Cytometric Data

3.7.1. Single-Parameter DNA Measurements

1. For single-parameter DNA measurements, analysis at least 10,000 cells for each sample using flow cytometer (*see* **Note 13**).
2. Display the data as frequency histograms, showing the measurements of cell DNA content versus total number of cells. In this way, it is possible to know the number of cells in G1, S, G2+M phases of cell cycle and measure the growth fraction of S phase cell.
3. Collect data with the program software running on a computer system displaying results as mono parameter DNA profile in the different phases of cell cycle.
4. Compare the results related at cells in different time to isolation.

3.7.2. Biparametric Analysis

1. Analyze with a flow cytometry and measure for each sample a total of $10–20^4$ cells.
2. Perform with Partec II cytometer two-parameter flow Cytometry analysis (FITC-green vs propidium iodide-red) of BrdU (or another proliferation marker, i.e., PCNA, Ki-67, etc.) incorporation and of nuclear DNA content.
3. Evaluate fluorescence intensity signals of BrdU immunostaining in each phase of the cell cycle by gating the cells according to their DNA content value, as determined by PI fluorescence.
4. Use electronic gating provided by instruments software program (Consort 30 software) for exclusion of cell doublets.
5. Collect data with the program software running on a computer system displaying results as dual-parameter contour density plots. Plots show the cell cycle distribution of immunofluorescence relative to specific protein in the different phases of cell cycle (G1, S, G2+M phases).

4. Notes

1. Do not filter solution.
2. CEC medium is CEC+ medium without fetal bovine serum.
3. *Caution*: DAB is classified as potential carcinogens and can cause skin irritation upon contact.
4. Respect the transitional sequence, step by step.
5. Specific dilution for primary antibodies: 1:100 for vimentin, filaggrin, p63, involucrin, integrin β1 subunit; 1:200 for pan-cytokeratin, laminin, and integrin α2β1 subunit. All antibodies may be diluted in PTA buffer.
6. The slides are viewed under phase contrast microscopy (to describe cell morphology) or fluorescence microscopy (to locate the cells and identify the focal plane). Anyway, the three-dimensional epidermal construct can also be analyzed under laser scanning confocal microscopy.
7. TBS or methanol may be used instead of water to dilute H_2O_2. Methanol is itself a blocker of peroxidase and may be used with up to 3% H_2O_2 for persistent enzyme activity.
8. For staining procedure with monoclonal antibody to BrdU it is possible to pre-treat the sections with HCl or, in alternative, pre-digest them with nuclease.
9. All antibodies may be diluted in specific buffer.
10. Since propylene oxide is much more volatile than ethanol or acetone be careful not to allow the sample to be exposed to the air as damage will occur due to the rapid evaporation of the solvent.
11. Remember to label the samples.
12. Must be carried out in the dark as uranyl acetate is photo-reductive and will precipitate.
13. It is possible to analyze the cells with a Partec II. However, other flow cytometers may be used, obviously. The instrument is equipped with an argon laser tuned at 488 nm for fluorescence excitation and electronic correction for doublets was used. Statistical analysis of the percentage of cells in each phase of the cell cycle was performed by the software provided with each instrument.

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Chapter 4

Matched Cultures of Keratinocytes and Fibroblasts Derived from Normal and NER-Deficient Mouse Models

Alex Pines and Claude Backendorf

Abstract

The uppermost layer of our skin, the epidermis, is formed largely of keratinocytes which constitute the skin's major barrier function and the first line of defence against environmental physical, chemical and biological agents. The subsequent layer, the dermis, which is mainly formed by fibroblasts, has a more supportive function, containing large amounts of collagen, blood vessels and nerve endings and is less directly affected by external insults. Hence it is likely that keratinocytes and fibroblasts have evolved different strategies to cope with the dangers of the environment. Mouse models with various genetic backgrounds in genome care-taking systems, such as DNA repair processes, are well suited to study differences between these two cell types and their implications for cancer and aging. In this chapter we describe a simple procedure to establish long-term keratinocyte and fibroblast cultures from, respectively, the epidermis and dermis of normal or NER-deficient newborn mice. The importance of the external O_2 pressure during the establishment and maintenance of these matched cultures is discussed.

Key words: Murine long-term cell cultures, Keratinocyte, Fibroblast, Calcium, Oxygen, KGF, EGF, Proliferation, Differentiation.

1. Introduction

The epidermis and dermis of the skin are constituted predominantly of keratinocytes and fibroblasts, respectively. Keratinocytes constitute the outermost living cell layer of our body. They play an essential role at various physiological levels, such as the maintenance of the body's water balance, the communication with the outside world via their immunological and sensory functions, but above all the protection of the organism from adverse environmental impacts (either physical, chemical, mechanical or microbial). The impact of

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UV light and UV-induced DNA damage on these cells is best demonstrated by the human syndrome xeroderma pigmentosum (XP), which is caused by severe deficiencies in nucleotide excision repair (NER). XP patients show hypersensitivity to UV light and a high incidence of keratinocyte-derived skin cancers such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) in sun-exposed parts of the body. The complexity of the disease is reflected by the identification of seven complementation groups (from A to G) and a post-replication repair-deficient variant group (XPV) (1). Deficiencies in NER can also lead to the premature aging disorders as for instance in Cockayne syndrome (CS) and trichothiodystrophy (TTD) (2). The majority of the studies on DNA damage responses have been performed by using human skin fibroblasts with the idea to extrapolate this knowledge to other cell types. However, only few studies have expanded the analysis to keratinocytes although these cells are more relevant as far as the cancer incidence is concerned (3–6), certainly in comparison to dermal fibroblasts. Nevertheless, these cell type-specific differences in the molecular processes that protect against cancer and aging are interesting to study in order to fully appreciate the protective functions that are specifically necessary to keratinocytes due to their environmentally exposed position within our body. A large number of mouse models have been generated to study the impact of NER deficiencies in vivo, and data, obtained from different NER-deficient long-term in vitro keratinocyte cultures, were consistent with and extended in vivo observations (7–9). Only more recently we have directly compared keratinocyte with fibroblast cultures derived from the same knock-out mouse (6).

The establishment of murine keratinocyte cultures that can be passaged and stored by freezing, while retaining their proliferation potential and differentiation capacity, in the same way as shown already in 1975 for their human counterparts (10), was difficult until recently (7, 11, 12). In this chapter we describe a simple procedure to establish long-term keratinocyte and fibroblast cultures from, respectively, the epidermis and dermis of normal or NER-deficient newborn mice. For the isolation, maintenance and differentiation of keratinocytes derived from mouse skin the calcium concentration in the culture medium is especially important. Calcium is indeed a major determinant of the differentiation state of mammalian keratinocytes, which is reflected by the presence of a calcium gradient in the epidermis in vivo (13). Low calcium concentrations (0.05–0.07 mM) are required to establish and maintain both short-term (14) and long-term keratinocyte cultures (7). Another important factor discussed here is the ambient O_2 pressure in the culture incubator. The physiological level of O_2 at the dermo-epidermal junction, where proliferating keratinocytes are located, is approximately 6% (15), and high oxygen levels have been shown to promote keratinocyte terminal differentiation in

vitro (16). For these reasons we studied the effect of the ambient O_2 pressure on murine keratinocyte and fibroblast cultures, in order to ask whether some of the earlier difficulties, encountered specifically in passaging murine keratinocyte cultures, might have been due to the high oxygen concentration (20%) that was generally used. Newborn pups were euthanized and the isolated cells were cultured at 3, 6 and 20% O_2. For each condition, the keratinocyte cultures were grown in the absence of added growth factors or in the presence of either epidermal growth factor (EGF) or keratinocyte growth factor (KGF). Our data clearly show that the use of lower (physiological) levels of O_2 (6%) during keratinocyte culture increased the probability of the primary cultures to be passaged successfully (**Fig. 4.1**). Successful passaging depends however also on the specific mouse strain that is used. Whereas keratinocyte cultures derived from the C57BL/6 mouse strain can be obtained in a 20% O_2 atmosphere (7), keratinocyte cultures derived from hairless SKH-1 albino strain mice were derived much easier by culturing the cells at a 6% O_2 pressure. Alternatively, fibroblasts from both strains grew optimally at 3% O_2. The described methodology for establishment, propagation and analysis of matched fibroblast and keratinocyte cultures has been applied by the authors for cells derived from both normal and

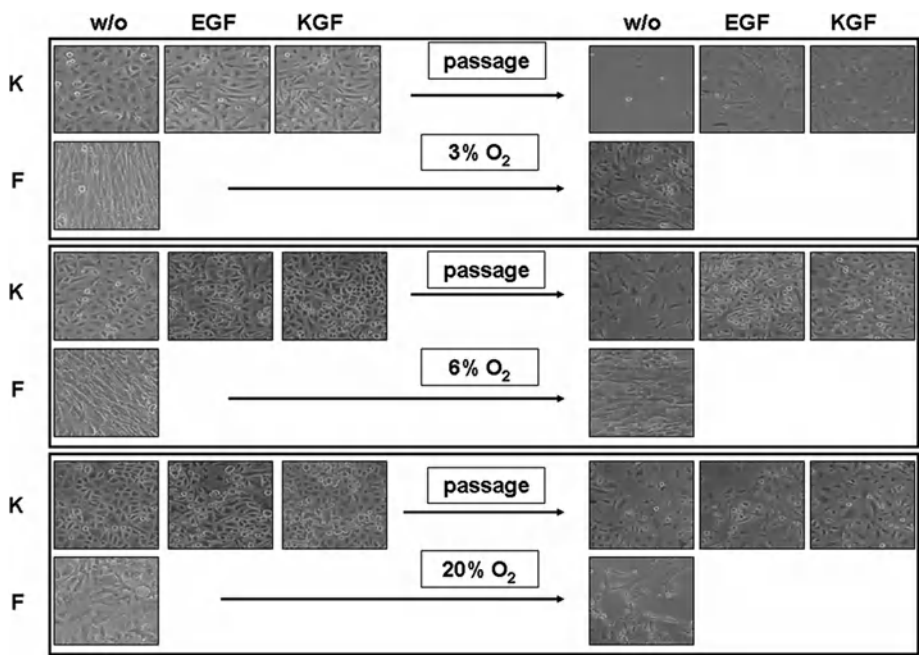

Fig. 4.1. Growth and passaging of primary cultures of murine keratinocytes (**K**) and fibroblasts (**F**) under various external oxygen concentrations (3, 6 and 20%) and either in the absence of added growth factors (w/o) or in the presence of 10 ng/ml epidermal growth factor (EGF) or 1 ng/ml keratinocyte growth factor (KGF) (for the keratinocyte cultures).

DNA repair-deficient mice of the C57BL/6 and SKH-1 strains. It is however important to realize that it might not apply to other inbred mouse strains.

2. Materials

2.1. Serum Chelexing

1. Chelex 100 resin (200–400 dry mesh, sodium form; Bio-Rad, Hercules, CA); store at room temperature
2. 47 mm Glass fibre filters (Millipore, Billerica, MA)
3. 47 mm Millipore membrane filters (5 and 0.45 μm)
4. 0.2 μm Filtropur L 0.2 S pressure filtration system (Sarstedt, Nümbrecht, Germany)
5. MilliQ water (Millipore, Billerica, MA)
6. Fetal Calf Serum (*see* **Note 1**), store at –20°C
7. HCl (1 N) and NaOH (1 N)
8. 70% ethanol

2.2. Coating Solution

1. Leibovitz L-15 culture medium (BioWhittaker, Lonza, Basel, Switzerland); store at 4°C (*see* **Note 2**).
2. Fibronectin (human) (1 mg, Becton Dickinson, Franklin Lakes, NJ); store at –20°C.
3. Collagen I (bovine) (30 mg, Becton Dickinson, Franklin Lakes, NJ); store at –20°C.
4. BSA (Sigma-Aldrich, Saint Louis, MO, Cat. No. A-7888) (1 mg/ml in PBS); store at –20°C.
5. 1 M Hepes (Duchefa Biochemie B.V., Haarlem, Netherlands); store at –20°C.
6. Tissue culture dishes (60×15 mm, 100×20 mm, 150×20 mm) (Sarstedt, Nümbrecht, Germany).
7. 100 mg/ml Penicillin G sodium, 100 mg/ml streptomycin-sulphate (Duchefa Biochemie B.V., Haarlem, Netherlands) in H_2O (PenStrep:1000 ×).

2.3. Keratinocyte and Fibroblast Isolation

1. Newborn mice (2 days after birth).
2. Tissue culture dishes (*see* **Section 2.2**).
3. 0.5% aqueous solution of Betadine (VWR, West Chester, PA), store at 4°C.
4. 70% EtOH; store at 4°C.
5. Eagle's minimal essential medium without calcium (EMEM-Ca) (BioWhittaker, Lonza, Basel, Switzerland).

6. Dulbecco's minimal essential medium, high glucose 4.5 g/l (DMEM) (PAA, Pashing, Austria).
7. 0.25% Trypsin solution (Gibco®, Invitrogen, Carlsbad, CA) in EMEM-Ca; store at –20°C.
8. Keratinocyte LoCal medium (0.05 mM calcium), 500 ml: 460 ml EMEM-Ca; 32.5 ml chelexed FCS (*see* **Section 3.1**); 7.5 ml FCS, 0.5 ml PenStrep (1000 ×), 1 ng/ml keratinocyte growth factor (KGF); store at 4°C for max. 1 month (*see* **Note 3**).
9. Keratinocyte HiCal medium (1.3 mM calcium), 100 ml: 92 ml EMEM-Ca; 8 ml FCS; 115 µl $CaCl_2$ (1 M); 0.1 ml PenStrep (1000 ×), store at 4°C for max. 1 month.
10. Keratinocyte 0.2Cal medium (0.2 mM calcium) 100 ml: 92 ml EMEM-Ca; 1.7 ml chelexed FCS (*see* **Section 3.1**); 6.3 ml FCS, 0.1 ml PenStrep (1000 ×); store at 4°C for max. 1 month.
11. 50 ml sterile centrifuge tube (red cap) (Sarstedt, Nümbrecht, Germany).
12. Fibroblast medium, 500 ml: 425 ml DMEM, 75 ml FCS, 0.5 ml PenStrep (1000 ×); store at 4°C.
13. 10 µm nylon cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ).
14. Keratinocyte growth factor (KGF) (Sigma-Aldrich, Saint Louis, MO): 10 µg/ml dissolved in PBS containing 1 mg/ml BSA (*see* **Note 4**). Store at –20°C.
15. Epidermal growth factor (EGF) (Sigma-Aldrich, Saint Louis, MO).
16. Refrigerated Eppendorf 5810 R centrifuge.
17. Nitrocellulose marking pen (Schleicher & Schuell, Keene, NH).

2.4. Passaging Mouse Keratinocyte and Fibroblast Cultures

1. Phosphate buffered saline (PBS) (Gibco®, Invitrogen, Carlsbad, CA) (*see* **Note 5**)
2. PBS containing 0.1 mM EDTA (PBS-EDTA)
3. PBS with 10% chelexed FBS
4. 0.25% Trypsin solution (Gibco®, Invitrogen, Carlsbad, CA) in a 1:1 mixture of PBS and PBS-EDTA
5. Bovine calf serum (BCS) (Hyclone, Thermo Fisher Scientific, Logan, UT)
6. 0.2Cal and LoCal keratinocyte medium (*see* **Section 2.3**), fibroblast medium (*see* **Section 2.3**)
7. Coating solution (*see* **Section 3.2**)

3. Methods

The extracellular calcium concentration is a major determinant for the differentiation state of mammalian keratinocytes and a low calcium concentration (0.05 mM) is required to establish proliferating murine keratinocyte cultures (7, 14). To obtain culture medium with the requested calcium concentration, the foetal calf serum has to be treated with a Chelex ion-exchange resin to remove the calcium from the serum. In LoCal culture medium, also non-chelexed serum is added to reach the requested 0.05 mM calcium concentration and to compensate for the loss of other important bivalent ions (e.g. magnesium and zinc) that are also removed by the Chelex resin (*see* **Note 3**). The methodology that is described has been used to isolate keratinocytes and fibroblasts from the C57BL/6 J and SKH-1 albino strains of mice. The total isolation procedure requires three consecutive working days from the isolation of the skins until separate keratinocyte and fibroblast cell cultures are obtained (**Fig. 4.2**).

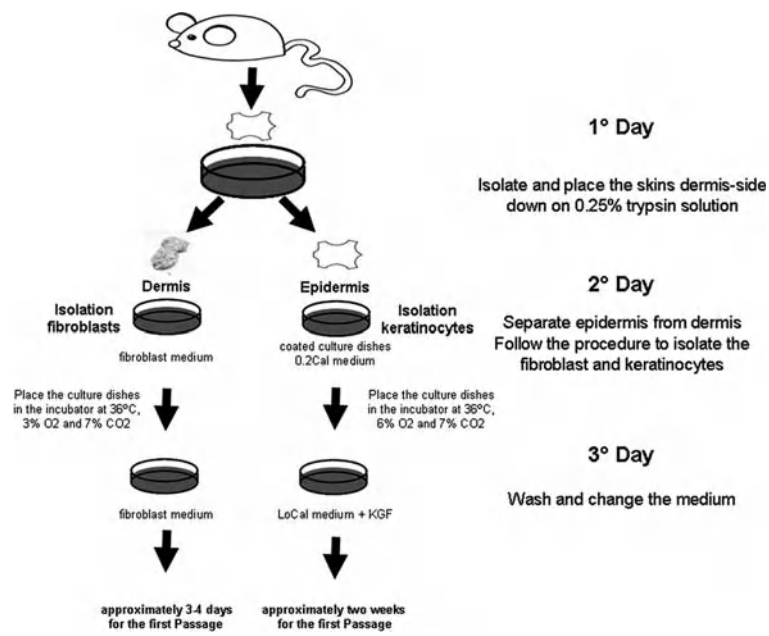

Fig. 4.2. Schematic representation of the culturing procedures for epidermal keratinocytes and dermal fibroblasts derived from a single murine skin biopsy.

3.1. Serum Chelexing

The procedure is largely based on the method first described by Brennan and co-workers (17):

1. Resuspend 200 g Chelex resin in 1 l of milliQ water and adjust the pH to 7–8 with HCl.

2. Collect the resin by vacuum filtration through a glass fibre filter by using a Millipore glass filter holder.
3. Add the resin to 500 ml serum and stir gently for 1 hour at room temperature.
4. Collect the FCS by subsequent vacuum filtrations through a glass fibre filter, a Millipore 5 µm membrane filter and a 0.45 µm Millipore membrane filter (*see* **Note 6**).
5. Sterilize the FCS by pressure filtration through a 0.2 µm Filtropur L 0.2 LS filter, mounted directly onto a sterile bottle.
6. Store the chelexed FCS in 40 ml aliquots at –20°C (*see* **Note 7**).

3.2. Chelex Regeneration

1. Wash the used resin several times extensively in milliQ water and recover the resin by vacuum filtration through a glass fibre filter.
2. Resuspend the resin in 1 l of 1 N HCl and filter the resin through (volume decreases by half).
3. Wash extensively with milliQ water and filter the resin through a glass fibre filter.
4. Resuspend the resin in 1 N NaOH and filter through a glass fibre filter. Volume increases.
5. Wash extensively with milliQ water, recover the resin by glass fibre filtration and suspend in 70% EtOH. Store at 4°C.

3.3. Coating Solution

1. Dissolve 1 mg of lyophilized fibronectin in 1 ml of sterile MilliQ water. Do not mix or shake, but leave for half an hour at room temperature.
2. To 100 ml of Leibovitz L-15 medium add 2 ml of 1 M HEPES, 100 µl BSA (1 mg/ml), 1 ml collagen I (3 mg/ml), 100 µl PenStrep antibiotics and fibronectin solution prepared above.
3. Mix gently and store 4°C.

3.4. Coating Culture Dishes

1. Place all culture dishes in a sterile flow cabinet.
2. Add sufficient coating solution to completely cover the bottom of a first culture dish.
3. Remove the coating solution immediately and add to a second dish.
4. Proceed in this way until all dishes have been treated. The remaining solution can be stored at 4°C and saved for future use (*see* **Note 8**).
5. Place the culture dishes in the 36°C O_2/CO_2 incubator at for least 30 minutes.
6. Use immediately for seeding keratinocytes.

3.5. Isolation of Keratinocytes and Fibroblasts from the Same Skin Biopsy

1st Day:

1. Euthanize newborn mice (preferentially 2 days after birth) by CO_2 asphyxiation (*see* **Note 9**).
2. Mark the mice on the abdomen with a nitrocellulose marking pen and remove tails for PCR genotyping (leave stump of at least 1 mm).
3. Wash the mice two times for 15 minutes by rocking in 0.5% Betadine.
4. Put the mice into a sterile 150 mm culture dish, rinse once with sterile MilliQ water and twice with 70% EtOH.
5. Put the dish with the mice on ice in a sterile flow cabinet, open the cover and wait until the EtOH is largely dried up.
6. Amputate the limbs and the tail, leaving visible stumps (*see* **Fig. 4.3A–C**) (*see* **Note 10**).
7. Make a longitudinal dorsal incision from the tail to the snout. Dislocate the skin, mainly at the back in order to be able to hold the hips with forceps (*see* **Fig. 4.3D**).
8. Pullout the back legs first, hold the skin over the whole width and pull towards snout (*see* **Fig. 4.3E,F**).
9. Place skin, dermis-side down, on a new sterile culture. Stretch the skin completely flat and leave the skins for 10–15 minutes on ice in the flow cabinet (*see* **Note 11**).
10. Transfer the skins, dermis-side down, onto a 0.25% trypsin solution (in EMEM-Ca). Up to eight skins can be floated on 40 ml trypsin solution in a 15 cm dish (*see* **Note 12**). Leave 18 hours (overnight) at 4°C (*see* **Fig. 4.3G**).

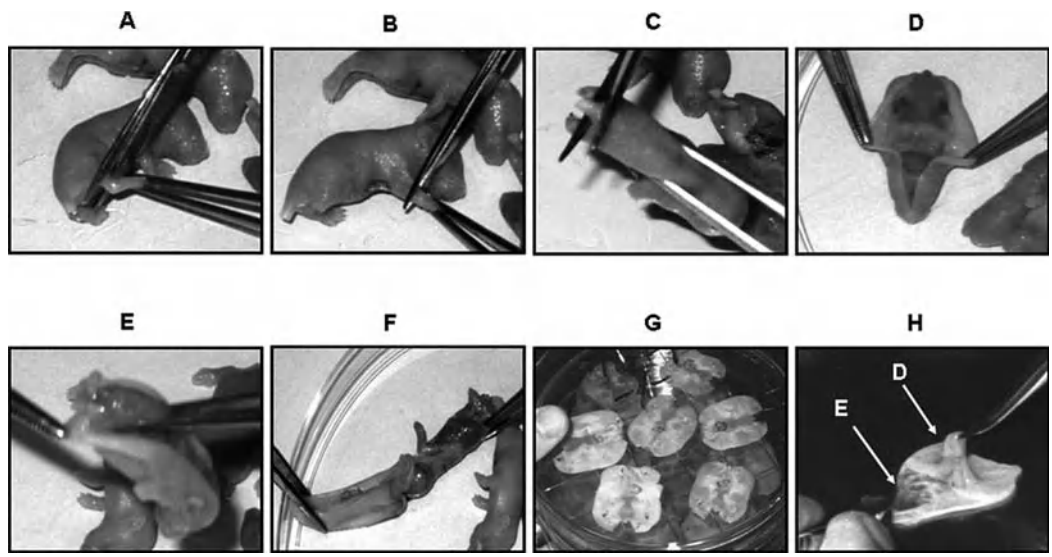

Fig. 4.3. Distinct consecutive steps in the isolation of murine dermis and epidermis from neonatal murine skin: **(A–C)** limb and tail removal; **(D–F)** skin dislodgement; **(G, H)** trypsinization and dermal–epidermal separation. In panel H: **E** = epidermis; **D** = dermis.

2nd Day:

11. Place skin, epidermal-side down on a dry spot of a sterile culture dish and peel off the dermis with sharp forceps (*see* **Fig. 4.3H**).
12. Place the dermis from a single skin into a sterile 50 ml Sarstedt centrifuge tube and keep on ice until proceeding with step 19.
13. Put the corresponding epidermis into a 6 cm culture dish, add 1 ml of HiCal medium and mince the epidermis with sterile curved scissors. If a fine suspension is obtained, add 10 ml HiCal medium and vigorously pipette 20–30 times up/down with a 10 ml sterile disposable pipette.
14. Filter through a 10 μm Falcon nylon cell strainer and recover the cells in a sterile 50 ml Sarstedt centrifuge tube.
15. Wash the filter with 10 ml of HiCal medium.
16. Centrifuge at 1000 rpm for 5 minutes at 4°C in an Eppendorf 5818 R centrifuge, discard the supernatant and take up the cells in 10 ml of 0.2Cal medium.
17. Seed the cells derived from a single epidermis into two 6 cm fibronectin/collagen-coated culture dishes (*see* **Section 3.4**). Allow cell attachment over night (at least 6 hours).
18. Place the culture dishes into an incubator set at 36°C, 6% O_2 and 7% CO_2 (**Fig. 4.4**).
19. Proceed with the isolated dermis (step 12) by adding 10 ml of fibroblast medium and vortex vigorously for 20–30 seconds.
20. Filter through a 10 μm Falcon nylon cell strainer and recover the fibroblasts into a 50 ml Sarstedt centrifuge tubes.

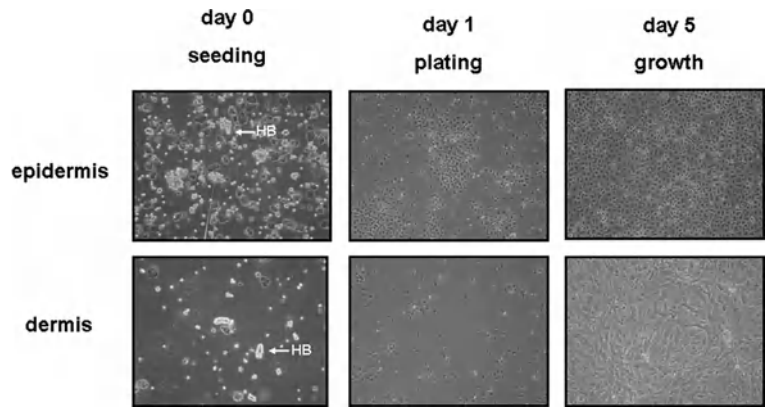

Fig. 4.4. Different aspects of keratinocytes (epidermis) and fibroblasts (dermis) at different time points during the setting up of matched long-term cultures. Note that the majority of keratinocytes plate as small clones (day 1), indicating that they are derived from epidermal hair follicle buds (HB).

21. Wash the filter with 10 ml fibroblast medium.
22. Centrifuge the filtrate at 2000 rpm for 10 minutes at 4°C.
23. Discard the supernatant and resuspend the pellet in 20 ml of fibroblast medium.
24. Seed the cells derived from a single dermis into two 100 mm culture dishes.
25. Place the dishes into an incubator set at 36°C, 3% O_2 and 7% CO_2 (**Fig. 4.4**).

3rd Day:

26. Wash all cultures twice with PBS to discard dead cells (*see* **Note 13**).
27. Add 5 ml of LoCal medium, supplemented with 1 ng/ml KGF, to the keratinocyte cultures (*see* **Note 14**) and 10 ml of fibroblast medium to the fibroblast cultures.
28. Fresh medium is supplied each second day until the culture reaches confluence.

3.6. Passaging Mouse Keratinocyte Cultures

It is imperative not to passage keratinocyte cultures too early. In general, we waited approximately 2 weeks before the first passage. Confluent culture dishes are characterized by the presence of loosely floating cells which consist of a mixture of differentiated, possibly apoptotic but also mitotic cells. A convenient way to determine whether the culture is ready to be passaged is to seed the "floaters" in 0.2 mM calcium medium onto a new coated culture dish. If after an overnight incubation ≈10% of the cells are attached, the culture is ready to be trypsinized. At early passages ($p<4$) the cultures should be split 1:2 (maximally 1:3), but this dilution can be gradually increased at later passages until 1:10 at $p>10$. We have observed that keratinocyte cultures become readily near-tetraploid already after a few passages (discussed in **Chapter 7**).

1. Wash the cells with 5 ml of PBS and then with 5 ml of PBS-EDTA.
2. Add 0.5 ml of trypsin solution (in PBS/PBS-EDTA) for a 60 mm culture dish.
3. Incubate at 37°C for 5–7 minutes. Check microscopically whether the cells have detached.
4. Add two times 5 ml PBS containing 10% chelexed bovine calf serum and collect the cells in a 50 ml Sarstedt centrifuge tube.
5. Centrifuge at 1000 rpm for 10 minutes.
6. Resuspend the pellet in 10 ml of 0.2Cal medium.
7. Seed the cells in two 60 mm fibronectin/collagen-coated culture dishes.

8. Incubate at 36°C, 6% O_2, 7% CO_2 for approximately 18 hours (overnight).
9. Wash the cells with 5 ml of PBS and add 5 ml of LoCal medium supplemented with 1 ng/ml KGF.

3.7. Passaging Mouse Fibroblast Cultures

In general the fibroblast cultures are confluent 3-4 days after isolation and adopt a typical spindle-like morphology (bundle-like alignment) (*see* **Fig. 4.1**). The cultures can than be split 1:3 without any specific precautions.

1. Wash the cells two times with 10 ml PBS.
2. Add 1 ml of trypsin solution (in PBS/PBS-EDTA) for a 90 mm culture dish.
3. Incubate at 37°C for 3–5 minutes.
4. Add 10 ml PBS containing 10% BCS to the cells, resuspend by pipetting several times and put into a 50 ml Sarstedt centrifuge tube.
5. Centrifuge at 1000 rpm for 10 minutes.
6. Resuspend the pellet with 30 ml of fibroblast medium.
7. Seed 10 ml of cell suspension into a 100 mm culture dish (3 ×).
8. Incubate at 36°C, 3% O_2, 7% CO_2 for approximately 18 hours (overnight).

4. Notes

1. It is advisable to test several batches of serum, as it turns out that various batches, even from a same brand, can differ substantially in plating efficiency
2. The Leibovitz L-15 medium contains calcium (140 mg/l, which corresponds to 1.26 mM). Calcium in the coating solution promotes cell attachment.
3. The calcium concentration in the LoCal medium, prepared with chelexed serum, is adjusted (0.05 mM) by the addition of non-chelexed serum (approximately 3 mM Ca^{2+}). This has the advantage to compensate, at least in part, for the loss of other essential bivalent ions that are also removed by the Chelex resin (approximately 99% decrease in magnesium and zinc concentrations). Chelexed serum has a calcium concentration of approximately 0.01 mM. Element analysis was performed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) using the Vista-MPX device from Varian Inc. (Mulgrave, Australia).

4. The inclusion of KGF in LoCal medium dramatically increased the probability of the primary cultures to be passaged successfully. KGF is a member of the fibroblast growth factor (FGF) family that functions as a potent mitogen for epithelial cells and plays an important role in tissue repair and cyto-protection (18, 19).
5. Be careful not to use PBS brands that contain $CaCl_2$.
6. Pre-rinse the Millipore membrane filters with sterile milliQ water before use as new filters release calcium at first use.
7. The calcium concentration in FCS is approximately 3 mM; the calcium concentration in chelexed FCS should range between 0.01 and 0.03 mM.
8. The coating solution can be stored and used successfully for up to 2 years when kept sterile.
9. When older pups are used, the keratinocyte cultures are more likely to be contaminated by melanocytes.
10. Perform skin removal procedures using sterile dissecting tools on the inner surface of the lid of a sterile 150-mm culture dish.
11. Slight drying out of the skin and proper stretching at this stage make subsequent floating on trypsin easier.
12. Do not allow edges to curl under, the epidermis will not separate properly.
13. Primary fibroblast cultures may still contain keratinocyte clones derived from dermal hair follicle buds. These cells will, however, terminally differentiate (due to the higher calcium concentration in the fibroblast medium) and be completely overgrown by proliferating fibroblasts (*see* **Fig.** 4.4).
14. In several control experiments we have substituted KGF by EGF (epidermal growth factor). At higher oxygen concentrations (20%) EGF performed less well than KGF as far as the ability to passage keratinocytes was concerned (*see* **Fig.** 4.1). These differences were less obvious in a 3 or 6% oxygen atmosphere.

Acknowledgements

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Chapter 5

Establishment of Spontaneously Immortalized Keratinocyte Lines from Wild-Type and Mutant Mice

Julia Reichelt and Ingo Haase

Abstract

A considerable number of transgenic or knockout mice in which epidermal keratinocytes have been targeted die shortly after birth due to barrier defects. In this case, recovery and cultivation of keratinocytes from these animals provide an opportunity for in vitro studies. Working with isolated keratinocytes is also interesting for certain experiments which cannot be performed in live animals.

Primary human keratinocytes can be kept in culture for a variable number of passages and then senesce. Immortalization can be achieved by transduction with constructs encoding viral genes. Murine keratinocytes can be kept in culture as primary cells. Naturally the numbers of cells obtained by direct isolation from mouse epidermis is restricted and sometimes not sufficient for certain biochemical analyses. To overcome this restriction some permanent murine keratinocyte lines have been generated by transfection with SV40T or HPV E6E7 genes. This is, however, not suitable if established or hypothetical biochemical links exist between these genes and the pathways or processes to be analysed in the respective experiment.

We describe an easy and reproducible method of establishing permanent keratinocyte lines from spontaneously immortalized primary murine keratinocytes. This method employs co-cultivation of keratinocytes with 3T3-J2 fibroblast feeder cells for several passages during which immortalization occurs. The resulting keratinocyte lines do not only grow infinitely but, in many cases, individual lines from the same genetic background also exhibit similar growth characteristics, hence they are especially valuable for comparative studies.

Key words: Mouse keratinocytes, Immortalization, 3T3-J2, Fibroblast feeder cells.

1. Introduction

The methods currently used to isolate and cultivate murine keratinocytes for experimental purposes can have major disadvantages.

Although primary keratinocytes are probably closest to the in vivo situation, their isolation and cultivation in sufficient numbers requires large amounts of neonatal mice bred just for this purpose.

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Furthermore, these cultures are not sustainable. Primary keratinocytes tend to differentiate rapidly in culture and cannot be propagated for long in order to yield a sufficient pool of comparable cells for large-scale experiments. Furthermore, the fact that primary epidermal cultures may harbour other epidermal cell types, e.g. melanocytes, is often disregarded.

Mouse keratinocytes have been immortalized in the past by using transfection with recombinant retroviruses encoding HPV-16 E6 and E7 genes (1) or by isolating cells from transgenic mice carrying an inducible SV40 T antigen gene (2) (ImmortoMouse, Charles River Laboratories). However, these immortalized keratinocytes harbour the risk of displaying unexpected or unwanted modifying effects caused by the transforming gene or the viral vector (3, 4).

Here, we describe a simple method to establish spontaneously immortalized keratinocyte lines from wild-type or from genetically modified mice. Several of the keratinocyte lines generated in this way have by now been serially cultivated for more than 250 passages with no obvious changes in morphological or growth characteristics.

The protocol starts with the isolation of keratinocytes from neonatal mouse skin and their co-cultivation with 3T3 fibroblast feeder cells for four to eight passages (1–3 months). From these isolated cells multiple keratinocyte clones emerge which proliferate and continue growing as immortalized keratinocyte lines without feeder cells after the initial four to eight passages.

We compared several wild-type keratinocyte lines obtained from BABL/c mice with each other and with keratin 10 (K10)-deficient keratinocyte lines isolated from K10 knockout mice (5–8). K10 expression is restricted to differentiated keratinocytes present in the suprabasal epidermis or in differentiating keratinocytes grown in high-calcium medium. In low-calcium medium K10 is not expressed and therefore K10-deficient keratinocytes should behave like wild-type cells. We analysed several lines of wild-type and $K10^{-/-}$ keratinocytes and found that all tested lines showed the same growth and migration characteristics (*see* **Fig. 5.1**). Importantly, the spontaneous immortalization did not impair the intrinsic differentiation potential of the keratinocytes. Switching confluent cultures of spontaneously immortalized keratinocytes from low- to high-calcium medium induces differentiation and stratification.

The presented method has been used to establish epidermal as well as oesophageal keratinocyte lines from distinct wild-type mouse strains (e.g. BALB/c and C57Bl/6) as well as from genetically altered mouse strains (e.g. $K10^{-/-}$, K10T, K14-Cre/$Ikk2^{fl/fl}$ (9) and N17Rac1 transgenic mice (10)).

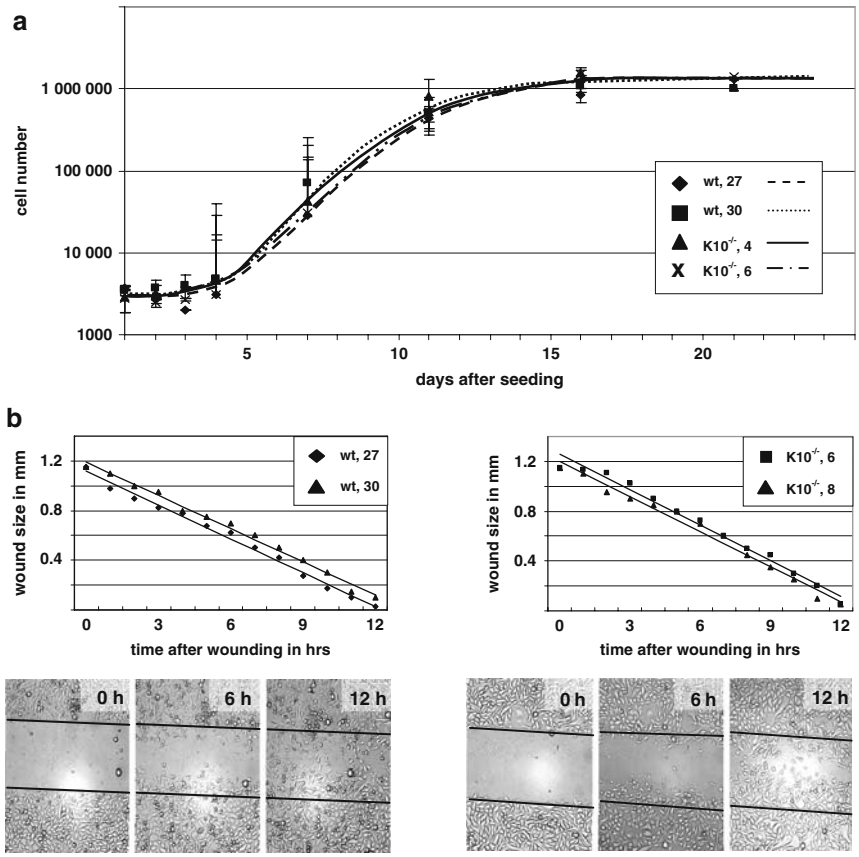

Fig. 5.1. Characterization of wild-type and keratin 10-deficient murine keratinocyte lines. Characterization was initiated at passage 20. (**a**) For the generation of a growth curve 2×10^3 keratinocytes/cm^2 were seeded onto collagen I-coated 35 mm dishes. Cell numbers of duplicates were determined at 1, 2, 3, 4, 7, 11, 16 and 21 days after seeding by counting trypsinized cells in a counting chamber. Plating efficiency was low due to sparse seeding but comparable in the tested keratinocyte lines (28%). Growth rates were determined in the exponential growth phase and saturation density was given by the maximum cell number obtained at the end of the growth curve (2.2×10^5 keratinocytes/cm^2). The growth curves of distinct wild-type (wt) and K10$^{-/-}$ keratinocyte lines showed no differences. The keratinocyte clones were numbered consecutively when established and these numbers are given in the legend. (**b**) Scratch assays were used in order to determine the migration potential of keratinocytes from different lines. Keratinocytes were seeded onto collagen I-coated 35 mm dishes and grown to confluency. Prior to the analysis of migration, cells were treated with mitomycin C (*see* **Section 3.1.2**) to inhibit cell proliferation. The monolayers were scratched in the middle of the dish with a yellow micropipette tip to yield a gap of about 1.2 mm width. The closure of the gap was followed by measuring the distance of the wound edges every hour over a period of 15 hours, during which the cells were kept at 32°C and 5% CO_2 in an incubation chamber on the microscope. Migration rate was about 0.1 mm/hour in wt and keratin 10$^{-/-}$ keratinocyte lines (two lines per genotype were tested). Figure (a) by Monika Loeher and (b) by Heike Stachelscheid.

The major advantages of this method for producing spontaneously immortalized mouse keratinocyte lines over primary keratinocytes are absence of other contaminating epidermal cells (e.g. melanocytes, dendritic T lymphocytes), the indefinite proliferation capacity and reproducibility of experiments due to stability of the keratinocyte characteristics. In contrast to keratinocytes

immortalized through transduction with viral genes the spontaneously immortalized keratinocyte lines presented here show no unwanted effects due to the presence of foreign protein or DNA.

2. Materials

2.1. Feeder Cell Cultivation

1. 3T3-J2 fibroblasts feeder cells were a generous gift of Fiona Watt, Cambridge, UK (11).
2. Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL) supplemented with 10% foetal calf serum (FCS) and 100 U/ml penicillin and 100 µg/ml streptomycin (supplied as 100 × stock solution, Invitrogen).
3. Mitomycin C (Applichem, Germany). Prepare 100 × stock solution (0.4 mg/ml) in PBS and store aliquots at –20°C. Add 10 µl/ml DMEM + 10% FCS.
4. 0.05% Trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA, Gibco/BRL).

2.2. Recovery of Neonatal Mouse Skin

1. Betaisodona, 10% iodine (Mundipharma), which is equivalent to povidone-iodine, PVP-I.
2. Dispase (Gibco/BRL), prepare 5 U/ml or 10 mg/ml solution in PBS and sterile filter.
3. 35 mm plastic dishes or six-well plates (Nunc/Nunclon, Falcon or TPP).
4. Sterile instruments (forceps, scissors), PBS (phosphate buffered saline) and 70% ethanol.

2.3. Culture of Keratinocytes

1. Sterile instruments (forceps, scalpels), PBS and 70% ethanol.
2. 35 mm plastic dishes or six-well plates (Nunc/Nunclon, Falcon or TPP).
3. Collagen I from rat tail (Becton Dickinson), 50 µg/ml 0.02 M acetic acid. The diluted solution is stored at 4°C and can be used several times for coating.
4. FCS Gold (PAA), chelex treated to remove Ca^{2+}: 20 g chelex 100 (Bio-Rad)/500 ml FCS Gold is left to rotate O/N at 4°C, then pre-cleared by paper filtration, sterile filtered and stored in 50 ml aliquots at –20°C.
5. FAD medium: DMEM/HAM's F12 3.5:1.1, low-calcium (0.05 mM Ca^{2+}) (custom made by Biochrom, Berlin, Germany).

6. FAD medium is supplemented to yield "complete FAD medium" before use with 10% chelex-treated FCS gold, 0.18 mM adenine, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin (all from Sigma), 10 ng/ml EGF (Invitrogen), 2 mM glutamine, 1 mM pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (all supplied as 100 × stock solutions, Invitrogen).

   Supplement stock solutions are prepared as follows either under sterile conditions or sterile filtered and stored in aliquots at –20°C unless otherwise indicated:

   a) Adenine (250 ×): 45 mM in 50 mM HCl. Add 2 ml stock solution/460 ml medium.

   b) Hydrocortisone (2000 ×): 1 mg/ml in ethanol. Dilute 1:4 with FAD medium. Add 1 ml stock solution/460 ml FAD medium.

   c) Insulin (1000 ×): 5 mg/ml in 5 mM HCl. Add 0.5 ml stock solution/460 ml FAD medium.

   d) EGF (1000 ×): 10 μg/ml in FAD medium. Add 0.5 ml stock solution/460 ml FAD medium.

   e) Cholera toxin (10^{-5} M): 1 mg/1.18 ml in sterile water, stored at 4°C. Add 5 μl stock solution/460 ml FAD medium.

7. 0.05% Trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA, Gibco/BRL).
8. 0.02% EDTA in PBS.
9. Keratinocyte freezing solution: 90% chelex-treated FCS Gold, 10% dimethyl sulphoxide (DMSO, cell culture grade, Applichem, Germany); aliquots are stored at –20°C.
10. CO_2 incubator at 32°C.

3. Methods

3.1. Feeder Cell Cultivation

Feeder cell expansion must be well timed with mouse breeding to make sure enough feeder cells are available on the day after birth of pups and thereafter. For a litter of six pups, one just confluent 10 cm Petri dish should be available on the day after mouse skin recovery to be plated at a density of $1.5–3 \times 10^4$ cells/cm^2 (*see* **Note 1**).

The 3T3-J2 subclone of Swiss mouse 3T3 fibroblasts has been widely used to support human keratinocyte cultivation (12, 13) as well as other epithelial cells (14–16) and has also proven useful in our hands for the establishment of permanent murine keratinocyte lines.

1. 3T3-J2 fibroblasts are routinely grown in DMEM + 10% FCS. They are split twice weekly when just confluent at no more than 1:3 (*see* **Fig. 5.2b** and **Note 2**).
2. In order to use 3T3-J2 feeder cells in co-culture with keratinocytes, culture a confluent Petri dish for 2–3 hours with DMEM + 10% FCS containing mitomycin C at 37°C (*see* **Note 3**).

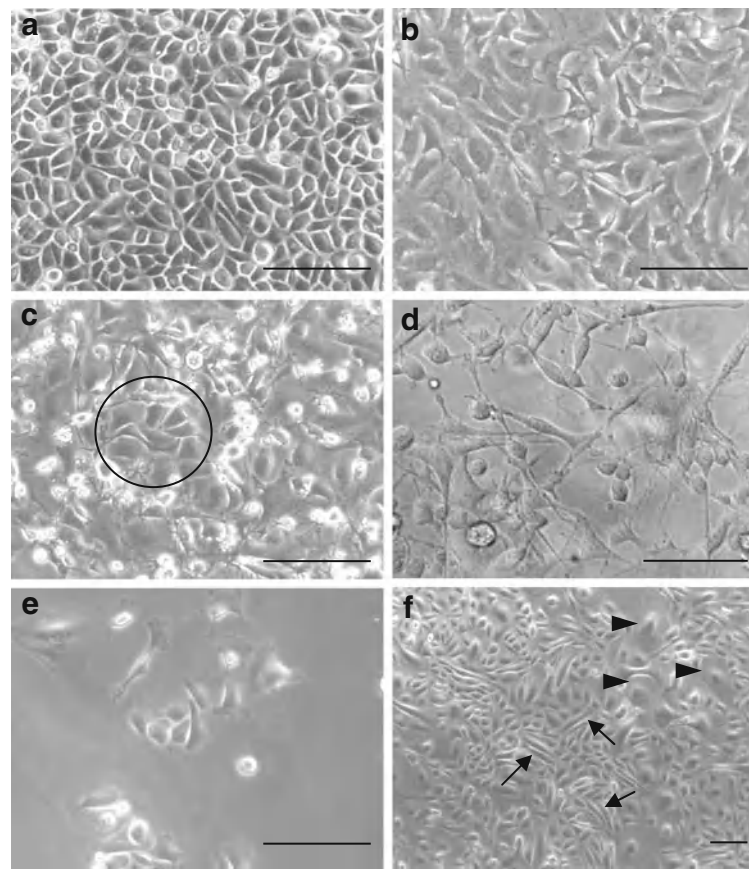

Fig. 5.2. Morphology of murine keratinocytes and 3T3-J2 fibroblast feeder cells. (**a**) Confluent keratinocyte monolayers show the typical cobblestone-like pattern in low-calcium FAD medium. (**b**) The photo shows confluent 3T3-J2 fibroblast. At this cell density, feeder cells should be routinely split or used for mitomycin C treatment and co-culture with keratinocytes. (**c**) A colony of keratinocytes (encircled) emerging 12 days after co-cultivation of primary keratinocytes with feeder cells. (**d**) 3T3 feeder cells 3 days after mitomycin C treatment. (**e**) Typical morphology of spontaneously immortalized murine keratinocytes about 12 hours after splitting. A group of cells in the centre show the typical cobblestone morphology of keratinocytes growing at higher density. (**f**) Splitting at too high dilutions often causes differentiation of keratinocytes. Differentiated keratinocytes may appear enlarged (arrowheads) or appear spindle shaped (arrows) causing an irregular pattern at confluency. Bars, 100 μm. Images were taken by Dr. Anne Vollmers.

3. Before adding trypsin solution, wash cells thoroughly twice with PBS to remove mitomycin C.
4. Wash the fibroblasts once with trypsin/EDTA solution, add fresh trypsin/EDTA and incubate the cells for about 2 min at 37°C. Resuspend the detached cells subsequently in keratinocyte medium (complete FAD medium) and centrifuge for 5 min at 220*g*. Suspend the cell pellet in fresh complete FAD medium by pipetting three to four times and seed the fibroblasts then directly onto collagen I-coated dishes at a density of 1.5–3 $\times$ $10^4/cm^2$. When first seeding primary keratinocytes and for the first passage using the higher density of feeder cells (approximately 3 $\times$ 10^4 cells/cm^2) is recommended.
5. The feeder cells may be seeded either prior to or together with the keratinocytes.

3.2. Recovery of Neonatal Mouse Skin

1. Decapitate neonatal mice and place them on ice for 1 hour (*see* **Note 4**).
2. From this point, a sterile laminar flow cabinet as well as sterile solutions and instruments are required.
3. Use one mouse after the other and keep the remaining corpses on ice.
4. Disinfect mouse corpses with Betaisodona (1:1 diluted with PBS) for 1 min (e.g. in a Petri dish), rinse two times briefly in PBS, place for 1 min in 70% ethanol and finally rinse briefly in PBS.
5. Remove extremities and cut the skin dorsally from head to tail. Peel the skin off in one piece using forceps and the dull parts of scissors.
6. Spread the skin on a 35 mm plastic dish with the epidermis side facing upwards.
7. Add 2 ml of dispase solution carefully from the side so that the skin floats.
8. Seal the dish with parafilm and incubate O/N (or at least for 7 hours) at 4°C (*see* **Note 5**).

3.3. Culture of Keratinocytes

If keratinocytes from transgenic mice and wild-type control mice or keratinocytes from distinct wild-type mouse strains are to be grown at the same time, cross-contaminations must be carefully avoided at any stage. Established keratinocyte lines should be regularly checked, e.g. by PCR genotyping, to ensure their purity.

1. Before starting, treat feeder cells with mitomycin C for 2–3 hours (*see* **Section 3.1**).

2. Cell culture dishes (35 mm) have to be coated with collagen I solution for 1 hour at room temperature and then washed carefully two times with PBS before cells are plated (*see* **Note 6**).
3. After incubation with dispase (*see* **Section 3.2.7**), remove the epidermis from the dermis with forceps (*see* **Note 7**).
4. Mince the epidermis in a Petri dish by moving two scalpels against each other in opposite directions for 1–3 min to yield a mush. The cells and tissue pieces must not dry out.
5. Suspend the minced epidermis of each mouse in 1.5 ml complete FAD medium and agitate for 30 min at 1000 rpm in a microcentrifuge tube.
6. Seed keratinocytes and epidermal fragments from each individual mouse separately on collagen I-coated dishes together with 3T3-J2 feeder cells. A subconfluent 10 cm dish of mitomycin C-treated feeder cells is sufficient for six to twelve 35 mm dishes of keratinocytes.
7. Grow the keratinocytes at 32°C and 5% CO_2.
8. Primary keratinocytes can be seen on the following day, but many of them will differentiate and die.
9. Change medium twice or three times weekly and add feeder cells at least at every medium change and if required also in-between medium changes. In low-calcium medium, the mitomycin C-treated feeder cells usually die after 3-4 days (*see* **Fig. 5.2d**) and therefore care must be taken to ensure the presence of sufficient feeder cells.
10. Usually, after about 1–3 weeks keratinocyte clones with the typical cobblestone-like appearance will be observed (*see* **Fig. 5.2c** and **Note 8**). Epidermal cell cultures of dark coated mouse strains may appear increasingly black during the first passages due to melanocyte growth (*see* **Note 9**).
11. At the beginning, split keratinocytes at 1:1. At later passages (4–5), splitting at 1:2 or 1:3 is possible. The day after splitting, the sparsely seeded keratinocytes do not show the typical cobblestone pattern (*see* **Fig. 5.2e**) which only develops when the cultures grow and become more confluent.
12. For splitting, wash keratinocytes with PBS, incubate with EDTA solution for 5 min at RT and finally incubate for 8–12 min with trypsin/EDTA solution at 37°C until they come off easily with or often without gently knocking on the side of the flask.
13. Add complete FAD medium and resuspend the cells by pipetting up and down three to five times. After centrifugation for 5 min at 220*g* resuspend the pellet in complete FAD medium and seed the cells onto collagen I-coated dishes.

14. The keratinocytes have to be grown with feeder cells for at least 4–8 passages before trying to omit the feeder cells (*see* **Note 10**).
15. Keratinocytes may be frozen at a density of 1–1.5 $\times$ 10^6 cells/ml in keratinocyte freezing solution.
16. After thawing, keratinocytes should be seeded densely (about 10^5 cells/cm^2) (*see* **Note 11**).
17. The spontaneously immortalized mouse keratinocytes may be differentiated by addition of calcium to the complete FAD medium at a final concentration of 1.2 mM (high-calcium medium). Confluent cultures stratify when grown in high-calcium medium.

4. Notes

1. 3T3-J2 fibroblasts should be split routinely or used for co-culture with keratinocytes as soon as they get confluent (*see* **Fig. 5.2b**).
2. 3T3-J2 fibroblasts are subject to normal ageing. Make sure to note passage numbers and to freeze enough aliquots at low passage. Cultures of 3T3-J2 fibroblasts should not be used any longer if the typical phenotype changes and cells acquire a spindle cell phenotype as this can be a sign of transformation.
3. About 5 ml of mitomycin C-containing medium is sufficient for a 10 cm Petri dish.
4. Neonates on days 0 or 1 postpartum yield the highest number of isolated keratinocytes. According to our experience, efficiency of isolation worsens dramatically when mice after day 3 postpartum are used. Keratinocytes from unborn mice at E18 or E19 can be isolated using the same protocol.
5. It is best to use the litter immediately after birth which is often in the morning. Then incubate with dispase for 7 hours during the day and go on with the protocol on the same day. If you start the recovery of neonatal mouse skin in the afternoon, terminate the digestion with dispase early the next morning.
6. The washed dishes can be left with traces of PBS at room temperature for a few hours until the keratinocytes are ready to be seeded. Alternatively, pre-coated dishes (e.g. Biocoat six-well plates, Beckton Dickinson) may be used.
7. The epidermis comes off in one piece as a thin white layer, whereas the residual tissue appears pink, shiny and slimy.

8. Depending on the mouse strain used as well as the nature of the mutation and age variations it might take up to 2 months before keratinocyte colonies become visible.
9. During the first eight or more passages, the cultures still contain melanocytes and possibly other resident epidermal cell types. These cells cannot always be easily distinguished without specific staining in cultures of white mouse strains, e.g. BALB/c, but melanocytes are clearly recognizable as black cells in cultures from coloured mouse strains, e.g. C57Bl/6 mice. The melanocytes are gradually depleted with increased passaging.
10. If high amounts of large, differentiated or irregularly shaped keratinocytes become a problem in an established mouse keratinocyte line (compare **Fig. 5.2f and a**), the addition of feeder cells for 1–2 passages and/or seeding at a higher density (less that 1:1) may be helpful.
11. In order to enhance plating efficiency after thawing, keratinocytes may optionally be seeded together with feeder cells irrespective of their passage number. One subconfluent to confluent 10 cm dish of 3T3-J2 feeder cells ($0.8–1.6 \times 10^6$) is sufficient for twelve 35 mm dishes. The feeder cells are lost at the first passage after thawing and may subsequently be omitted.

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Chapter 6

Study of Epidermal Differentiation in Human Keratinocytes Cultured in Autocrine Conditions

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Abstract

This chapter deals with protocols to set up human keratinocyte cultures in serum-free conditions and lead them to autocrine autonomously growing conditions. These conditions have proven adequate for studies of epidermal differentiation by measurements of the expression of typical early and late differentiation markers. The chapter also deals with the use of quantitative RT-PCR in order to determine the epidermal marker gene expression levels by comparison with adequate housekeeping genes.

Key words: Keratinocytes, Autocrine culture, Cell density, Epidermal differentiation markers, Quantitative RT-PCR.

1. Introduction

Since the development of efficient culture conditions for the growth of human epidermal keratinocytes (1, 2), the utilization of such in vitro techniques in order to study the control of keratinocyte proliferation and differentiation by its own environment has become very common. However, among the multiple compounds that are part of the complex culture medium described for this cell type, several molecules (e.g. epidermal growth factor (EGF), vitamin A, insulin) are well known as potential controllers of the epidermal differentiation process itself. The researchers who have used those culture techniques for studying epidermal differentiation either did not take into account the possible interference of the molecular controllers on their results or described procedures where these molecules were withdrawn for a few hours

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preceding the study of this material. Consequently, these procedures had to deal with the cellular response induced in keratinocytes when deprived of certain essential compounds and with the inductions and/or inhibitions due to those controller molecules during the culture period that preceded their withdrawal.

A solution to these problems was brought when autocrine culture conditions were discovered after removing growth factors from the medium of proliferating keratinocytes grown up to a certain cell density (3). Indeed, these keratinocytes deprived of exogenous growth factors continue to grow rapidly until culture confluence is reached, thanks to the endogenous production of amphiregulin (AR) at a rate that is high enough to sustain autocrine activation of the EGF receptor (4, 5). The autocrine culture conditions maintain rapid cell growth and high clonogenic potential during the subconfluent period of the culture until the culture plate is fully covered by tightly packed keratinocytes, when the keratinocytes confluence is reached. The absence of growth factors in autocrine conditions is absolutely required for the elevated expression of epidermal differentiation markers, when the commitment to differentiate is triggered by culture confluence (6). Moreover, the induction of differentiation started at confluence of keratinocytes is pursued toward a more complete program of epidermal differentiation during postconfluence (7), leading to characteristic expression of later markers in those conditions, as revealed for instance by the occurrence of unique alternative splicing of the Ecm1 gene (8).

In this chapter, we describe how autocrine conditions can be obtained in our model of human keratinocyte cultures, and we present analysis of multiple markers of early and late epidermal differentiation by the quantitative RT-PCR approach (qRT-PCR).

2. Materials

1. Solution A: Washing solution for tissue and cells: 10.0 mM glucose, 3.0 mM KCl, 130.0 mM NaCl, 1.0 mM $Na_2HPO_4.7H_2O$ (or anhydrous), 0.0033 mM Phenol Red, 30.0 mM Hepes. Dissolve Hepes in distilled H_2O and adjust pH at 7.4 with 10 M NaOH. Then, dissolve the other compounds and check pH before adjusting the final volume. Solution A is sterilized through a Sterivex^{TM} – GP 0.22 μm filter (Millipore cat. no. SVGP01015) and stored refrigerated at 4°C.
2. Trypsin solution for epidermal tissue disruption: Trypsin (Sigma T-9201 trypsin from bovine pancreas) is dissolved at 0.17% (weight/volume) into ice-cold solution A. The

solution is then sterilized through a Millex^{TM}-GP 0.22 μm filter (Millipore cat. no. SLGP033RB), aliquoted as 40 ml samples and stored frozen at –20°C.

3. Trypsin/EDTA (0.025%/0.01%) solution for culture passage: Dissolve EDTA di-sodium salt into solution A and adjust pH at 7.4 if necessary. The solution is then chilled on ice before addition of 0.025% (weight/volume) trypsin (Sigma cat. no. T-9201) and then sterilized through a Millex^{TM}-GP 0.22 μm filter (Millipore cat. no. SLGP033RB), aliquoted as 8 ml samples and stored frozen at –20°C.

4. Dialyzed fetal calf serum: For neutralization of trypsin solutions, a dialyzed serum containing no calcium is required. Dialysis tubing MWCO-12,000–14,000 Da (Dialysis tubing – Visking Medicell International Ltd) is prepared by 15 min boiling in a 0.1% EDTA, 0.1% Na_2CO_3 solution, followed by two 15 min washings in boiling distilled H_2O.

   100 ml Fetal calf serum (BioWhittaker® cat. no. 14-471F) is introduced into the dialysis tubing which is then sealed before against 10 l of stirred 4°C phosphate buffered saline (PBS) solution without calcium (0.2 g KCl, 0.2 g KH_2PO_4, 8 g NaCl, 1.44 g $Na_2HPO_4.2H_2O$ in 1 l distilled H_2O and adjusted at pH 7.4). The PBS solution is replaced four times approximately every 12 hours.

5. Antibiotics and antifungal agent: A penicillin/streptomycin (PS) solution is prepared by dissolution of 50,000 U/ml penicillin G (Sigma cat. no. P-3032) and 50,000 μg/ml streptomycin (Sigma cat. no. S-9137) into solution A before sterilization through a Millex^{TM}-GP 0.22 μm filter (Millipore cat. no. SLGP033RB). This PS solution is diluted 1,000 × for use in medium.

   Gentamycin (50 mg/ml, BioWhittaker® cat. no. 17-518Z), fungizone (250 μg/ml, Sigma cat. no. A-9528), and ampicillin (250 μg/ml, sodium salt, Sigma cat. no. A-2804) are also diluted 1,000 × for final use.

6. Hydrocortisone: First, 25 mg of hydrocortisone (Sigma cat. no. H-4001) is dissolved in 5 ml ethanol, filtered through a Millex^{TM}-GP 0.22 μm filter (Millipore cat. no. SLGP033RB), and stored at 4°C. Then, 0.4 ml of this solution is mixed with 9.6 ml of solution A, stored at –20°C, and finally diluted 1,000 × in culture medium.

7. Amino acids: A concentrated solution of amino acids is prepared by dissolution of 1.677 g L-histidine (Sigma cat. no. H-5659), 3.280 g L-isoleucine (Sigma cat. no. I-2752), 0.448 g L-methionine (Sigma cat. no. M-2893), 0.306 g de L-tryptophan (Sigma cat. no. T-8941), 0.453 g L-tyrosine (Sigma cat. no. T-1020) into 1,000 ml distilled H_2O (*see*

Note 1). The solution is sterilized through filtration on a Sterivex™-GP 0.22 μm filter (Millipore cat. no. SVGP01015) and then stored at –20°C. Dilute 1:33 for final use in culture medium.

8. Medium for culture settings: For the settings of keratinocyte cultures, the basal medium KBM®-2 (Clonetics® cat. no. CC-3103) is supplemented with SingleQuots® KGM-2® (Clonetics® cat. no. CC-4152) in order to contain at final concentrations 50 μg/ml bovine pituitary extract (BPE), 10 ng/ml EGF, 5 μg/ml insulin, 5×10^{-7} M hydrocortisone, and 5 μg/ml transferrin. For the settings of the primary culture, fungizone, gentamycin, and ampicillin are added to this medium.

   For secondary cultures, the PS solution of antibiotics is added to the same medium.

9. Medium for culture growth: After settings of the cultures, a keratinocyte growth medium is used: Epilife® medium (Cascade Biologics™ cat. no. M-EPI-500-CA) supplemented with HKGS (Cascade Biologics™ cat. no. S-001-5) in order to contain at final concentrations 0.2% BPE, 0.2 ng/ml human recombinant EGF, 0.18 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin. For primary cultures, fungizone, gentamycin, and ampicillin are added to this medium.

   For secondary cultures, the PS solution is added to the same medium.

10. Medium for autocrine growth of human keratinocytes: For autocrine growth of keratinocytes, the basal Epilife® medium (Cascade Biologics™ cat. no. M-EPI-500-CA) supplemented with amino acids, hydrocortisone, and PS antibiotics is used.

11. Freezing solution: For cryopreservation of cells in ice-cold cryopreservation tubes (Nunc CryoTube™ Reference 366656), twice concentrated medium containing 60% basal Epilife® medium (Cascade Biologics™ cat. no. M-EPI-500-CA), 20% dimethylsulfoxide, and 20% dialyzed fetal calf serum is used.

12. RNA extraction: To isolate Total RNA, RNeasy mini kit (Qiagen cat. no. 74106) and QIAshredder spin column (Qiagen cat. no. 79656) are used using the spin technology protocol according to the instructions of the manufacturer.

13. cDNA synthesis: To produce cDNA, Oligo$(dT)_{12\text{-}18}$ primer (Invitrogen cat. no. 18418-012), dNTP (Invitrogen cat. no. 10297-018), and SuperScript II RNAse H-Reverse transcriptase kit (Invitrogen cat. no. 18064-014) are used.

3. Methods

3.1. Primary Culture of Human Adult Epidermal Keratinocytes

1. The normal skin is obtained from excessive tissue removed during abdominoplasty of adult patients and superficially sliced with a dermatome in order to reduce the thickness of the dermal tissue. The samples are stored at 4°C in physiological solution.
2. After transportation to the culture room, skin samples are spread in large plastic Petri dishes containing solution A and cut with a scalpel into pieces of an approximate surface of 1–2 cm^2.
3. They are then transferred as floating pieces on the cold (4°C) trypsin solution supplemented with gentamycin, ampicillin, and fungizone. The hydrophobic stratum corneum is exposed to air on the surface, while the dermis is immersed in the trypsin solution during the overnight incubation at 4°C.
4. On the next morning, the epidermis and dermis are separated using thin forceps and the epidermal tissue is placed in the medium for culture settings containing 2% of dialyzed fetal calf serum. The epidermal cells are then dissociated mechanically by stirring of the medium. The cell suspension is then filtered through a Cell Strainer (Falcon cat. no. 352350) and the eluate recovered in a conical sterile centrifugation tube. The cell suspension is then centrifuged 10 min at 1,000 rpm and 4°C with the Allegra X-15R centrifuge (Beckman Coulter). The pellet is resuspended in 10 ml of the medium described in Medium for culture settings and cells are counted.
5. In 175 cm^2 culture flasks, 25 μl of dialyzed fetal calf serum is layered and then the cell suspension is seeded at an approximate density of 46,000 cells/cm^2. The addition of 25 ml of the culture medium allows a good dispersion of cells and the flasks are incubated for 3 days in the culture incubator at 37°C in a humidified atmosphere and 5% CO_2.
6. Finally, the medium is aspirated and replaced by the medium described in Medium for culture growth. This medium is changed every other day. After a couple of weeks, keratinocytes cover 50–60% of the surface of the flask and are ready for subculture.

3.2. Passage of Serum-Free Cultured Human Keratinocytes

1. 20 ml of the medium for culture setting containing 2% of dialyzed fetal calf serum is prepared for each culture flask. This solution is then kept on melting ice until use.
2. A volume of 8 ml of trypsin solution described in Medium for autocrine growth of human keratinocytes is melted up to room temperature. The culture medium is aspirated from each flask and replaced by the 8 ml of trypsin solution. After 5 min, the trypsin solution is

aspirated, leaving approximately 1 ml to cover the cells. This removes the majority of fibroblasts and melanocytes present in the primary culture.

3. The cell detachment is regularly monitored under phase-contrast microscopy. After approximately 30 min, cells are clearly detached from the plastic substratum. The flask is then immediately hit by hand laterally in order to detach all the cells and 10 ml of the ice-cold blocking solution containing the dialyzed serum is rapidly added to the cell suspension. This suspension is then rapidly transferred into a 50 ml centrifugation tube on melting ice. The flask is rinsed with 10 ml of the blocking solution and the rinsing suspension added to the centrifugation tube.
4. The suspension is then centrifuged 10 min at 233 g and 4°C (*see* **Note 2**). The supernatant is discarded and the pellet is resuspended in 10 ml of the medium described in Medium for culture growth. The cells are then counted and plated into 175 cm^2 flasks at a density of 10,000 cells/cm^2. The medium is changed every other day. When a cell density of 60% of confluence is reached, the cells are ready for freezing.

3.3. Cryopreservation of Keratinocytes

1. Cells are detached as described as for the passage of serum-free cultured human keratinocytes. After counting, keratinocytes are suspended at 2×10^6 cells/ml into cold EpiLife medium (Medium for autocrine growth of human keratinocytes). An equivalent volume of freezing solution is added in order to obtain a cell density of 1×10^6 cells/ml.
2. Aliquots of 1 ml are placed in ice-cold cryopreservation tubes. The tubes are placed in a polystyrene isolating box and transferred to –80°C for 24 hours minimum. Finally, the tubes are transferred in liquid nitrogen for further preservation.
3. For thawing of keratinocytes, CryoTubes are incubated in a bath at 37°C. When the suspension is totally liquid, it is diluted in KGM-2 medium. Cells are then counted and seeded at cell density between 5000 and 7000 cells/cm^2.

3.4. Secondary Cultures of Keratinocytes

1. Cells are cultured for 24 hours in KGM-2 medium, then this medium is replaced by complete EpiLife medium. This medium is replaced every 2 days.
2. When the proliferative keratinocytes cover approximately 50% of the culture substratum, the cells are washed twice in solution A and then cultured in EpiLife medium containing no peptide prepared as described above. This medium is renewed every 2 days and allows keratinocytes to continue growth and proliferation. From that point, the culture condition is described as autocrine since keratinocytes provide themselves the growth factors necessary for their proliferative phenotype.

3. When the whole culture surface is covered by keratinocytes (usually 3–4 days later), mitotic figures can still be observed for additional 24 hours. On the next day, the culture is growth-arrested and considered as absolutely confluent. This type of culture can be kept in the same autocrine medium for several days. We typically studied cultures as post-confluent 4 days after the day they had become confluent. Some cell stratification can then be observed (**Fig. 6.1**). For study of differentiation in this model, four different culture densities of keratinocytes have been chosen around culture confluence. Typically, cells from four different normal donors are studied. In this case, 7000 cells/cm^2 are plated in 35-mm dishes. Two days before confluence (C-2d), at confluence (C), or both 2 and 4 days after confluence (C+2d and C+4d), the medium is removed and dishes are stored frozen at –80°C before further analysis.

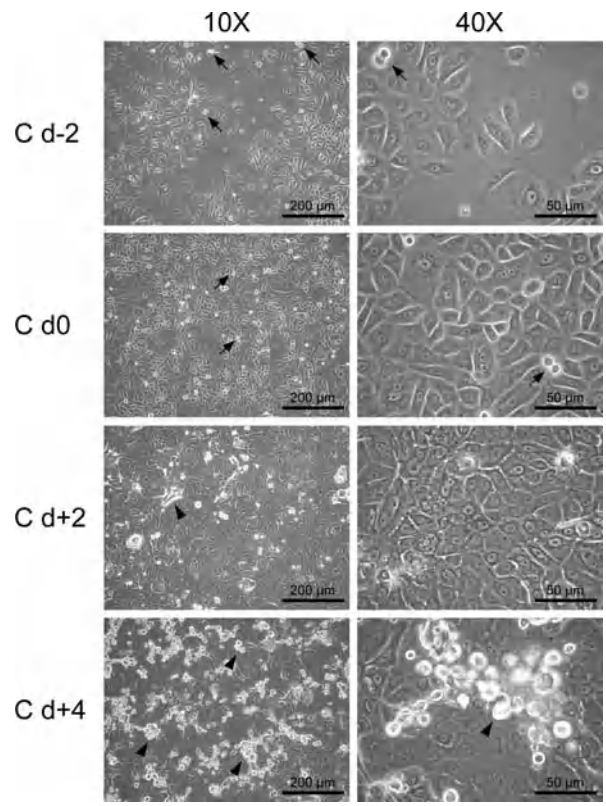

Fig. 6.1. Morphology of autocrine cultures of human keratinocytes at various cell densities. Autocrine keratinocytes were photographed at two different magnifications in EpiLife medium containing no peptide using inverted phase-contrast microscopy at different times of culture of keratinocytes (C-2d, 2 days before culture confluence; C, confluence; C+2d, 2 days after confluence; C+4d, 4 days after confluence). Arrows indicate mitotic figures and arrowheads indicate cellular stratification.

3.5. Analysis of Keratinocyte Expression of Epidermal Differentiation Markers by qRT-PCR

3.5.1. RNA Extraction

1. Before RNA extraction, the cell-culture dishes are thawed on ice (*see* **Note 3**).
2. Total RNA is then isolated at room temperature using the RNeasy mini kit. Cells plated in 35-mm dishes are lysed with 350 µl of buffer RLT containing 10 µl of β-mercaptoethanol per 1 ml buffer RLT. Lysate is homogenized into a QIAshredder spin column. This column is centrifuged for 2 min at full speed in microcentrifuge and 350 µl of 70% ethanol is added to the homogenized lysate. Each sample is mixed by pipetting. A volume of 500 µl of the sample is transferred to an RNeasy spin column placed in a 2 ml collection tube. The remaining volume of about 200 µl is centrifuged afterward in the same RNeasy spin column. To wash the spin column membrane, 650 µl of buffer RW1 is first added to the RNeasy spin column which is centrifuged for 30 sec at 9,300 g. Second, the column is washed twice using the same procedure with 500 µl of RPE buffer. To eliminate any possible carryover of the RPE buffer, the column is centrifuged at full speed for 1 min. To elute the RNA, the column is placed in a new 1.5 ml collection tube and 40 µl of RNAse-free water is added directly to the spin column membrane which is centrifuged for 1 min at 9,300 g.
3. The concentration of RNA in each sample is measured spectrophotometrically with 3 µl of RNA diluted in 87 µl of RNAse-free water. For each sample, the concentration is measured in duplicate and the mean is validated if the values vary by less than 10%. If not, duplicate measurements are repeated until validated. Total RNA is stored at a concentration of 100 ng/µl, frozen at –80°C.

3.5.2. Reverse Transcription

1. A volume of 10 µl of total RNA (i.e., 1µg) is mixed with 2 µl Oligo$(dT)_{12\text{-}18}$ primer (50 ng) and 1 µl of dNTP Mix (10 mM each) in a 100 µl PCR tube. Mixture is heated to 65°C for 5 min using the Gene Amp PCR System 2400 (Perkin Elmer) and then quickly chilled on ice.
2. The tube content is collected by a brief centrifugation using Galaxy mini centrifuge (VWR). A volume of 4 µl of 5 × first-strand buffer is added with 2 µl of DTT (0.1 M) and 1 µl of SuperScript II RNAse H-Reverse transcriptase (10 units). The mixture (20 µl of volume) is then incubated at 50°C for 50 min and the reaction is stopped by heating at 70°C for 15 min using the Gene Amp PCR System 2400 (Perkin Elmer). The produced cDNA is diluted 1:50 and stored at –20°C. For each qPCR, 5 µl of this product is used.

3.5.3. Quantitative PCR (qPCR)

1. Primers (Sigma-Genosys) were designed using Primer Express software and optimized to work with universal assay conditions. Using MicroAmp™ Optical 96-well reaction plate (Applied

Table 6.1
Sequences of primers

Gene symbol	Forward primer	Reverse primer
RPL13A	CTCAAGGTCGTGCGTCTGAA	TGGCTGTCACTGCCTGGTACT
TBP	TCAAACCCAGAATTGTTCTCCTTAT	CCTGAATCCCTTTAGAATAGGGTAGA
KRT10	AATCAGATTCTCAACCTAACAAC	TTCCTCTTGCTTTGATGGG
LOR	TCATGATGCTACCCGAGGTTTG	CAGAACTAGATGCAGCCGGAGA
FLG	GGGCACTGAAAGGCAAAAAG	CACCATAATCATAATCTGCACTACCA
TGM-1	GTCGTCTTCCGGCTCGAA	TCACTGTTTCATTGCCTCCAAT
KRT14	CGATGGCAAGGTGGTGTC	GGGTGAAGCAGGGTCCAG
IVL	TGAAACAGCCAACTCCAC	CTCATCCAGCACCCTACG
PCNA	CCACTCTCTTCAACGGTGACACT	TCCCATATCCGCAATTTTATACTCT
RPLP0	ATCAACGGGTACAAACGAGTC	CAGATGGATCAGCCAAGAAGG

Biosystems, cat. no. N801-0560), 2.5 µl of forward primer (2.4 µM) and 2.5 µl of reverse primer (2.4 µM) designed for the gene of interest (**Table 6.1**) are mixed with 10 µl of Power SYBR Green PCR master Mix (Applied Biosystems cat. no. 4368708) in each well. A volume of 5 µl of the 1:50 diluted template cDNA is then added and the plate is finally coated with an optical adhesive cover (Applied Biosystems cat. no. 4311971) (*see* **Note 4**). The plate is centrifuged for 3 min at 1,000 rpm with the Allegra X-15R centrifuge (Beckman Coulter) and then placed in the 7300 Real-Time PCR machine (Applied Biosystems) to perform the amplification. All samples are run in duplicates (or in triplicates for the housekeeping genes) and the mean of duplicated values is validated if the dCt standard error, measured by the 7300 Real-Time PCR software, is lower than 0.200. In case of failure, the duplicate measurements for the gene of interest, in parallel with housekeeping genes *TBP* and *RPL13A*, are repeated until validated (*see* **Note 5**).

2. Universal conditions of amplification assay are: 50°C, 2 min

$$\left.\begin{array}{l} 95^{\circ}\mathrm{C},\ 10\ \mathrm{min} \\ 95^{\circ}\mathrm{C},\ 15\ \mathrm{sec} \\ 60^{\circ}\mathrm{C},\ 1\ \mathrm{min} \end{array}\right\} 40\ \mathrm{cycles}$$

3. All mRNA levels are normalized relative to the geometric mean of TATA-binding protein (*TBP*) and ribosomal protein large 13A (*RPL13A*) expression levels, two housekeeping

genes selected with the *geNorm* analysis program version 3.5 because of their optimal stabilities during the differentiation of human epidermal keratinocytes (Minner and Poumay, 2009) (*see* (9) and **Note 6**).

4. Statistical analysis can be performed with deltaCt values by the paired Tukey–Kramer Multiple Comparisons Test (One-way Anova) using GraphPad InStat 3.0. In this epidermal differentiation model, previous data have shown that mRNA expression of keratin-14 (*KRT-14*), an early marker detectable in the basal layer in the epidermis, remains stable, independently of state of confluence of keratinocyte culture. Conversely, the

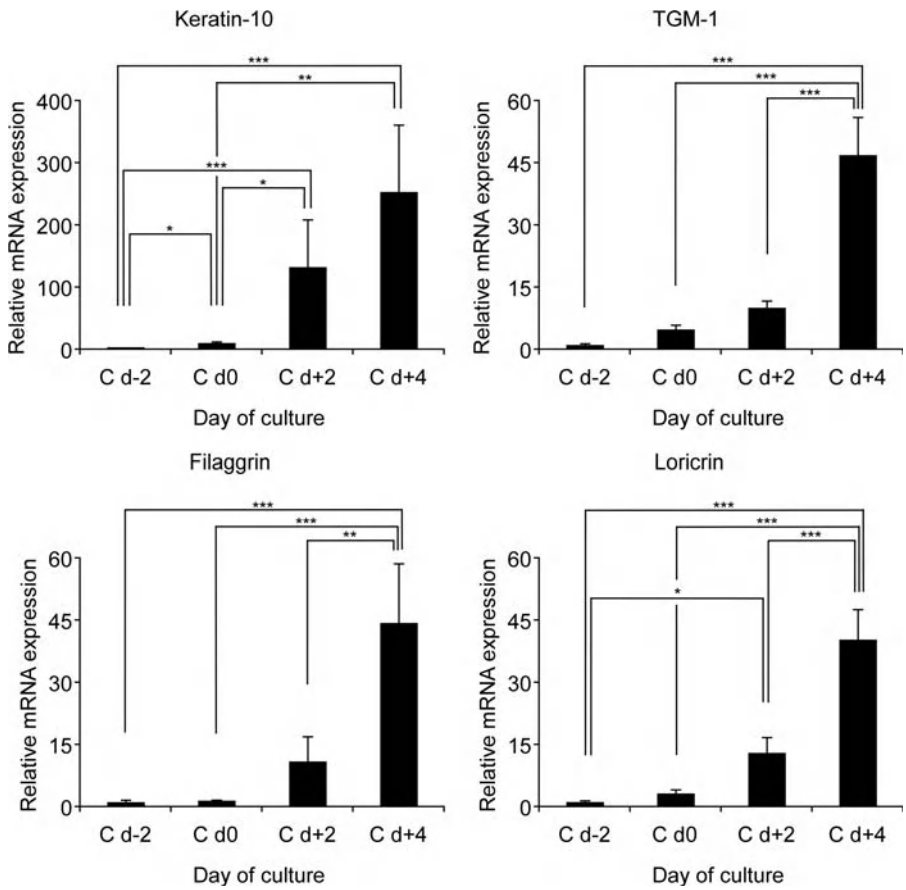

Fig. 6.2. Relative mRNA expression normalized to *RPL13A* and *TBP*. The mRNA expression determined in human keratinocytes obtained from four different normal donors at four different times of culture was normalized relative to the geometric mean of *RPL13A* and *TBP* expression levels. The cell density of keratinocytes (C-2d, 2 days before culture confluence; C, confluence; C+2d, 2 days after confluence; C+4d, 4 days after confluence) reveals a progressively increased relative expression of *KRT10*, *LOR*, *FLG*,and *TGM-1*. For each sample, total RNA was extracted, reverse transcription was performed, and cDNA was analyzed in duplicate by real-time PCR. Statistical analysis was performed with deltaCt values by the paired Tukey–Kramer Multiple Comparisons Test (One-way Anova). Data are shown as means ± SEM ($n = 4$) (*$p<0.05$; **$p<0.01$; ***$p<0.001$) and C-2d is chosen as reference.

mRNA expression of keratin-10 (*KRT10*) and of involucrin (*IVL*) increases significantly with confluence of the culture, in relation with the duration of the culture, whereas the cell proliferation decreases after confluence, as revealed by the lowered expression level of the marker Proliferating Cell Nuclear Antigen (*PCNA*) in these conditions (9).

5. This model can also be used for studies of the expression of later markers of differentiation: Loricrin (*LOR*), Filaggrin (*FLG*), and Transglutaminase-1 (*TGM-1*) can be analyzed and compared with *KRT10* expression (**Fig. 6.2**). Results show that these epidermal genes (*LOR*, *FLG,*and *TGM-1*) are very significantly expressed 4 days after confluence in comparison with subconfluent conditions, whereas *KRT10* is already significantly expressed concomitantly with cell growth arrest at culture confluence. Two days after confluence, the expression of *KRT10* becomes stable. These observations confirm that, in this model as in vivo, the expression of *KRT10* can be used as an early marker of differentiation and suggest the possible analysis of *LOR*, *FLG,*and *TGM-1* as late markers of differentiation.

4. Notes

1. The dissolution of amino acids is obtained by heating at 55°C approximately and slow stirring for 1–2 hours (until complete dissolution).
2. The centrifuge must be turned on well ahead of its utilization in order to be sure that the temperature is actually 4°C.
3. Stability and activity of ribonucleases (RNAses) require keeping an RNAse-free environment before the RNA extraction. It is crucial to use sterile and RNAse-free plasticware and/or baked glassware for all applications. Diethyl pyrocarbonate (DEPC) is able to modify RNAses by covalent modification. Glassware and plastic microtubes (0.5 or 1.5 ml) are systematically treated with 0.1% of DEPC (0.1% in water), minimum overnight at room temperature and then autoclaved to eliminate residual DEPC. It is also crucial to wear latex or vinyl gloves. The purified RNA is always kept on ice for downstream analysis.
4. The use of powder-free gloves is recommended because the presence of powder on the optical adhesive cover can perturb the light detection in the 7300 Real-Time PCR system (or other systems).

5. Many events can lead to a mistake during pipetting and thus to erroneous results. We really stress the importance of being perfectly constant during pipetting. For this reason, the use of volumes under 5 µl is not advised. We recommend the preparation of a first mix containing primers and Power SYBR Green PCR master Mix in order to make available a sufficient volume for all samples tested, and we recommend to pipet 15 µl of this solution in relevant wells of the MicroAmp^{TM} Optical 96-well reaction plate.
6. As already recently demonstrated (*see (9)*), ribosomal protein large P0 (*RPLP0*), also called *36B4*, can also be chosen as housekeeping gene in this model of epidermal differentiation.

Acknowledgments

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Chapter 7

Directed Differentiation of Human Embryonic Stem Cells to Epidermal Progenitors

Christian M. Metallo, Lin Ji, Juan J. de Pablo, and Sean P. Palecek

Abstract

Human embryonic stem (hES) cells can differentiate into virtually all somatic cell types. In order to incorporate these derivatives into scientific or clinical applications, efficient methods of directing hES cell differentiation to pure subpopulations are required. Here we describe a robust strategy for generating cytokeratin 14+ (K14+)/p63+ keratinocyte progenitors from hES cells through stage-specific application of retinoic acid (RA) and bone morphogenetic protein-4 (BMP4). Induction of undifferentiated hES cells with RA stimulates expression of epithelial genes such as K18 and p63. Subculture of RA-treated cells in defined keratinocyte medium enables isolation of relatively pure K14+ epithelial populations; these cells also retain the capacity to terminally differentiate. The use of defined media throughout differentiation allows for detailed characterization of keratinocyte lineage specification from hES cells through the use of gene expression and immunofluorescence analyses.

Key words: Human embryonic stem cells, Directed differentiation, Ectoderm, Retinoic acid, Bone morphogenetic protein, p63, Epithelial progenitors, Keratinocytes.

1. Introduction

Human embryonic stem (hES) cells are capable of proliferating extensively and differentiating to form cells of the three embryonic germ layers (1). As such, these pluripotent cells can serve as a model system for studying early events during human development and are a powerful resource of non-transformed human cells for use in diagnostic and potentially therapeutic applications. Successful exploitation of hES cell derivatives requires the ability to direct hES cell differentiation to specific lineages in defined, efficient, and scalable systems (2). While researchers have

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identified strategies of obtaining endodermal, mesodermal, and neuroectodermal progenitors from hES cells (3–5), efficient methods of deriving non-neural epithelia have only recently been identified (6, 7). The method described here involves treatment of hES cells with retinoic acid (RA) and bone morphogenetic protein-4 (BMP-4) in defined medium at specific stages of differentiation to direct hES cells to form relatively pure populations of epithelial cells and more definitive keratinocytes.

Early induction of undifferentiated hES cell cultures is necessary to ensure efficient differentiation to epithelial lineages by RA and BMP4. This process is marked by significant increases in cytokeratin 18 (K18) and p63 transcription and relative decreases in pluripotency (e.g., Oct-4, SSEA-4, Nanog) and neural gene transcription (7), as measured by quantitative PCR or flow cytometry. These initial findings are supported by a previous study in mice that identified a role for RA and retinoic acid receptor α (RARα) in regulation of p63 (8), which, in turn, is involved in ectodermal fate choices (neural vs. non-neural epithelia) in the zebrafish (9). After early induction with RA and BMP4, these K18+/p63+ cells are subcultured in a defined epithelial medium and ultimately give rise to K14+/p63+ keratinocytes, as demonstrated by immunofluorescent staining and flow cytometry. A consistent epithelial morphology and lack of detectable Brachyury (T) or FOXA2 transcription throughout the differentiation process provide evidence that these K14+ epithelia are of ectodermal origin (7). However, markers associated with various epithelial tissues are detected in culture, demonstrating multipotent potential of these epithelial progenitors (7). This highly efficient process enables robust generation of keratinocyte progenitors that may be incorporated into engineered tissues for clinical or analytic use.

2. Materials

2.1. Cell Growth and Differentiation

1. hES cell growth medium: Dulbecco's Modified Eagle's Medium (DMEM)/F12 (1:1) (Invitrogen, Carlsbad, CA) supplemented with 20% Knockout Serum Replacer (KSR, Invitrogen), 1 × MEM non-essential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma).
2. Basic fibroblast growth factor (bFGF, Invitrogen): added to conditioned hES cell growth medium at a concentration of 4 ng/ml.

3. Matrigel (BD Biosciences, San Jose, CA). Store at –80°C in single-use aliquots. Thaw at 4°C; all manipulations must be conducted on ice using chilled pipettes to avoid gelation of Matrigel.
4. Dispase (Invitrogen). Reconstituted in DMEM/F12 at 2 mg/ml; store aliquots at –20°C.
5. Gelatin powder (Sigma) dissolved in water at 0.1% (w/v).
6. Differentiation medium: DMEM/F12 supplemented with 1 × N2 supplement (Invitrogen), 1 μM all-trans retinoic acid (RA, Sigma), and 25 ng/ml BMP-4 (R&D Systems, Minneapolis, MN) (*see* **Note 1**). RA is sensitive to light, heat, and oxidation and should be reconstituted in sterile DMSO at a stock concentration of 10 mM and stored at –20°C for up to 6 months. Prior to use make working stock by diluting stock 10-fold in DMSO (can be stored at 4°C for up to 2 weeks); dilute working stock 1:1000 in culture medium.
7. Defined keratinocyte serum-free medium and supplement (DSFM, Invitrogen).
8. Trypsin (0.05%)-ethylenediamine tetraacetic acid (EDTA, 1 mM) from Invitrogen.
9. Soybean trypsin inhibitor (Invitrogen) dissolved in PBS at 0.5 mg/ml and sterile filtered.

2.2. Quantitative RT-PCR

1. RNeasy Mini Kit (Qiagen, Valencia, CA).
2. Omniscript RT reverse transcriptase (Qiagen).
3. Oligo-dT primers at 50 μM (Invitrogen).
4. Quantitect SYBR Green qPCR kit (Qiagen).
5. Fluorescein sodium salt (Sigma) (Fisher).
6. Primers (**Table** 7.1) reconstituted in water to 20 μM and stored at –20°C (IDT DNA, Coralville, IA).

2.3. Immunofluorescent Staining

1. IF fixation buffer: 16% (w/v) paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA) diluted to 4% (v/v) in PBS prior to use.
2. Quenching buffer: PBS with 100 mM glycine.
3. Blocking buffer: PBS with 5% chick serum (Sigma) and 0.4% (v/v) Triton X-100 added.
4. Primary antibodies (recommended dilution): mouse anti-p63 monoclonal antibody (mAb, 1:100), rabbit anti-K14 polyclonal antibody (pAb, both from Lab Vision, Fremont, CA) (1:200), mouse anti-β-catenin mAb (1:200, BD Biosciences), goat anti-involucrin pAb (1:100), goat anti-filaggrin pAb (1:100, both from Santa Cruz Biotechnology, Santa Cruz, CA).

Table 7.1
Primers used for quantitative PCR analysis using SYBR Green-based fluorescent quantification

Gene	Forward primer	Reverse primer	Amplicon size
GAPDH	5′-caccgtcaaggctgagaacg-3′	5′-gccccacttgattttggagg-3′	91
FOXA2	5′-gggagcggtgaagatgga-3′	5′-tcatgttgctcacggaggagta-3′	89
Brachyury (T)	5′-tgcttccctgagacccagtt-3′	5′-gatcacttctttcctttgcatcaag-3′	121
Nestin	5′-tgaagggcaatcacaacagg-3′	5′-tgaccccaacatgacctctg-3′	136
Sox1	5′-caatgcggggaggagaagtc-3′	5′-ctctggaccaaactgtggcg-3′	464
Pax6	5′-ggcaggtattacgagactgg-3′	5′-cctcatctgaatcttctccg-3′	427
K18	5′-ccgtcttgctgctgatgact-3′	5′-ggccttttacttcctcttcgtg-3′	200
TAp63	5′-aagatggtgcgacaaacaag-3′	5′-agagagcatcgaaggtggag-3′	234
ΔNp63	5′-ggaaaacaatgcccagactc-3′	5′-gtggaatacgtccaggtggc-3′	294
K14	5′-gaccattgaggacctgagga-3′	5′-attgatgtcggcttccacac-3′	157

5. Secondary antibodies: chick anti-mouse IgG Alexa 488-conjugated antibodies, donkey anti-rabbit IgG Alexa 594-conjugated antibodies, donkey anti-goat IgG Alexa 488-conjugated antibodies (all from Invitrogen).
6. Hoechst 33342 nuclear staining solution (Sigma, 10 mg/ml stock diluted 1:5000 in water).

2.4. Flow Cytometry

1. FC fixation buffer: 1% (v/v) PFA in PBS.
2. Methanol (Fisher).
3. FACS buffer: PBS with 2% (w/v) fetal bovine serum (FBS, Invitrogen), 0.1% (v/v) Triton X-100 (Sigma), and 0.1% (w/v) sodium azide (Sigma) added. Store at 4°C for up to 2 weeks.
4. Primary antibodies: mouse anti-K18 (DC10) mAb, rabbit anti-K14 pAb (both from Lab Vision), mouse anti-nestin mAb (Santa Cruz Biotechnology).
5. Secondary antibodies: chick anti-mouse IgG Alexa 647-conjugated antibodies, goat anti-rabbit IgG Alexa 488-conjugated antibodies (both from Invitrogen).

3. Methods

High efficiency directed differentiation of hES cells requires precise manipulation of the cellular microenvironment. Specification of hES cells to epithelial lineages can be effectively accomplished

through time- and concentration-dependent application of RA and induction of BMP signaling. Early treatment of undifferentiated hES cells stimulates expression of epithelial genes, including the transcription factor p63, which is involved in maintenance of epithelial progenitors in a variety of tissues (10, 11). Subsequent culture of RA-induced hES cells in defined keratinocyte medium allows for propagation of definitive keratinocytes capable of undergoing terminal differentiation.

The method described here enables production of high-purity epithelial populations from hES cells. Initial cultures entering the differentiation process must be pure and undifferentiated, as spontaneously differentiating cultures may not be restricted to epithelial lineages, compromising the ultimate purity of keratinocytes obtained. Procedures for the characterization of differentiating cultures are provided, including quantitative RT-PCR, fluorescence microscopy, and flow cytometry, which provide quantitative and qualitative measures of gene expression. Given the use of defined culture media throughout differentiation, this system provides a unique means of investigating the molecular events involved in early epithelial differentiation during human development.

3.1. Cell Growth and Differentiation

1. hES cells are cultivated in hES cell growth medium that has been conditioned by irradiated mouse embryonic fibroblasts (MEFs). To condition medium, plate irradiated MEFs (5000–10,000 rads, varies from lot-to-lot) at a density of 5×10^4 cells/cm^2 and incubate with fresh hES cell growth medium for 18–24 hours. After conditioning, bFGF is added and complete conditioned medium is sterile filtered. hES cells are routinely passaged every 5–6 days (1:3 or 1:4 split) on Matrigel-coated plates using Dispase to remove cell colonies (*see* **Note 2**).
2. Begin treatment with differentiation medium after approximately 4 days (prior to cells reaching confluence), adding 2–3 ml/well for 6-well plates. Change medium daily for 7 days. Significant levels of cell death may be observed, though healthy differentiating cells should remain attached.
3. Aspirate medium and treat cultures with Dispase for 3–5 minutes at 37°C. When epithelial sheets begin to detach, gently aspirate Dispase and resuspend colonies in DSFM by scraping plate with a glass pipette. Centrifuge differentiated cell colonies at 200*g* for 4 minutes, resuspend in DSFM, and centrifuge again. Colonies are then resuspended in DSFM and distributed to gelatin-coated plates at a split ratio of 1:3 (*see* **Note 3**). After attachment, culture medium (DSFM) should be changed every other day (2 ml/well for 6-well plate).

4. After 3–4 weeks of cultivation in DSFM, tightly packed epithelial colonies that express high levels of nuclear p63 and cytoplasmic K14 should be observed. At this point one can subculture the differentiated cells via trypsinization for 5–10 minutes, inactivation with an equal volume of trypsin inhibitor, and two subsequent washes via centrifugation. Plate cells at 10,000 cells/cm^2 on gelatin-coated tissue culture plates in DSFM (*see* **Note 4**).
5. Epithelial monolayers obtained at this stage express K14 at high purity (>90%) and can be passaged several times before undergoing senescence. These cells can also be incorporated into a variety of epithelial growth and differentiation assays.

3.2. Quantitative RT-PCR

1. Cells at all stages of differentiation are lysed directly on the culture plate (or in trypsinized cell pellets) for total RNA extraction using the RNeasy kit according to the manufacturer's instructions (*see* **Note 5**). On-column DNase treatment is recommended to reduce amplification of genomic DNA in subsequent PCR reactions.
2. cDNA is generated immediately using the Omniscript RT kit. Briefly, 1 μg total RNA is mixed with 10 × RT buffer, oligo-dT primers (1 μM final concentration), RNase inhibitor (0.5 U/μL final concentration), 1 μL RT, and water to 20 μL and incubated for 1 hour at 37°C. cDNA can be stored for at least 1 year at –20°C.
3. For quantitative analysis of RNA levels duplicate qPCR reactions are run for each experimental gene and sample along with triplicate reactions for the reference gene GAPDH using the Quantitect SYBR Green kit. Combine SYBR Green qPCR Master Mix with gene specific primers (0.4 μM), 1 μL cDNA, and water to 50 μL for each reaction and initiate reactions on an iCycler or equivalent thermocycler. When using the iCycler, 10 nM fluorescein must be added to the reaction mixture for well-factor normalization. Gene-specific primers of interest and their associated amplicon sizes are listed in **Table** 7.1. The annealing temperature for all reactions is 54°C, and 40 cycles are sufficient to detect signal. The iCycler is used to monitor fluorescence throughout the reaction and conduct melt curve analysis of all samples to verify specificity. Thermocycler program is listed below.
   a) Hot start incubation – 15 minutes at 95°C.
   b) Denaturation – 15 seconds at 94°C.
   c) Annealing – 30 seconds at 54°C.
   d) Extension and data acquisition – 30 seconds at 72°C.
   e) Repeat steps (b)–(d) 39 times to complete reaction.

4. The iCycler iQ software generates cycle threshold (CT) values for all samples; be sure to manually set the threshold level to the exponential phase of background subtracted data. Relative gene expression (to GAPDH) is subsequently calculated via the following equation (*see* **Note 6**):

$$Relative\ gene\ expression = 2^{\wedge}[\Delta CT_{Gene} - \Delta CT_{GAPDH}]$$

3.3. Immunofluorescent Staining

1. Aspirate culture medium from cells and rinse 1 × with PBS.
2. Fix cells for 15–20 minutes at room temperature (RT) in IF fixation buffer.
3. Rinse cells 1 × with PBS and incubate in quenching buffer for 10 minutes at RT.
4. Rinse cells 1 × with PBS and incubate culture in blocking buffer for 2 hours at RT or up to 72 hours at 4°C.
5. Incubate cells with primary antibodies in fresh blocking buffer at the appropriate dilution (see Materials) overnight at 4°C.
6. Rinse cells 5 × with PBS.
7. Incubate cells with secondary antibodies at a dilution of 1:500 in blocking buffer for 30–60 minutes at RT.
8. Rinse cells 2 × with PBS then incubate for 5 minutes with nuclear staining solution.
9. Rinse cells 2 × with PBS and view on an epifluorescent microscope, using a mercury lamp and dichroic filters to excite Alexa 488 and 594 dyes as well as Hoechst stain. *See* **Fig.** 7.1 for representative images of immunofluorescent stains.

3.4. Flow Cytometry

1. Incubate cells with trypsin-EDTA for 5–10 minutes to detach from plate.
2. Inactivate trypsin with soybean trypsin inhibitor, disperse cells with vigorous pipetting, and pass them through a 40 μm filter to remove large clumps.
3. Centrifuge samples at 200*g* for 4 minutes and pour off supernatant.
4. Resuspend cell pellet in 1 ml FC fixation buffer and incubate at 37°C for 10 minutes.
5. Chill tubes on ice for 1 minute and centrifuge at 200*g* and pour off supernatant.
6. Resuspend cell pellet in 2 ml ice-cold methanol and hold on ice for 30 minutes (*see* **Note 7**).
7. Centrifuge cells as above, pour off supernatant, and resuspend pellet in 2 ml FACS buffer. Cells should be counted and aliquoted to 10^5 cells per tube.

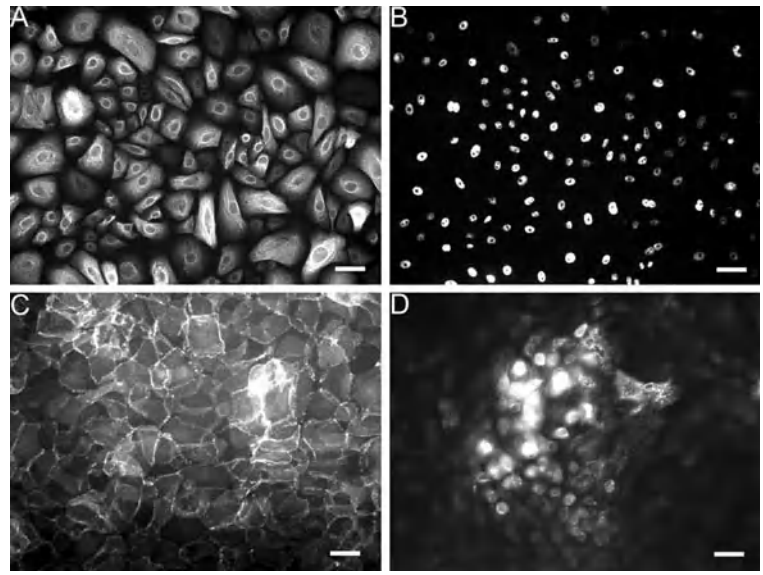

Fig. 7.1. Immunofluorescent staining of hES cell-derived keratinocyte progenitors. (**A, B**) Subcultured keratinocytes (as in step *3.1.5* of protocol) immunostained against the cytoskeletal protein K14 (**A**) and the nuclear transcription factor p63 (**B**). (**C**) Differentiated hES cells (after 3 weeks culture in step *3.1.4* of protocol) stained against β-catenin; note punctate staining at membranes. (**D**) Keratinocyte progenitors cultured to confluence and treated with 0.8 mM Ca^{2+} (after 4 weeks of culture in step *3.1.4* of protocol), then stained against the terminal differentiation marker filaggrin; note localization within suprabasal layers. Scale bar denotes 50 μm.

8. Centrifuge cells and pour off supernatant, add 2 ml FACS buffer.
9. Centrifuge cells and again pour off supernatant, leaving approximately 100 μL in each tube.
10. Pre-dilute primary antibodies (1 μL per sample) in 50 μL FACS buffer and add to each tube while resuspending pellet. Incubate overnight at 4°C. Be sure to include controls lacking primary antibodies in each combination for sample compensation.
11. Add 2 ml FACS buffer to each sample, centrifuge, and pour off supernatant, leaving 100 μL.
12. Pre-dilute secondary antibodies (0.2 μL per sample) in 50 μL FACS buffer and add to all tubes while resuspending pellet. Incubate for 30–45 minutes at RT.
13. Add 2 ml FACS buffer to each sample, centrifuge, and pour off supernatant, leaving 100 μL.
14. Resuspend each sample in approximately 300 μL FACS buffer and hold on ice until analysis.
15. Analyze samples on a BD FACSCalibur or equivalent cytometer capable of excitation at 488 nm and 630 nm wavelengths. Fixed cells preclude gating of live/dead populations,

though the appropriate populations should be gated under forward scatter vs. side scatter plots to eliminate cellular debris from analysis. Use of Alexa 488 and 647 fluorophores limits crossover emission; however, fluorescence compensation may need to be applied after measuring control samples (no primary and single-stained samples).

16. Appropriately gated populations enable quantitative comparisons of marker expression in samples under various treatment conditions or at specified time points.

4. Notes

1. The described Differentiation Medium is advantageous to use for investigation of signaling events during differentiation given its chemically defined composition. Alternatively, unconditioned hES cell growth medium lacking bFGF and supplemented with 1 μM RA only may be substituted to provide similar results (high-purity K14+ populations). The presence of BMP activity in the KSR supplement has been previously demonstrated (12), and the omission of BMP-4 is a cost-effective alternative if use of defined medium is not necessary.
2. Cultivation of hES cells in TeSR, a chemically defined medium (13), prior to use of Differentiation Medium yields similar results to those described here. Please note that *pure, undifferentiated hES cell cultures must be treated with RA-containing medium*; appreciable levels (>10%) of spontaneously differentiated cells significantly reduces the purity of epithelial populations ultimately generated using this protocol.
3. Attachment and growth of hES cells and their derivatives may vary from passage to passage; as such, the split ratio of differentiated cultures may need to be modified slightly. A variety of matrices are effective in this protocol, including Collagen I and Collagen IV; in some cases use of alternative coatings improves adhesion. Finally, we have successfully differentiated cells by adding DSFM directly to the confluent cell layer without passaging on day 7.
4. The extent of cell attachment may vary at this stage. However, attached cells will begin dividing within 24–72 hours and proliferate extensively for 3–4 passages before undergoing senescence (7).
5. For time course experiments, cell pellets or lysates can be stored at –70°C for simultaneous RNA extraction of all samples within an experiment.

6. The equation presented assumes all PCR reactions occur at 100% efficiency. If desired, standard curve reactions can be used to generate efficiency values for each primer set and incorporated into the equation as described by Pfaffl (14).
7. Cells in methanol may be held at –20°C for several days. Methanol fixation/permeabilization can be omitted when staining immediately with the listed antibodies.

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Chapter 8

Expression and Analysis of Exogenous Proteins in Epidermal Cells

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Abstract

In this chapter we review protocols for transient transfection of primary keratinocytes. The ability to transfect primary epidermal cells regardless of their differentiation status allows the biochemical and molecular characterization of multiple proteins. We review methods to analyze exogenous protein abundance in transfected keratinocytes by immunoblot and immunoprecipitation. We also present protocols to determine the subcellular distribution of these proteins by indirect immunofluorescence microscopy approaches.

Key words: Transfection, Epidermis, Keratinocytes, Gene transfer.

1. Introduction

The ability to modify cellular functions by modulating gene expression is key to understanding fundamental biological processes and is being increasingly used for cell therapies and tissue engineering. Epidermal keratinocytes are very accessible and have high proliferative capacity, characteristics that make them potential vehicles for gene therapies. Virus-mediated gene transfer is very efficient in epidermal keratinocytes (1–4). However, non-viral methods of gene delivery have met more limited success (5, 6). In this chapter, protocols for efficient non-viral gene transfer in cultured primary keratinocytes using polyethyleneimine (PEI) and their application to study exogenous protein expression are presented. Methods to analyze the abundance and subcellular localization of these proteins are also presented. Although the protocols

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described for isolation of primary cultures are developed for mouse keratinocytes, transfection and subsequent analyses are applicable to primary mouse and human keratinocytes, as well as established epidermal cell lines.

2. Materials

2.1. Keratinocyte Culture

1. Scissors, scalpel, and two forceps.
2. Bacterial-grade Petri dishes.
3. Sterile gauze (5 × 5 cm pads).
4. 50-ml conical tubes.
5. Culture dishes for primary cells (e.g., BD Primaria, BD Biosciences).
6. 70-μm nylon cell strainer (BD Falcon, BD Biosciences).
7. Eagle's minimum essential medium without $CaCl_2$ (EMEM without $CaCl_2$, BioWhittaker Lonza Cat. No. 06-174G).

2.2. PEI Transfection Mix

1. Polyethyleneimine, 25 kDa linear (Polysciences # 23966).
2. HCl (36.5% w/v).
3. Sterile 150 mM NaCl solution.
4. Sterilizing filters (0.2 μm pore size, PALL, Life Sciences # 4652).
5. 20-ml sterile syringe.
6. Sterile 1.5-ml microfuge tubes.

2.3. Keratinocyte Lysates

1. Triton X-100 (EMD # CATX 1568).
2. 100 mM NaF (Sigma # S-6521) stock solution, diluted in ddH_2O, stored at 22°C.
3. 200 mM Na_3VO_4 (Bioshop # SOV664-10) stock solution, diluted in ddH_2O, stored at –20°C.
4. 1 mg/ml Aprotinin (Bioshop # APR600) stock solution, diluted in ddH_2O, stored at –20°C.
5. 1 mg/ml Leupeptin (Bioshop # LEU001) stock solution, diluted in ddH_2O, stored at –20°C.
6. 1 mg/ml Pepstatin (Bioshop # PEP 605) stock solution, diluted in ddH_2O, stored at –20°C.
7. 100 mM Phenylmethylsulfonyl fluoride (PMSF, Bioshop # PMS123.5) stock dissolved in methanol and stored at –20°C.
8. Teflon scraper (Fisher Scientific # 08-773-1).

2.4. Denaturing Gel Electrophoresis

1. 30% Acrylamide:bis-acrylamide stock solution (37.5:1) (Bio-Rad # 161-0158).

2. 10% Ammonium persulfate (Sigma # A9164) stock solution, diluted in ddH_2O, immediately frozen and stored at –20°C in single-use aliquots.
3. TEMED (N,N,N,N′-tetramethyl-ethylenediamine) (Sigma # T9281); stored at 22°C, preferably in a dessicator.
4. Pre-stained protein molecular weight markers (Fermentas # SM0671).

2.5. Electrotransfer of Proteins, Immunoblotting, and Immunoprecipitation

1. Polyvinylidene fluoride (PVDF) membrane (Millipore # IPVH 00010) or nitrocellulose membrane (Invitrogen # LC2000).
2. Blotting paper (VWR # 28298-020).
3. BSA (bovine serum albumin, Bioshop # ALB001).
4. Protein A/G Sepharose slurry (Pierce Laboratories # 53132).
5. ECL (enhanced chemiluminescence kit) (GE Healthcare # RPN2132).
6. X-ray film (BioMax-MR, Kodak # 8701302).

2.6. Laminin 332 Matrix

1. G804 rat bladder epithelial cells (obtained from Dr. J. C. R. Jones).
2. T75 culture flasks.
3. Dulbecco's Modified Eagle's Medium (DMEM, Hyclone # SH30565-01) supplemented with 8% fetal bovine serum (FBS).
4. EMEM without $CaCl_2$ (BioWhittaker Lonza # 06-174G).
5. Sterilizing filters; 0.4 μm pore size (PALL Life Sciences # 4654).

2.7. Immuno fluorescence Microscopy

1. Solution of trypsin (0.25%) and EDTA (ethylenediamine tetraacetic acid; 1 mM) (Invitrogen # 25200).
2. Paraformaldehyde (PFA, Fisher Scientific # AC416780030).
3. Glycine (Bioshop # GLN001).
4. Hoescht 33258 (Sigma # 14530).
5. Goat serum (Invitrogen # 16210-064).
6. Mounting medium (Immumount, Thermoscientific # 9990412).

2.8. Antibodies for Immunoblotting and Immunofluorescence Microscopy

1. Primary antibody against the exogenous protein expressed or a tag present in the protein (e.g., anti-V5 tag antibody, Invitrogen # R960).
2. Horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 115-035-146).
3. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 111-035-144).

4. Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes/Invitrogen # A11001).
5. Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes/Invitrogen # A11032).
6. Alexa 350-conjugated goat anti-mouse IgG ((Molecular Probes/Invitrogen # A11045).

2.9. PEI Transfection Reagent Stock Solution (1 mg/ml)

Dissolve 20 mg PEI in 18 ml distilled water and adjust solution to pH 7.0 with HCl (*see* **Note 1**), add distilled water to a final volume of 20 ml, filter sterilize through 0.22-μm membrane. Aliquot into sterile microfuge tubes in 500-μl portions and store at –20°C (*see* **Note 2**).

2.10. Isolation and Culture of Primary Mouse Keratinocytes

1. Chelex-treated FBS: The FBS is pre-treated with Chelex 100 chelating resin (200–400 mesh, sodium salt, Bio-Rad Laboratories) to remove Ca^{2+} ions. Stir the FBS with the resin (40 g resin/500 ml serum) at room temperature for 1 h. Filter through filter paper to remove the resin and follow by 0.45-μm filter sterilization. Store at –20°C in 40-ml aliquots.
2. Hydrocortisone (Sigma # H4001; 500 μg/ml dissolved in EtOH, stored at –20°C).
3. 5 mg/ml Insulin (Sigma # I6634), dissolved in dH_2O, pH adjusted to 3.0 with HCl, stored at –20°C in single-use 500-μl aliquots.
4. 1 mg/ml Cholera toxin (List Biological Laboratories Inc. # 100) dissolved in dH_2O, stored at –20°C.
5. 340 μg/ml Triiodothyronine (T3, Sigma # T6397) dissolved in 0.1 N NaOH, stored at –20°C.
6. 100 μg/ml Epidermal growth factor (EGF, Sigma # E4127) dissolved in sterile dH_2O, stored at –20°C in single-use aliquots.
7. 10,000 μ/ml Penicillin and 10 mg/ml streptomycin (Invitrogen # 15070-063).
8. Keratinocyte growth medium: EMEM without $CaCl_2$, containing 8% chelex-treated FBS, 100 μ/ml penicillin and 0.1 mg/ml streptomycin, hydrocortisone (74 ng/ml), insulin (5 μg/ml), cholera toxin (9.5 ng/ml), triiodothyronine (6.7 ng/ml), and EGF (5 ng/ml).
9. 0.4% Trypan blue stain (ICN BioMed # 194600), dissolved in 0.85% NaCl.

2.11. Cell Lysis and Denaturing Gel Electrophoresis

1. Lysis buffer: 10 mM HEPES pH 7.5 (with HCl), 150 mM NaCl, 1% Triton X-100, 5 mM NaF, 5 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM PMSF (*see* **Note 3**).

2. Ca^2-free phosphate buffered saline (PBS).
3. 4 × Separating buffer: 1.5 M Tris-HCl pH 8.8, 0.4% SDS. Store at 22°C.
4. 4 × Stacking buffer: 1.5 M Tris-HCl pH 6.8, 0.4% SDS. Store at 22°C.
5. 5 × Electrophoresis buffer: 125 mM Tris, 960 mM glycine, 0.5% (w/v) SDS. Do not adjust pH (pH should be 8.8–8.9) and store at 22°C.
6. 10% Resolving polyacrylamide mix: 10 ml of 30% Acrylamide:bis-acrylamide stock solution, 7.5 ml of 4 × separating buffer, 12.5 ml ddH_2O, 100 µl of 10% APS.
7. Stacking polyacrylamide mix: 1.8 ml of 30% Acrylamide:bis-acrylamide stock solution, 2.5 ml of 4 × stacking buffer, 5.7 ml ddH_2O, 75 µl of 10% APS.

2.12. Immunoblot Analysis

1. TBST: 100 mM Tris-HCl pH 7.5, 0.9% NaCl, 0.1% Tween 20.
2. 4 × sample buffer: 100 mM Tris-HCl (pH 6.8), 4% SDS, 40% glycerol, 40 mM DTT, 0.2% bromophenol blue.
3. 2 × sample buffer: 50 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 20 mM DTT, 0.1% bromophenol blue.
4. 5% BLOTTO: 5% powdered skim milk dissolved in TBST.

2.13. Immunofluorescence Microscopy

1. 20% PFA: Add 20 g PFA to 80 ml PBS. With constant stirring, adjust the pH to 7.0 by dropwise addition of 10 N NaOH. Once the PFA has completely dissolved, adjust the final volume of the solution to 100 ml. Store at –20°C in single-use 10-ml aliquots.
2. 4% PFA, prepared by diluting the 20% PFA solution with PBS (*see* **Note 4**).
3. Glycine quench solution: 100 mM glycine dissolved in PBS.
4. Permeabilizing solution: 0.1% Triton X-100 and 0.1% BSA dissolved in PBS.
5. Hoescht 33258 (1.2 mg/ml stock solution).

3. Methods

3.1. Isolation and Culture of Primary Murine Keratinocytes

1. Following euthanasia by CO_2 inhalation, 1–3-day-old mice are immersed in 70% ethanol for at least 10 min and kept on ice.
2. Dispense 6 ml of freshly thawed 0.25% trypsin into a 10-cm bacterial-grade Petri dish.

3. Under sterile conditions, remove the head, limbs, and tail from the animals using scissors.
4. Using a scalpel, a longitudinal incision through the skin is made all along the back of the carcass.
5. With a pair of forceps, the skin is gently removed from the carcass, starting at the incision point.
6. The stretched skin is floated with the dermis side down on the 0.25% trypsin at 4°C for 15–20 h. Ensure that the skin is freely floating and the edges are not folded.
7. Replace the trypsin with fresh 0.25% trypsin and incubate the tissues at 37°C for 0.5–1.5 h, depending on the thickness of the skin (*see* **Note 5**).
8. Remove the skin from the trypsin and briefly place on a square of sterile gauze to remove excess liquid. Place the trypsinized skin on a Petri dish with the epidermis side down. Gently pull off the dermis with the tip of a forceps.
9. Place the epidermis in a bacterial culture dish containing 10 ml of keratinocyte growth medium. Mince with sterile scissors and transfer to a conical tube.
10. Incubate at 37°C with very gentle rocking for 20 min. To remove tissue fragments, filter the cell suspension through a 70-μm nylon cell strainer into a clean, sterile conical tube. Mix a 50-μl aliquot of the filtered cell suspension with 50 μl of trypan blue stain and determine the number of viable cells/ml of suspension using a hemocytometer.
11. Plate the keratinocytes on culture dishes at a density of $2–3 \times 10^5$ cells/cm^2. This will yield a culture 40–60% confluent the following day.
12. One day after plating, remove the culture medium and non-adherent cells and replace with fresh keratinocyte growth medium. Replace the culture medium every other day.

3.2. Transient Transfection

1. Prior to transfection, replace the culture medium of the keratinocytes with 0.9 ml fresh medium. Culture the cells for 2–4 h (*see* **Note 6**).
2. Thaw an aliquot of PEI transfection reagent on ice.
3. In a sterile microfuge tube, dilute 2 μg of DNA (1 μg/μl stock) with 92 μl 150 mM NaCl. Mix well (*see* **Note 7**).
4. Add 6 μl of PEI transfection reagent (1 mg/ml) and vortex immediately (*see* **Note 8**).
5. Briefly centrifuge (5–10 sec, 20,800 × *g*).
6. Let stand at room temperature for 10 min (*see* **Note 9**).
7. Add the DNA/PEI mix dropwise to the cells, swirling the culture dish to ensure thorough mixing (*see* **Note 10**).

8. Culture the cells in the presence of DNA/PEI mix for 4–16 h.
9. Remove the DNA/PEI-containing medium and replace with fresh growth medium.
10. Rinse the cells once with Ca^2-free PBS.
11. Add growth medium and culture the cells for 24–48 h and process as appropriate.

3.3. Analysis of Exogenously Expressed Proteins by Immunoblot and Immunoprecipitation

3.3.1. Preparation of Cell Lysates

1. Remove the growth medium and rinse the cells once with ice-cold PBS. Keep the culture dishes and cells on ice at all times.
2. Add 5 ml of PBS to the culture dish. Harvest the keratinocytes by gently scraping them off the dish with a teflon cell scraper, maintaining the dish on ice. Transfer the cell suspension to a 15-ml conical centrifuge tube.
3. Centrifuge the cells (4°C, 500 × *g* for 5 min). Remove the supernatant without disturbing the cell pellet.
4. Thoroughly suspend the cell pellet in 500 µl lysis buffer. Incubate lysate on ice for 30 min, with occasional mixing.
5. Transfer to a microfuge tube and pellet cell debris by centrifugation at 20,800 × *g* for 10 min at 4°C and transfer the supernatant to a new tube. Determine the protein concentration of the lysates or store at –80°C until use.

3.3.2. Analysis of Exogenous Proteins in Cell Lysates by Immunoblot

1. Prepare lysate samples for electrophoresis: To denature the proteins in the lysates, add 1/3 volume of 4 × sample buffer or 1 volume of 2 × sample buffer (final sample volume is 50 µl), boil for 5 min, and microfuge at 20,800 × *g* for 3 min (*see* **Note 11**).
2. Wash the glass plates for electrophoresis, wipe with 70% ethanol, allowing to dry completely. Assemble the plate sandwich using 1.5-mm spacers, according to manufacturer's instructions.
3. Prepare the resolving acrylamide mix and add 10% ammonium persulfate (300 µl/30 µl acrylamide mix) and TEMED (20 µl/30 ml acrylamide mix) to begin polymerization. Immediately cast the resolving gel mix into the sandwich, leaving 2 cm for the stacking gel.
4. Overlay the resolving gel with either 2-propanol, 70% ethanol, or 1% SDS (*see* **Note 12**). Allow polymerization for 30 min at room temperature.
5. Once the resolving gel has polymerized, discard the overlaying solution, rinsing several times with deionized water. Remove any remaining fluid with a piece of blotting paper.
6. Prepare the stacking acrylamide mix and add 10% ammonium persulfate (80 µl/30 µl acrylamide mix) and TEMED (10 µl/10 ml acrylamide mix). Immediately cast the stacking gel and insert the 1.5-mm comb, avoiding introduction of bubbles.

7. Allow the stacking gel to polymerize for 30 min.
8. Assemble the gel unit and pour 1 × electrophoresis buffer in the chambers. Carefully remove the comb and rinse the wells several times with the buffer, to remove unpolymerized acrylamide.
9. Load the protein samples and pre-stained molecular mass marker. Load 50 µl of 1 × sample buffer in any unused wells.
10. Start electrophoresis with a constant voltage of 120 V (if using a minigel). Once the samples concentrate in the stacking gel, increase current to 180–200 V.
11. Once the desired resolution has been achieved, as monitored by the separation of the pre-stained molecular mass markers, stop electrophoresis, remove the gel from the plates, and assemble for immunoblotting, as described in **Section 3.3.3**.

3.3.3. Semidry Electroblotting

1. Prepare a PVDF membrane and six sheets of blotting paper just slightly larger than the polyacrylamide gel. Wet the PVDF membrane with methanol for 2 min. Rinse the membrane with distilled water and incubate in transfer buffer for at least 5 min (*see* **Note 13**).
2. Soak the sheets of blotting paper in transfer buffer.
3. Remove the polyacrylamide gel from the glass plates, discarding the stacking gel. Soak the separating gel in transfer buffer for 1–2 min.
4. Sequentially place three sheets of blotting paper on the anode of the transfer system. Gently roll a serological pipet over the entire surface of the paper to extrude any air bubbles that may form between each pair of sheets. Place the PVDF membrane, followed by the gel, avoiding formation of air bubbles. Sequentially place the remaining three sheets of blotting paper.
5. Assemble the blotting unit and transfer at a constant current of 4.5 mA/cm^2 for 60 min.
6. Stop transfer and rinse membrane thrice with TBST buffer.

3.3.4. Immunodetection

1. After rinsing, block the membrane by incubation in 5% BLOTTO for 2 h at 22°C, or 16 h at 4°C, followed by three 10-min TBST washes.
2. Probe the blots by incubation for 1–2 h at 22°C with primary antibody diluted in TBST containing 0.1% bovine serum albumin.
3. Wash the membrane thrice with 10–15 ml TBST, 15 min each wash.
4. Incubate the membrane with horseradish peroxidase-conjugated secondary antibody diluted 1:5000 in TBST containing 5% BLOTTO.

5. Wash the membrane thrice with 10–15 ml TBST, 15 min each wash.
6. Remove the excess buffer by rolling a clean serological pipet over the PVDF membrane. Incubate the membrane with ECL blotting substrate for 2 min.
7. Remove excess fluid as above, wrap the membrane in plastic wrap, and expose to X-ray film for 30 sec–2 h, depending on the signal strength (*see* **Note 14**).

3.3.5. Analysis of Exogenously Expressed Proteins by Immunoprecipitation

1. Wash 20 µl of Protein A/G sepharose slurry in 100 µl lysis buffer. Centrifuge (20,800 $\times g$, 1 min), remove the supernatant, and repeat wash. Resuspend in 30 µl lysis buffer.
2. Preclear 1 ml of cell lysate by incubation with 30 µl of the washed Protein A/G sepharose slurry for 1 h at 4°C, with constant rocking.
3. Pellet sepharose beads by centrifugation at 20,800 $\times g$ for 1 min at 4°C.
4. Transfer supernatant to a clean tube. Add 1–3 µg antibody and incubate 4–16 h at 4°C, with constant rocking (*see* **Note 15**).
5. Add 20 µl of Protein A/G sepharose slurry prepared as in item 1. Incubate for 1 h at 4°C, with constant rocking.
6. Pellet sepharose beads by centrifugation at 2,700 $\times g$ for 1 min at 4°C and wash by resuspending the beads in 1 ml of ice-cold lysis buffer. Pellet the beads and remove the supernatant. Wash 5–9 more times.
7. Elute immunocomplexes with 20 µl of 2× SDS sample buffer, boiling the samples for 5 min, and centrifuging at 20,800 $\times g$ for 2 min.
8. The supernatants can be directly loaded onto denaturing polyacrylamide gels.

Electrophoresis, transfer to PVDF membranes and immunoblotting are conducted as described in **Sections 3.3.2** to **3.3.4**.

3.4. Subcellular Localization of Exogenous Proteins by Immunofluorescence Microscopy

3.4.1. Isolation of Laminin 332 Matrix and Coating of Culture Surfaces for Keratinocytes

1. Seed two T-75 tissue culture flasks, with 1×10^6 G804 cells each (*see* **Note 16**).
2. Culture the cells in normal growth medium (DMEM with 8% FBS) for 48 h. At this time, a completely confluent monolayer should be present.
3. Remove the growth medium, rinse with Ca^2-free PBS thrice, and add 10–15 ml of Ca^2-free EMEM without any supplements. Culture the cells for 48 h.
4. Transfer the EMEM-conditioned medium (which now

contains laminin 332 matrix secreted by the G804 cells) to a conical tube. Centrifuge at 1000 $\times g$ for 5 min to remove cells and debris.

5. Filter sterilize conditioned medium through membranes with 0.4 μm pore size. Use immediately to coat keratinocyte growth surfaces or store at –20°C (*see* **Note 17**).
6. To coat plastic or glass surfaces with laminin 332 matrix, incubate with sufficient G804-conditioned medium to completely cover the surface for 1–2 h at 37°C. Completely remove the conditioned medium just prior to plating keratinocytes on the treated surface. No rinsing prior to seeding the cells is necessary

3.4.2. Culture of Transiently Transfected Keratinocytes on Laminin 332 Matrix-Coated Surfaces

1. Twenty-four to forty-eight hours following transfection of keratinocytes, remove the growth medium and rinse the cultures twice with Ca^2-free PBS.
2. Add trypsin/EDTA solution (3 ml in a 100-cm dish) and incubate at 37°C for about 5 min (*see* **Note 18**). Examine the cells periodically to verify the extent of detachment from the culture surface.
3. As soon as ~80% of the keratinocytes have detached, transfer the cell suspension into a conical tube. Briefly rinse the culture dish with 3–5 ml pre-warmed keratinocyte growth medium, immediately adding it to the conical tube containing the cells.
4. Pellet the cells by centrifugation at 130 $\times g$ for 5 min at 22°C. Remove the supernatant.
5. Suspend the cell pellet in pre-warmed Ca^2-free EMEM supplemented with 25 μg/ml BSA and plate on laminin 332 matrix-coated surfaces at a density of 2,500–5,000 cells/cm^2 (*see* **Note 19**).
6. Culture the cells at 37°C for the appropriate time (2–8 h) and process for fluorescence microscopy as described in **Section 4.4.3**.

3.4.3. Analysis of Exogenous Proteins in Keratinocytes by Indirect Immunofluorescence Microscopy

1. Place culture dishes on ice and remove culture medium from the keratinocytes.
2. Gently rinse the cultures with ice-cold Ca^2-free PBS.
3. Fix the cells by incubation with freshly diluted 4% PFA in ice-cold PBS for 40 min on ice (*see* **Note 20**).
4. Rinse the cells thrice with ice-cold PBS.
5. Quench the PFA by incubating with 100 mM glycine at 22°C for 15 min.
6. Permeabilize the cells by incubation with constant rocking with Triton X-100 permeabilizing solution, 20 min at 22°C. Remove the permeabilizing solution.

7. Repeat PBS rinses as in step 4. Remove the PBS.
8. Incubate with constant rocking with 1% BLOTTO containing 1% goat serum in PBS. Rock 1 h at 22°C. Remove the blocking solution.
9. Rinse the cells thrice with ice-cold PBS.
10. Incubate with constant rocking with primary antibody diluted in PBS containing 5% goat serum (or serum derived from the same species in which the secondary antibody was raised) for 1 h at 22°C, or 16 h at 4°C. Use enough solution to completely cover the surface (e.g., 200–300 µl/well if using a 24-well plate).
11. Remove the primary antibody solution. Wash with PBS, rocking for about 10 min. Repeat the PBS wash three additional times (total is four washes over a 45-min period).
12. Remove the PBS and add a solution containing an Alexa fluorochrome-labeled secondary antibody diluted in PBS containing 1% goat serum (or serum from the same species in which this antibody was raised). Use 300 µl/well of a 24-well plate. Incubate with rocking for 1 h at 22°C protected from the light.
13. Remove secondary antibody solution. Wash with PBS, keeping the cells protected from the light.
14. To stain the DNA, add Hoescht 33258 (0.1–0.3 µg/ml, final) in PBS. Incubate with constant rocking for 5–15 min at 22°C, protected from the light.
15. Remove the Hoescht-containing PBS. Wash with PBS three times, over a 25-minute period, and protected from the light.
16. Mount with mounting medium. Allow to dry for 16 h at 22°C in the dark. Long-term storage of mounted specimens should be at –20°C protected from the light.

4. Notes

1. The pH of the solution is critical to ensure optimal transfection efficiency.
2. The PEI stock solution will store well at –20°C for about 6 months, 2–3 months longer at –80°C. Over more prolonged storage, the pH of the solution can change, resulting in poor transfection efficiency and cytotoxicity.
3. Protease inhibitors are thawed on ice and added to the lysis buffer immediately prior to use.

4. To prepare 4% PFA, dilute an aliquot of 20% PFA with PBS immediately prior to use. This is a single-use solution. Do not re-freeze and discard according to institutional regulations.
5. Check periodically to verify extent of tissue digestion. When the epidermis appears as a thin translucent sheet overlaying the thick, pink dermis it is ready for isolation. Do not continue the incubation with trypsin longer than necessary, as overdigestion of the tissue substantially reduces keratinocyte viability.
6. For optimal transfection efficiency and viability, the cells are cultured with DNA/PEI mixture for 16–18 h.
7. The amount given of DNA, 150 mM NaCl, and PEI transfection reagent are optimal for one well in a 24-well dish (2 cm^2). For a 100-mm culture dish containing 4.5 ml growth medium, add 60 μl PEI transfection reagent to a mix containing 8 μg DNA diluted with 432 μl 150 mM NaCl. The volume of the DNA/PEI mix should be 1/10 of the total volume of medium used for the cells (i.e., DNA/PEI plus the culture medium).
8. It is critical to add the PEI to the diluted DNA mix. Do not mix concentrated DNA with either dilute or concentrated PEI.
9. The interaction between DNA and PEI is based on their electrostatic properties, giving rise to small, highly diffusible particles. It is very important to incubate the DNA and the PEI long enough to allow complex formation. However, longer incubation times (>15 min) give rise to particles that exhibit substantially reduced transfection efficiency.
10. Optimal results are obtained when cells are ~70% confluent at the time of transfection.
11. Generally, 10–25 μg protein from lysates are sufficient to analyze by immunoblot exogenous proteins expressed from vectors with strong promoters, such as CMV, RSV, or SV40.
12. Be careful not to mix the gel solution with the overlaying solution. This can be easily accomplished by using a spray bottle to dispense the overlaying solution.
13. The use of PVDF membranes allows for stripping and/or re-probing of the blots.
14. Many types of X-ray films can be used. Bio-Max ML is one of the most sensitive types, allowing detection of proteins with low abundance.
15. The length of incubation depends on the specific antibody used and must be empirically determined.

16. G804 rat bladder epithelial cells secrete laminin 332 matrix into their culture medium (7). Conditioned medium from these cells can be used to efficiently coat cell culture surfaces that promote epidermal keratinocyte attachment, spreading, and migration.
17. Coating of culture surfaces with laminin 332-containing medium either fresh or stored at –20°C for a week is equally effective.
18. It is very important to avoid over-trypsinization of the cells, as they will be damaged and will not attach upon re-plating.
19. Re-plating keratinocytes at a density of 2,500–5,000 cells/cm^2 will result in a sub-confluent cell population that will allow analysis of attachment, spreading, and movement of individual cells.
20. Fixation at 4°C will ensure that the protein analyzed will not have any possibility of changing subcellular localization.

Acknowledgments

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Chapter 9

Using siRNA Knockdown in HaCaT Cells to Study Transcriptional Control of Epidermal Proliferation Potential

Julie Wells and Xing Dai

Abstract

Compared to primary keratinocytes, HaCaT cells are easier to transfect and yet still maintain at least some features of normal epidermal proliferation and differentiation. This chapter describes methods used in our laboratory to maintain HaCaT cells in an undifferentiated state and to use the siRNA technology to efficiently deplete a gene product of interest from these cells. We also provide protocols on how to couple siRNA knockdown with a clonal assay to examine keratinocyte proliferation potential and a luciferase reporter assay to examine promoter regulation.

Key words: HaCaT, Cell culture, siRNA, Stem cells, Progenitor cells, Proliferation potential, Epidermis, Keratinocytes, Transcription factors, Luciferase reporter.

1. Introduction

Epidermal homeostasis is important to maintain the epidermis for the lifetime of an organism. In a widely-accepted model of epidermal homeostasis, stem cells of the epidermis are relatively quiescent in vivo and hence are able to maintain long-term proliferation potential (1, 2). These undifferentiated, self-renewing cells give rise to daughter transit amplifying (TA) cells which compose the majority of proliferating cells within the basal layer. Unlike stem cells, TA cells can only undergo a limited number of divisions before exiting the cell cycle and undergoing terminal differentiation (1, 3–5). Early experiments from Barrandon and Green described a clonal assay to decipher the difference in proliferation potential between stem cells and TA cells (6). Large colonies are each derived from a cell that has long-term proliferation potential

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and therefore is believed to be a stem cell. Smaller colonies are derived from a cell that has limited proliferation potential and therefore is thought to be a TA cell. This useful assay, if combined with gene manipulations, will allow the dissection of molecular mechanisms controlling epidermal proliferation potential. However, the use of this assay has been largely restricted to primary epidermal keratinocytes which are difficult to manipulate due to low transfection efficiency.

HaCaT cells are a spontaneously immortalized keratinocyte cell line that are easily transfectable. When transplanted onto either nude mice or grown in organotypic cultures, these cells are able to give rise to stratified epithelium that resembles the epidermis to a certain extent (7, 8). This demonstrates that HaCaT cells retain at least some of the functional differentiation properties of normal keratinocytes. Like primary keratinocytes in culture, HaCaT cells represent a heterogeneous cell population. When sorted on the basis of Desmoglein 3 (Dsg3) expression, cells that were $Dsg3^{dim}$ from both HaCaT and primary human keratinocytes possess higher colony forming ability than Dsg^{bri} (9, 10). This shows that HaCaT cells, similar to primary cells, contain cells with different proliferation potentials. These findings demonstrate that HaCaT cells can serve as a valid model to study epidermal keratinocyte proliferation and differentiation.

In this chapter, we first provide a protocol to prepare low-calcium medium in order to maintain HaCaT cells in an undifferentiated state. We then describe how to efficiently deplete a gene product of interest from HaCaT cells using the siRNA technology and how to examine the effect of the depletion on keratinocyte proliferation potential and target promoter activity.

2. Materials

2.1. Cell Culture

1. Chelex 100× Resin (Bio Rad; cat. no. 142-2842).
2. Fetal Bovine Serum, characterized (Hyclone; cat. no. SH30071.03). Store at –20°C away from light (*see* **Note 1**).
3. Millipore Steritop sterile filter bottle tops, 500 mL volume, GP Express Plus membrane, 45 mm neck size, 0.22 μm filter (Fisher; cat. no. SCGP T05 RE).
4. Insulin from bovine pancreas (Sigma Aldrich; cat. no. I5500), 5 mg/mL in 0.1 N HCl, store at –20°C.
5. Apo-transferrin human (Sigma Aldrich; cat. no. T2252), 5 mg/mL in 1× PBS, store at –20°C.

6. T3 (3,3′,5-Triiodo-L-Thyronine) (Sigma Aldrich; cat. no. T6937), 2×10^{-8} M. Weigh out 13.6 mg of T3 and dissolve into 100 mL of 0.02 N NaOH to give a 2×10^{-4} M T3 solution. Then take 0.1 mL of 2×10^{-4} M T3 and add 9.9 mL 1× PBS to make a 2×10^{-6} M T3 solution. Take 1 mL of 2×10^{-6} M T3 and add 99 mL 1× PBS to make a working stock of T3 of 2×10^{-8} M. Store at –20°C (*see* **Note 2**).
7. 100× Cocktail. Add 20 mL of 5 mg/mL insulin, 20 mL of 5 mg/mL transferrin, 20 mL of 2×10^{-8} M T3, and 140 mL of 1× PBS to a 250 mL plastic graduated cylinder. Mix well and aliquot 24 mL each in 50 mL conical tubes. Store at –20°C.
8. Cholera toxin (Fisher; cat. no. ICN15000501), 84 μg/mL in water, store at 4°C. TOXIC (*see* **Note 3**).
9. Hydrocortisone (Calbiochem; cat. no. 386698), 4 mg/mL in 95% ethanol, store at –20°C.
10. DMEM:F12 (3:1) no Ca^{2+} powder media (Gibco/Invitrogen; cat. no. 90-5010EA) (*see* **Note 4**).
11. 6-well sterile TC plate (Fisher; cat. no. 08-772-1B).
12. 12-well sterile TC plate (Fisher; cat. no. 08-772-29).
13. 10 cm sterile TC plate (Fisher; cat. no. 08-772-22).
14. Sterile 15 mL disposable conical tube (Fisher; cat. no. 05-539-5).
15. Sterile 50 mL disposable conical tube (Fisher; cat. no. 06-443-20).
16. 1.7 mL microfuge tubes (Fisher; cat. no. 50400413); autoclave before use.
17. Hemacytometer (Fisher; cat. no. 02-671-5).
18. Hand cell tally (Fisher; cat. no. NC9684786).
19. HaCaT human keratinocyte cell line is available in many tabs including ours (*see* **Note 5**).
20. Penicillin–streptomycin 100× liquid (Invitrogen; cat. no. 15140-122).
21. 1× sterile PBS.
22. 0.25% Trypsin-EDTA (Invitrogen; cat. no. 25200-056).
23. $NaHCO_3$ (Fisher; cat. no. BP-328-500).
24. Stir plate at 4°C.
25. Media bottles of various sizes (including 250 mL, 500 mL, and 1 L) (Fisher; cat. no. 06-414-1B, 06-414-1C, 06-414-1D).
26. 1 N HCl
27. 1 N NaOH

2.2. Transfection Reagents

1. OptiMEM I reduced serum medium 1× liquid (Invitrogen; cat. no. 31985-070).

2. Lipofectamine 2000 Transfection Reagent (Invitrogen; cat. no. 11668-019). Store at 4°C (Do *not* freeze).
3. Ambion siRNA (catalog number varies according to siRNA; visit www.ambion.com; *see* **Note 6**).
4. Negative control siRNA #1 (Ambion; cat. no. AM4635).
5. pGL3 basic vector (Promega; cat. no. E1751) (*see* **Note 7**).
6. β-Actin promoter – β-gal plasmid.
7. pEGFP-N1 plasmid (Clontech).
8. Filler DNA (any plasmid that does not code for a protein. This is used to bring the amount of DNA up to 1600 ng for efficient transfection).

2.3. Luciferase Assay

1. 1 M Na_2HPO_4. Add 14.196 g Na_2HPO_4 and bring the volume to 100 mL with milliQ water.
2. D-luciferin (Sigma; cat. no. L-9504). Make a 1 mM stock solution by adding 1.75 mL 0.2 M Na_2HPO_4 to the 10 mg bottle of D-luciferin. Mix well and transfer to a graduated cylinder. Bring the volume to 35 mL with milliQ water (*see* **Note 8**).
3. Tricine (Sigma; cat. no. T0377). Make a 1 M Tricine-Tris buffer, pH 7.8 stock solution by adding 89.5 g Tricine to 450 mL milliQ water. Adjust the pH to 7.8 with Tris (about 30.38 g). Bring the volume to 500 mL.
4. 1 M $MgSO_4$. Add 24.65 g $MgSO_4$ and bring the volume to 100 mL with milliQ water.
5. 1 M $MgCl_2$. Add 20.3 g $MgCl_2$ and bring the volume to 100 mL with milliQ water.
6. 0.5 M EDTA, pH 8.0. Add 18.6 g EDTA to 90 mL milliQ water. Adjust the pH to 8.0. Bring the volume to 100 mL with milliQ water.
7. Three vials ATP (Sigma; cat. no. A1852). Make a 0.1 M ATP solution by adding 544 μL milliQ water to each vial that contains 30 mg ATP.
8. Sodium pyrophosphate (PPi) (Fisher; cat. no. S390-500). Make a 0.5% PPi stock solution by adding 0.5 g PPi and bring the volume to 100 mL with milliQ water.
9. Luciferase assay substrate. Add the following to a 250 mL graduated cylinder: 35 mL 1 mM D-luciferin, 3.5 mL 1 M Tricine-Tris pH 7.8, 938 μL 1 M $MgSO_4$, 210 μL 1 M $MgCl_2$, 140 μL 0.5 M EDTA pH 8.0, 70 μL 1 M DTT, 1.4 mL 0.1 M ATP, and 350 μL 0.5% PPi. Bring the volume to 140 mL with milliQ water. Mix well and make 1 mL aliquots. Store at –80°C (*see* **Note 9**).

10. Reporter Lysis buffer 5× (Promega; cat. no. E397A); store for long term at –20°C (*see* **Note 10**).
11. Luminometer with printer (Fisher; cat. no. 12-200-17).
12. 12 × 75 mm polystyrene test tubes for luminometer (Fisher; cat. no. 22-170-142).
13. Cell scraper.

2.4. CRPG Assay (β-Galactosidase Assay)

1. CRPG (chlorophenol red-β-D-galactopyranoside monosodium salt) (Roche Molecular; cat. no. 10884308001). Make a 40 nM CRPG stock solution by adding 10.29 mL milliQ water to the bottle with 250 mg CRPG. Mix well and aliquot in 1.5 mL Eppendorf tubes. Store at –20°C.
2. 1 M NaH_2PO_4. Add 11.99 g NaH_2PO_4 and bring the volume to 100 mL with milliQ water.
3. 2× CRPG buffer. Add 1.4 mL 1 M Na_2HPO_4, 526 μL 1 M NaH_2PO_4, and 15 mL milliQ water. Adjust the pH to 7.3 and then bring the volume to 19.4 mL with milliQ water. Add 283.2 μL β-mercaptoethanol and 360 μL 1 M $MgCl_2$. Good for 2 weeks when stored at 4°C.
4. 37°C water bath.
5. Disposable polystyrene 1.5 mL cuvettes (Fisher; cat. no. 14-385-942).
6. Spectrophotometer.

2.5. Clonal Assay

1. 4% paraformaldehyde (*see* **Note 11**).
2. Rhodamine B (Sigma Aldrich; cat. no. R6626-100G) and Nile Blue A (Sigma Aldrich; cat. no. N5632-25G). Make a 1% Rhodamine B/Nile Blue solution by adding 1 g Rhodamine B and 1 g Nile Blue and then bring volume to 100 mL with milliQ water.

3. Methods

3.1. Making Low Ca^{2+} Complete Media

3.1.1. Making Ca^{2+}-Free Serum

1. Weigh out 200 g Chelex 100× Resin and place in a 2 L beaker with a stir bar.
2. Mix well with 2 L milliQ H2O.
3. Adjust pH to 7.5 with 12 N HCl. Stir at room temperature for 1 hour.
4. Cover and place at 4°C for 4 hours or overnight to allow the beads to settle.
5. Decant the slurry and add 2 L fresh milliQ H2O. Allow the beads to come to room temperature.

6. Repeat pH adjustment to 7.5. Stir at room temperature for 1 hour.
7. Cover and place at 4°C for 4 hours or overnight.
8. Allow beads to come to room temperature. Check the pH. (If pH is not 7.5, repeat Steps 5.8.)
9. Carefully pipet out as much water as possible. Try not to disturb the beads.
10. Carefully pour in 500 mL characterized Fetal Bovine Serum.
11. Stir gently (no bubbles) at 4°C for 1 hour.
12. Let resin settle in cold room overnight.
13. Pipet out the serum.
14. Filter-sterile through 0.22 μm filter (use a 500 mL filter apparatus).
15. Aliquot 179 mL serum into sterile 250 mL TC bottles. Store at –20°C until needed.

3.1.2. Regenerating Chelex beads

The Chelex beads can be regenerated and used up to three times to chelate Ca^{2+} ions. Regenerate immediately after use.

1. Add 600 mL 1 N HCl to the beads. Stir at room temperature for 1 hour.
2. Cover and place at 4°C for 4 hours or overnight to allow the beads to settle.
3. Decant and add 2 L milliQ water. Stir at room temperature for 1 hour.
4. Cover and place at 4°C for 4 hours or overnight to allow the beads to settle.
5. Decant and add 600 mL 1 N NaOH. Stir at room temperature for 1 hour.
6. Cover and place at 4°C for 4 hours or overnight to allow the beads to settle.
7. Decant and add 2 L milliQ water. Stir at room temperature for 1 hour.
8. The beads are now ready to re-use. Start with step 5 for the "Making Ca^{2+}-free serum" protocol.

3.1.3. Making Ca^{2+}-Free Serum Mix

Do not store the serum mix. Make just enough for the amount needed for the final media preparation.

1. Thaw one bottle of 179 mL Ca^{2+}-free serum.
2. Add the following to the serum: 11.8 mL of 100× cocktail, 11.8 μL of 84 μg/mL cholera toxin, and 179 μL of 4 mg/mL hydrocortisone.
3. Mix well and store at 4°C until the low Ca^{2+} base media is ready.

3.1.4. Making Low Ca^{2+} Base Media (1 L)

1. Place 800 mL milliQ water in a 2 L beaker with a stir bar.
2. Add 1 bag of DMEM:F12 (3:1) no Ca^{2+} powder media. Rinse out the inside of the package with milliQ water to remove all traces of powder.
3. Add 3.07 g of $NaHCO_3$ and 10 mL of 100× pen/strep. Mix well.
4. Adjust the pH to 7.2 (*see* **Note 12**).
5. Bring volume up to 1 L with milliQ water.

3.1.5. Making Low Ca^{2+} Complete Media

1. Add the Ca^{2+}-free serum mix to 1 L of Low Ca^{2+} base media made in the 2 L beaker.
2. Mix gently, avoiding bubbles.
3. Filter media through a sterile 0.22 μm Millipore filter in the TC hood. Place in sterile TC bottles.
4. Store at 4°C away from light.

3.2. Titrating siRNA Knockdown

Test the efficiency of siRNA knockdown by transfecting cells with siRNA and then 72 hours later collecting the cells, making cell lysates, and performing a Western blot. The amount of siRNA used should be titrated first, followed by a time course assay to determine the length of knockdown. For each concentration of siRNA being tested, make sure to include a negative-control siRNA of the same concentration. The negative-control siRNA should be a siRNA that has a scrambled sequence that will not target any gene.

3.2.1. Plating HaCaT Cells (Day 0)

1. Wash plate with 8 mL 1× PBS. Aspirate off.
2. Add 2 mL 0.25% Trypsin-EDTA to the plate and swirl the plate. Place the plate in the 37°C incubator for about 15 minutes until the cells have lifted off the plate.
3. Inactivate the trypsin by adding 8 mL media to the plate. Pipet up and down to create a single cell suspension. Collect in a 15 mL conical tube.
4. Count the cells using a hemacytometer. Calculate the number of cells needed to plate 4×10^5 cells per 10 cm plate (*see* **Note 13**).
5. Pipet the number of cells needed into a new 50 mL conical tube.
6. Centrifuge the cells at 1500 rpm for 5 minutes at room temperature.
7. Aspirate off the old media. Resuspend the cells in the amount of media needed (add 10 mL of media for each plate that is to be plated).
8. Pipet 10 mL of cell suspension to each plate of a 10 cm dish.
9. Place the cells in the 37°C, 5% CO_2 incubator.

3.2.2. Transfecting siRNA (Day 1)

To titrate the amount of siRNA needed for efficient knockdown, use at least three different concentrations of siRNA. Start by using siRNA at a final concentration of 30 nM, 60 nM, and 90 nM. For most siRNAs, 60 nM will knockdown efficiently; however, this should be empirically determined for different siRNAs. Also, use a negative-control siRNA at each concentration to determine if there are any non-specific effects from the siRNA. **Table 9.1** shows the amounts needed for different size plates when using 60 nM siRNA (*see* **Note 14**).

1. For each transfection sample, prepare siRNA–Lipofectamine 2000 complex in a laminar flow hood as follows:
   a. Dilute siRNA into 1.5 mL OptiMEM I Reduced Serum Medium in a 15 mL conical tube (*see* **Note 15**). Mix gently.
   b. Mix Lipofectamine 2000 gently before use, then dilute 30 μL into 1.5 mL OptiMEM in a 15 mL conical tube. Mix gently and incubate for 5 minutes at room temperature (proceed to step c within 25 minutes).
   c. After the 5-minute incubation, combine the diluted siRNA with the diluted Lipofectamine 2000. Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy).
2. Add the siRNA–Lipofectamine 2000 complexes to each plate containing cells and medium. Mix gently by rocking the plate back and forth (*see* **Note 16**).
3. Place the cells in the 37°C, 5% CO_2 incubator.
4. Change the media 24 hours after transfection (*see* **Note 17**).
5. 72 hours after transfection, harvest the cells and perform a Western blot to determine the concentration at which there is efficient knockdown.
6. Titrate the length of knockdown by repeating the above section, but only use the concentration of siRNA that gives the most efficient knockdown. Then harvest the cells at

Table 9.1
Reagent amounts for siRNA transfection

Culture vessel	Surface area per well	Volume of plating media (mL)	Volume of OptiMEM	Volume of 50 μM siRNA (μL)	Volume of Lipofectamine 2000 (μL)
12 well	~4 cm^2	1	2 × 100 μL	1.44	2
6 well	~10 cm^2	2	2 × 250 μL	3	5
10 cm	~60 cm^2	10	2 × 1.5 mL	15.6	30

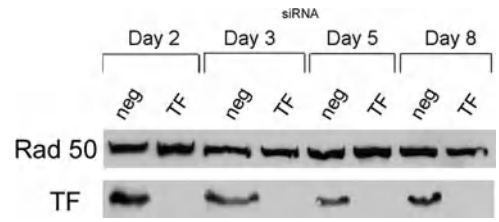

Fig. 9.1. Knockdown in HaCaT cells occurs efficiently and lasts for at least 6 days. Rad50 was used as a nuclear loading control. TF, transcription factor. Neg, negative control.

various days after transfection. **Figure 9.1** shows that this method results in efficient knockdown of a transcription factor (TF) that we study.

3.3. Clonal Assay

A clonal assay can be used to determine the proliferation potential of individual keratinocytes. By transfecting siRNA into HaCaT cells, genes can be knocked down and the effect on the proliferation potential of cells can be monitored. The results from the first 14 days uncover the initial effects from the siRNA. However, replating the cells from day 14 at clonal density allows the monitoring of longer-term proliferation potential. For example, **Fig. 9.2** shows that when a transcription factor that we study was knocked down, there was an initial increase in colony-forming ability. However, when colonies were harvested at the end of the first plating and equal number of knockdown and control cells was re-plated at clonal density and allowed to grow for an additional 14 days in the absence of siRNA, there was now a decrease in colony-forming ability in the knockdown. This result indicates that the expansion seen in the first-generation plating was likely due to an increase in the number of cells that are faster proliferating but have only limited proliferation potential (i.e., TA cells). Concurrently, there was a loss of cells with long-term proliferation potential, as demonstrated by reduced colony-forming ability in the second generation. This illustrates how a colony-forming assay

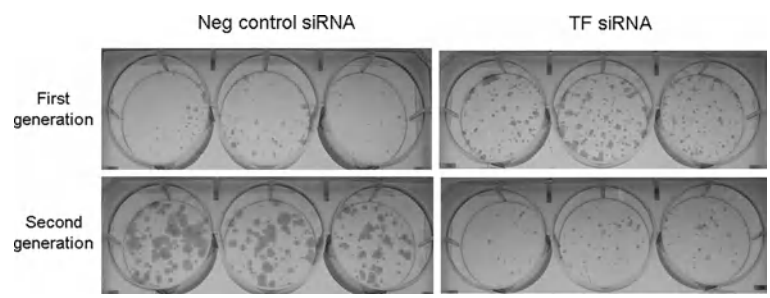

Fig. 9.2. HaCaT cells can be used in a clonal assay to determine the proliferation potential of individual cells.

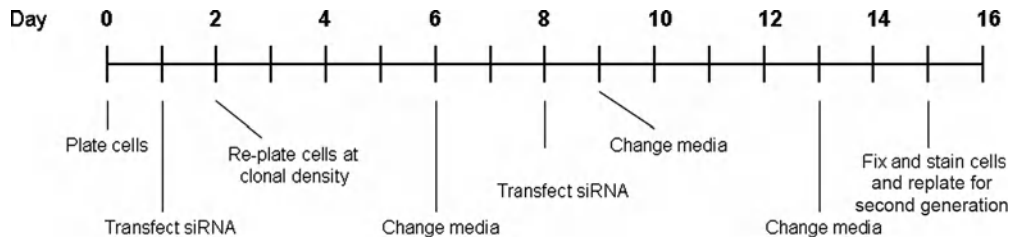

Fig. 9.3. Timeline for clonal assay.

can be used in conjunction with siRNA knockdown to study the molecular control of the keratinocyte proliferation potential (*see* **Fig. 9.3** for a timeline of the first generation assay).

3.3.1. Plating HaCaT Cells (Day 0)

1. Follow steps 1–3 of **section 3.2.1**.
2. Count the cells using a hemacytometer. Calculate the number of cells needed to plate 8×10^4 cells per well (*see* **Note 13**).
3. Pipet the number of cells needed into a new 15 mL conical tube.
4. Centrifuge the cells at 1500 rpm for 5 minutes at room temperature.
5. Aspirate off the old media. Re-suspend the cells in the amount of media needed (add 2 mL of media for each well that is to be plated).
6. Pipet 2 mL of cell suspension to each well of a 6-well plate.
7. Place the cells in the 37°C, 5% CO_2 incubator.

3.3.2. Transfect siRNA (Day 1)

1. Transfect cells using Lipofectamine 2000 according to **Section 3.2.2**. Make sure to adjust the volume for a 6-well plate.
2. Place cells in the 37°C, 5% CO_2 incubator.

3.3.3. Re-plate HaCaT at Clonal Density (Day 2)

1. Wash each well with 2 mL 1× PBS. Aspirate off.
2. Add 400 µL 0.25% Trypsin-EDTA to each well of the plate. Place the plate in the 37°C incubator for about 15 minutes until the cells have lifted off the plate.
3. Inactivate the trypsin by adding 600 µL media to each well. Pipet up and down to create a single cell suspension. Collect in a 1.5 mL microfuge tube.
4. Count the cells using a hemacytometer. Calculate the number of cells needed to plate 3500 cells per 6-well plate (this is for 500 cells/well with enough for one extra well) (*see* **Note 18**).
5. Add 3500 cells into a volume of 14 mL media in a 15 mL conical tube. Do this for each sample.

6. Place 2 mL of the diluted cell suspension to each well of a 6-well plate.
7. Place the cells in the 37°C, 5% CO_2 incubator.

3.3.4. Change Media (Day 6)

1. Aspirate off old media.
2. Add 2 mL of fresh media to each well.
3. Place cells in the 37°C, 5% CO_2 incubator.

3.3.5. Transfect siRNA (Day 8)

1. Transfect cells using Lipofectamine 2000 according to **Section 3.2.2**. Make sure to adjust the volume for a 6-well plate. Prepare a master mix for the samples and make enough for one extra well. For example, for the negative control siRNA you would make a master mix for the transfection that contains enough reagents for 7 wells.
2. Place cells in the 37°C, 5% CO_2 incubator.

3.3.6. Change Media (Day 9 and Day 13)

1. Aspirate off old media.
2. Add 2 mL of fresh media to each well.
3. Place cells in the 37°C, 5% CO_2 incubator.

3.3.7. Fix and Stain HaCaT Cells (Day 15)

1. For half of the wells, for each sample, follow **Section 3.3.3.** to replate cells at Clonal density for second generation. Change media every four days. Fourteen days after replating, follow step 2–13 below.
2. For the remaining wells, (wash) wash each well with 2 mL 1× PBS. Aspirate off.
3. Add 2 mL 4% paraformaldehyde to each well to fix the cells. Incubate at room temperature for 15 minutes.
4. Aspirate off.
5. Wash each well with 2 mL 1× PBS. Aspirate off.
6. Repeat step 4 two more times.
7. Add 2 mL ice-cold 90% methanol to each well to permeabilize the cells. Incubate at room temperature for 5 minutes.
8. Wash each well with 2 mL 1× PBS. Aspirate off.
9. Repeat step 7 two more times.
10. Stain the cells by adding 2 mL of the staining solution (1% Rhodamine B/1% Nile Blue). Incubate at room temperature for 30 minutes.
11. Wash plate with DI water several times until there is no background staining.
12. Pour off water and allow plates to air dry.
13. Take pictures of the plates and quantify the number and size of the colonies.

3.4. Luciferase and CRPG β-Galactosidase Assays

A luciferase assay is a reporter assay that allows one to determine if the transcription factor being studied regulates a specific promoter. By combining siRNA transfection with reporter plasmid transfection, one can assess the effect of gene depletion on reporter promoter activity. A CRPG β-galactosidase assay is performed to normalize the data for possible differences in transfection efficiencies. The CRPG assay can also be used to determine if experimental conditions may have non-specific effects on promoter activity. For example, if knockdown of a gene under study results in consistent dosage-dependent increase in both luciferase expression driven by the specific promoter and β-galactosidase assay driven by the control β-actin promoter, this could indicate that the siRNA being used is causing a non-specific activation of promoters. In this case, additional control promoters such as CMV should be tested. In addition to having samples that are transfected with the negative-control siRNA, there should also be samples that are transfected with an empty pGL3 basic vector. This control allows one to determine if the siRNA is having any promoter-independent effect on the luciferase read-out. A comparison between luciferase read-outs from pGL3 vector and pGL3 with target promoter will also ensure that the promoter is indeed active under the experimental conditions. **Table 9.2** provides examples of experimental conditions, with the proper controls included.

Table 9.2
Example of Luciferase assay experimental design

Sample #	siRNA	Promoter plasmid
1	30 nM neg	Empty pGL3
2	30 nM gene being studied	Empty pGL3
3	30 nM neg	Target promoter pGL3
4	30 nM gene being studied	Target promoter pGL3
5	60 nM neg	Empty pGL3
6	60 nM gene being studied	Empty pGL3
7	60 nM neg	Target promoter pGL3
8	60 nM gene being studied	Target promoter pGL3

Each condition being studied should be done in triplicate. In the example above you would need 24 wells (two 12-well plates). However, when preparing master mixes, make enough for one extra well (*see* **Fig. 9.4** for a timeline for this assay).

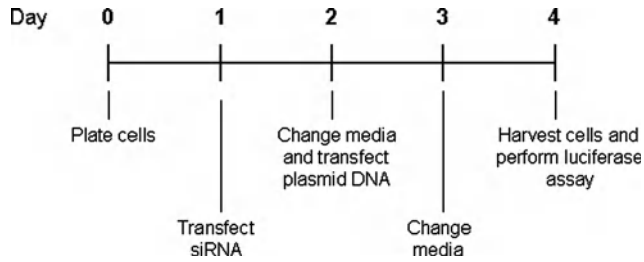

Fig. 9.4. Timeline for luciferase and CRPG β-galactosidase assay.

3.4.1. Plating HaCaT cells (Day 0)

1. Follow steps 1–3 of **Section 3.2.1.**
2. Count the cells using a hemacytometer. Calculate the number of cells needed to plate 7×10^4 cells per well (*see* **Note 13**).
3. Pipet the number of cells needed into a new 15 mL conical tube.
4. Centrifuge the cells at 1500 rpm for 5 minutes at room temperature.
5. Aspirate off the old media. Re-suspend the cells in the amount of media needed (add 1 mL of media for each well that is to be plated).
6. Pipet 1 mL of cell suspension to each well of a 12-well plate.
7. Place the cells in the 37°C, 5% CO_2 incubator.

3.4.2. Transfecting siRNA (Afternoon, Day 1)

1. Transfect cells using Lipofectamine 2000 according to **Section 3.2.2**. Make sure to adjust the volume for a 12-well plate and the samples are prepared in triplicate. Prepare a master mix for the samples and make enough for one extra well.
2. Change the media 24 hours after transfection.

3.4.3. Transfecting Plasmid DNA (Early Evening, Day 2)

The cells should be at least 80% confluent at the time of transfection to minimize the amount of cell death caused by Lipofectamine toxicity. The following plasmids will be transfected into HaCaT cells for the luciferase assay in triplicate for each sample (*see* **Note 19**):

Plasmid name	Amount
Reporter plasmid (i.e., promoter of interest-pGL3 basic)	800 ng
β-actin promoter – β-gal plasmid (for normalization)	300 ng
Filler DNA (empty vector plasmid)	500 ng
Total DNA	1600 ng

1. Calculate the amount of each plasmid needed to transfect the samples in triplicate, but add enough plasmid for one extra well. For example, if you have four samples, you would

prepare them each in triplicate. This means that you should have a master mix that contains enough DNA for 12 wells. However, to account for pipetting errors, you should prepare the DNA for 13 wells.

2. Calculate the amount to OptiMEM I Reduced Serum Medium needed to dilute both the DNA and the Lipofectamine 2000. For each you will need 100 µL per well. Using the example above, you would need 13 wells, therefore you would dilute the DNA in OptiMEM to make the final volume 1300 µL. You would also dilute the Lipofectamine 2000 in OptiMEM to make a final volume of 1300 µL.
3. Calculate the amount of Lipofectamine 2000 needed. You will need 4 µL per well. Using the example above for 13 wells, you would need to add 52 µL to 1248 µL OptiMEM media.
4. For each transfection sample, prepare DNA-Lipofectamine 2000 complex in a laminar flow hood as follows:
   a. Dilute the plasmids into OptiMEM I Reduced Serum Medium in a 15 mL conical tube. Mix gently.
   b. Mix Lipofectamine 2000 gently before use, then dilute into OptiMEM in a 15 mL conical tube. Mix gently and incubate for 5 minutes at room temperature (proceed to step c within 25 minutes).
   c. After the 5-minute incubation, combine the diluted DNA with the diluted Lipofectamine 2000. Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy).
5. Add the DNA–Lipofectamine 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
6. Place the cells in the 37°C, 5% CO_2 incubator.
7. Change the media 16 hours after transfection.

3.4.4. Luciferase Assay (Afternoon, Day 4)

1. Aspirate the media and add 0.5 mL 1× PBS to each well.
2. Scrape each well and collect each sample in a 1.7 mL microfuge tube.
3. Centrifuge for 1 minute at full speed at room temperature to pellet the cells.
4. Prepare 1× lysis buffer by diluting the 5× reporter lysis buffer down to 1× with milliQ water.
5. Aspirate the supernatant and re-suspend cells in 70 µL 1× lysis buffer.
6. Freeze-thaw the samples one time to lyse the cells by placing the samples at –80°C until samples are fully frozen (about 1 hour) and then thawing at room temperature (*see* **Note 20**).

7. While the freeze-thaws are occurring, thaw the luciferase substrate to room temperature.
8. Vortex the samples for 10 seconds.
9. Centrifuge at full speed at room temperature for 30 seconds. Make sure the hinge of the tube faces outward. The pellet will be on the same side as the hinge.
10. Transfer the supernatant for each sample into new 1.7 mL microfuge tubes.
11. Add 50 μL of cell lysate to a luminometer tube.
12. Add 150 μL of luciferase substrate to the first sample.
13. Read sample immediately in luminometer (*see* **Note 21**).
14. Repeat steps 11–13 for each sample.
15. Print the results from the luminometer readings.

3.4.5. CRPG β-Galactosidase Assay (Afternoon, Day 4)

1. For each sample, add 100 μL 2× CRPG and 55 μL milliQ water to a 1.7 mL microfuge tube. Prepare an extra tube for a blank that contains no cell lysate.
2. Then add 5 μL cell lysate to each tube.
3. Finally add 40 μL 40 mM CRPG and close the caps tightly.
4. Briefly vortex the sample.
5. Pulse spin the samples to get the sample to the bottom of the tube.
6. Incubate the samples at 37°C until color develops.
7. After the desired color is obtained (orange, but not brown or red), stop the reaction by adding 600 μL milliQ water.
8. Transfer the samples to the disposable cuvettes.
9. Read the O.D. of the samples in the spectrophotometer at 562 nm (*see* **Note 22**).
10. Print the results from the spectrophotometer readings.
11. To normalize the luciferase samples, divide the luciferase reading by the O.D. (β-gal) reading to give the "Relative Light Unit" (RLU). Do this for each sample. Then average the RLU for the triplicate samples and also determine the standard deviation. Prepare a graph of the average RLU for each sample.

4. Notes

1. Serum lots must be screened because they show variability in supporting keratinocytes growth.
2. When making T3 solutions, make sure to use only plastic. T3 will stick to glass and therefore glass items should be avoided.

3. Cholera toxin is extremely toxic. Make sure to take precautions when handling it.
4. This is a special formula media and therefore must be ordered in large quantities (100 L). Make sure to specify that the media needs to be calcium-free when ordering. The media comes with L-glutamine, but without calcium chloride, HEPES buffer, and sodium bicarbonate.
5. HaCaT cells should be maintained on a 10-cm plate in culture by preventing them from being confluent for longer than a day. Allowing them to be confluent for too long can cause the cells to undergo terminal differentiation. HaCaT cells can be grown in standard DMEM/F12 10% FBS medium. However, by growing in low-calcium medium as described in this protocol, the cells maintain a more basal-like phenotype and are more resistant to undergoing unwanted terminal differentiation. The cells grown in low-calcium medium can be switched to high-calcium medium (2.8 mM calcium) and will undergo normal terminal differentiation, with K1 protein expression occurring 4 days after calcium addition (*see* **Fig. 9.5**).

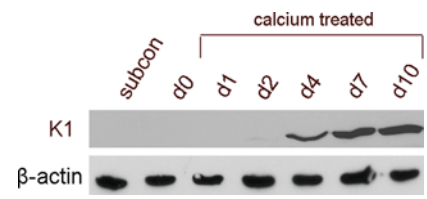

Fig. 9.5. K1 expression is upregulated 4 days after treatment with 2.8 mM calcium in HaCaT cells grown in low-calcium media complete. Subcon, sub-confluent.

6. Check Ambion's web site for availability of siRNA against your gene of choice. If there is not a validated siRNA for your gene, then you should order multiple siRNAs against the gene that you study. When initially screening siRNAs, order only 5 nmol standard purity and re-suspend the siRNA in the nuclease-free water supplied with the siRNA. Make sure to re-suspend the siRNA in the tissue culture hood to avoid contamination and then aliquot into sterile microfuge tubes in small amounts. Avoid repeated freeze-thaws of the siRNA to avoid degradation. Once the siRNAs are screened for efficient knockdown and low toxicity, then order larger scale of the one that works best.
7. Make sure that the DNA preparation is clean and free of contamination by taking O.D. readings at 260 nm and 280 nm using a spectrophotometer. The 260:280 ratio should be greater than 1.8 for good-quality DNA.

8. Protect from light. D-luciferin is light sensitive.
9. Protect from light. Do not allow the aliquots to undergo multiple freeze-thaws. An alternative to the homemade luciferase substrate is to buy a luciferase assay kit from Promega (cat. no. E4530) which also includes the 5× reporter lysis buffer. However, making the luciferase substrate is much more cost efficient.
10. Dilute the lysis buffer to 1× with water right before use. Only make enough for what you need right away. For example, if you have 24 wells, then you would need 1680 μL 1× lysis buffer. You want to make extra to account for pipetting error, so you could make 1800 μL (add 360 μL of 5× reporter lysis buffer to 1440 μL milliQ water). Do not store the 1× lysis buffer.
11. Paraformaldehyde is very toxic. Take precautions when handling it.
12. Media should be red-orange in color. If the media is pink, then de-gas the media.
13. Always calculate the amount of cells needed for one extra plate or well. This minimizes variability in the number of cells plated due to pipetting errors. Make sure that after you centrifuge the cells and aspirate the supernatant that you re-suspend the cells in enough medium for the extra plate. For example, if you need four 10-cm plates, then calculate the number of cells needed for five plates. Once the cells have been pelleted, re-suspend the cells in 50 mL low Ca^{2+} complete media and then add 10 mL of the cell suspension to each of the four 10-cm plates.
14. For example, if you are transfecting a 10-cm plate with 30 nM siRNA, then use only 7.8 μL siRNA. If using 90 nM siRNA, then use 23.4 μL siRNA.
15. Use only polypropylene tubes for the transfection mixes because Lipofectamine 2000 sticks to polystyrene.
16. The transfection mixes can be added directly to the plates with the E-low Ca^{2+} complete media. The serum and antibiotics in the media do not have an effect on transfection efficiency in HaCaT cells.
17. Aspirate off the old media and add fresh media.
18. Whenever handling small volumes, the pipetting error is likely to be high. Therefore, it is better to make dilutions first to reduce the variability between samples. For example, if you have a total of 25×10^4 cells, then you can make a dilution for each sample to achieve a concentration of 1×10^4 cells/mL (add 40 μL of cell suspension to 960 μL low Ca^{2+} complete media). Then to get 3500 cells, you would add 350 μL of the

diluted cell suspension to 14 mL media. Then 2 mL of the final dilution would be added to each well of a 6-well plate to give 500 cells per well.

19. The amount of reporter plasmid (promoter of interest-pGL3 basic) must be determined empirically. To titrate the reporter plasmid, follow the protocol for the luciferase and CRPG assays, except that you do not need to transfect in siRNA and you would add different amounts of the reporter plasmid. For example, one triplicate set of wells would contain no reporter plasmid (this is the background level of luciferase activity). You would then have a triplicate set of wells that contains the following amounts of reporter plasmid: 100 ng, 200 ng, 400 ng, 800 ng, 1000 ng. You would perform luciferase and CRPG assays. You should have a dose-dependent increase in basal promoter activity with increasing amounts of reporter plasmid. If you start to see the promoter activity plateau or decrease with higher amounts of reporter plasmid, then squelching has occurred and concentrations from this point on should not be used in future experiments. Pick a concentration of reporter plasmid that is in the linear range.
20. Do not leave the samples at room temperature for extended periods of time as this will allow for protein degradation. Once thawed, place the samples on ice.
21. Once the substrate is added to the cell lysate, the sample needs to be read immediately. The reaction occurs quickly and the luciferase activity will decrease over time.
22. The O.D. readings should be between 0.1 and 1.0 to be within the linear range of the spectrophotometer. If your samples are reading less than 0.1, allow the samples to incubate at room temperature until you see the color become more orange-red. Then repeat the readings in the spectrophotometer. If the readings are above 1.0, then dilute all your samples by adding about 100 μL water to each sample. Repeat the readings. If the samples are still at an O.D. above 1.0, then repeat the addition of water to all your samples until the readings are below 1.0.

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Chapter 10

RNA Interference in Keratinocytes and an Organotypic Model of Human Epidermis

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Abstract

Gene silencing approaches afford investigators the ability to gain important insight into the normal functional requirements of specific epidermal proteins and promise to yield a powerful therapeutic means to dampen the level of proteins that are mutated or frequently overexpressed in skin disease. The efficient and tractable delivery of siRNAs into epidermal keratinocytes is seminal to this process. Here, we describe techniques for transient and long-term silencing of a representative gene product, namely desmoglein 1, in primary human epidermal keratinocytes maintained as submerged cultures or three-dimensional organotypic raft cultures. As a complement to epidermal-specific gene targeting strategies in mice, these technical approaches permit relatively rapid loss-of-function studies purely in keratinocytes without some of the potential influences present in situ, such as an immune system or vasculature.

Key words: Desmoglein, Desmosome, Epidermis, Keratinocyte, Lamin A/C, miRNA, Organotypic, Retrovirus, siRNA.

1. Introduction

The epidermis comprises a specialized stratified epithelium containing proliferating keratinocytes in the basal layer that exit the cell cycle, stratify, and migrate through the suprabasal layers, ultimately giving rise to a cornified, dead cell layer (1). Several characteristic structural and biochemical changes occur during the keratinization process. For example, the composition and size of cell–cell junctions, known as desmosomes, varies as keratinocytes progress through the upper epidermal layers (2). Desmoglein 1, in particular, is a transmembrane protein

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concentrated in desmosomes of the suprabasal epidermis. Although desmoglein 1 can be detected in primary cultures of human epidermal keratinocytes that have been induced to differentiate, the morphological resolution of these classical, submerged culture systems is somewhat limited (3, 4). The development of three-dimensional, organotypic models of human epidermis permits the analysis of differentiation-specific epidermal proteins in an architecturally more relevant context than submerged cultures (5–9).

The advent of RNA interference (RNAi) technology, which takes advantage of the endogenous machinery of cells to silence gene expression, has revolutionized the ability of investigators to study gene function (10–12). A variety of experimental approaches have been developed to deliver small interfering RNAs (siRNAs) into mammalian cells for knockdown studies. Here, we describe protocols for the transient introduction of siRNA oligonucleotide duplexes into freshly isolated human epidermal keratinocytes. We further outline the development of a retroviral gene delivery system that allows for the expression of microRNA (miRNA)-like sequences which have been modified for more stable gene silencing in long-term cultures. Representative examples of these RNAi tools designed for desmoglein 1 knockdown studies in an organotypic raft model of human epidermis are detailed.

2. Materials

2.1. Primary Keratinocyte Cell Isolation and Culture

1. Neonatal foreskins (less than 48 h post-circumcision).
2. Foreskin Storage Medium: Hank's Balanced Salt Solution (HBSS; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone), and 10 μg/ml gentamicin/0.25 μg/ml amphotericin B (Sigma).
3. Phosphate-buffered saline (PBS; Sigma).
4. 10% bleach solution.
5. Sterile dissecting tools, forceps, and scissors.
6. 2.4 U/ml dispase II (neutral protease grade 2; Roche Applied Science). Store at –20°C.
7. Newborn Calf Serum (NCS; Invitrogen).
8. 0.25% and 0.05 % Trypsin/1 mM ethylenediamine tetraacetic acid (EDTA; Sigma).
9. 40 μm cell strainer (BD Falcon).

10. Keratinocyte growth medium: Medium 154-calcium free (M154CF; Cascade Biologics) supplemented with 0.07 mM $CaCl_2$, human keratinocyte growth supplement (HKGS; Cascade Biologics), and 10 μg/ml gentamicin/0.25 μg/ml amphotericin B. Store in the dark at 4°C.
11. J2-3T3 fibroblast growth medium: Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% NCS, 4 mM L-glutamine (Sigma), and 10 μg/ml gentamicin/0.25 μg/ml amphotericin B.

2.2. Organotypic Raft Cultures of Human Epidermis

1. J2 clone of 3T3 fibroblasts.
2. J2-3T3 growth medium (refer to item 11 of **Section 2.1**).
3. 0.05 % Trypsin/1 mM EDTA.
4. Rat tail collagen type I (5 mg/ml; BD BioScience).
5. 10 × Collagen Plug resuspension buffer (store aliquots at –20°C).

   Add 1.1 g $NaHCO_3$ and 2.39 g HEPES to 50 ml 0.05 N NaOH and pass through a 0.2 μm PES membrane filter (Millipore) to sterilize.
6. 10 × DMEM liquid (Sigma).
7. Tissue Culture-treated 6-well plates.
8. Low-passage primary human epidermal keratinocytes.
9. 2×10^{-8} M triiodothyronine (T_3; Sigma). To make a 20 ml solution
   a. dissolve 6.8 mg in 50 ml 0.02 N NaOH (2×10^{-4} M T_3);
   b. add 0.1 ml 2×10^{-4} M T_3 to 9.9 ml PBS (2×10^{-6} M T_3);
   c. add 0.2 ml 2×10^{-4} M T_3 to 19.8 ml PBS (2×10^{-8} M T_3)
10. 100 × E medium cocktail (store aliquots at –20°C). Add the following solutions to 120 ml of PBS and filter sterilize:
    a. Dissolve 486 mg adenine (Sigma) in 20 ml ddH_2O.

       Add ~0.5 ml 6 N HCl to get adenine into solution.
    b. Dissolve 100 mg bovine pancreatic insulin (Sigma) in 20 ml 0.1 N HCl.
    c. Dissolve 100 mg human apo-transferrin (Sigma) in 20 ml PBS.
    e. Add 20 ml 2×10^{-8} M T_3.
11. HEPES-buffered saline (HBS; 20 mM HEPES and 150 mM NaCl), pH 7.4.
12. 0.4 μg/ml hydrocortisone (1000 ×; Sigma). Store 1 ml aliquots at –20°C.

a. Dissolve 25 mg hydrocortisone in 5 ml 100% ethanol (5 mg/ml).

b. Dilute 3.6 ml 5 mg/ml hydrocortisone in 41.1 ml HBS.

13. 10 μg/ml cholera toxin (1000 × ; Sigma). Store in the dark at 4°C.
    a. Dissolve 0.5 mg cholera toxin in 0.5 ml sterile ddH_2O (1 mg/ml).
    b. Dilute 0.1 ml of 1 mg/ml cholera toxin in 9.9 ml sterile ddH_2O (10 μg/ml).
14. E medium DMEM:Ham's F-12 (3:1; Sigma) supplemented with 1 × E medium cocktail (180 μM adenine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml T_3), 0.4 ng/ml hydrocortisone, 10 μg/ml cholera toxin, 4 mM L-glutamine (Sigma), and 10 μg/ml gentamicin/0.25 μg/ml amphotericin B.
15. 5 μg/ml murine epidermal growth factor (1000 × mEGF; Chemicon). Add 5 μl of 1 mg/ml stock solution to 995 μl PBS containing 100 μg/ml bovine serum albumin (BSA; Sigma). Store at –20°C.
16. Sterilized forceps, spatula, and steel mesh grids (Purolator EFP; mesh size 40/0.010). Mesh grids bent at three corners to generate a raised surface when placed into a 100 mm tissue culture-treated plate.

2.3. siRNA Oligonucleotide Transfection in Primary Human Epidermal Keratinocytes

1. 20 μM Stealth siRNA duplex and RNAi Negative Control Duplex (Invitrogen). Aliquot and store at –70°C.
2. DharmaFECT 1 oligonucleotide transfection reagent (Dharmacon).
3. Low-passage primary human epidermal keratinocytes.
4. Keratinocyte growth medium: Complete M154 (refer to item 10 of **Section 2.1**), M154 lacking gentamicin/amphotericin B, and M154 lacking HKGS and gentamicin/amphotericin B (M154-SFM).
5. 0.05 % Trypsin/1 mM EDTA.

2.4. Retroviral EGFP-miRNA Plasmid Cloning and Production of Viral Supernatants

1. 25 nmol gene-specific sense and anti-sense DNA oligomers (Integrated DNA Technologies).
2. 10 mM Tris-HCl, pH. 7.4.
3. pMIRAGE and pLZRS-LINKER plasmids.
4. Annealing buffer: 30 mM HEPES, 100 mM potassium acetate, 2 mM magnesium acetate; pH 7.0.
5. Deoxynucleotide solution (dNTP; 10 mM of each; New England Biolabs/NEB).
6. 10 mM dATP (NEB).
7. T4 Polynucleotide Kinase with 10 × buffer (10,000 U/ml; NEB).
8. T4 DNA Ligase with 10 × buffer (400,000 U/ml; NEB).

9. Restriction enzymes with 10× buffers (NEB): *BamHI, BstXI*, *EcoRI, HindIII, XhoI*, *XbaI*.
10. Klenow large fragment (5,000 U/ml; NEB).
11. Calf intestinal phosphatase (CIP; 10,000 U/ml; NEB).
12. Mini-Elute Reaction Clean-up Kit (Qiagen).
13. Pure Yield Plasmid Mini-Prep Kit (Promega).
14. Competent bacteria (e.g., JM109). Store at –70°C.
15. Luria broth (LB; Difco) and LB plate with 50 μg/ml ampicillin (Sigma).
16. Low melting point agarose (Invitrogen).

2.5. Retroviral Transduction in Human Epidermal Keratinocytes

1. HEK-293-derived Phoenix amphotropic packaging cell line (ATCC; courtesy of Gary Nolan).
2. Phoenix cell growth medium: DMEM supplemented with 10% heat-inactivated FBS (Hyclone), 4 mM L-glutamine, and 10 μg/ml gentamicin/0.25 μg/ml amphotericin B.
3. PBS (Sigma).
4. 0.05 % Trypsin/1 mM EDTA.
5. Lipofectamine 2000 (Invitrogen).
6. Opti-MEM (Invitrogen).
7. 1 mg/ml puromycin (1000 × ; Sigma). Store at –20°C.
8. Low-passage primary human epidermal keratinocytes.
9. Keratinocyte growth medium: Complete M154 (refer to line 10 of **Section 2.1**).
10. 4 mg/ml Polybrene (Hexadimethrine bromide; Sigma). Store at –20°C.
11. Inverted immunofluorescense microscope.
12. Phoenix cell freezing medium: 90% FBS containing 10% dimethylsulfoxide (DMSO; Sigma).
13. 10% bleach solution diluted in H_2O.

3. Methods

3.1. Initiation and Maintenance of Primary Human Epidermal Keratinocyte Cultures from Neonatal Foreskins

Low-passage human epidermal keratinocytes are utilized for gene silencing in submerged and raft culture studies as described. For this purpose

1. Obtain de-identified neonatal foreskins from an IRB-approved tissue repository kept in 15 ml conical tubes containing 4 ml Storage Medium at 4°C for less than 48 h (*see* **Note 1**).

2. Remove and discard Storage Medium in a bleach containing solution and wash foreskin twice in 10 ml PBS.
3. Transfer foreskin into a 100 mm Petri dish containing 3 ml PBS. Using sterilized forceps and dissecting scissors, trim off excess dermal tissue including fat and blood vessels. Dissect foreskin into 0.25–0.5 cm^2 pieces.
4. Transfer foreskin pieces into a 60 mm tissue culture dish containing 3 ml 2.4 U/ml dispase II, epidermal side facing up. Incubate overnight at 4°C.
5. Using forceps, separate epidermal lining from underlying dermal tissue. Transfer epidermal sheets into a 100 mm dish containing 3 ml 0.25% trypsin/EDTA. Incubate for 10 min at 37°C.
6. Neutralize trypsin with 0.5 ml of NCS. Using forceps, scrape individual epidermal sheets against the surface of the Petri dish for 10–15 sec to dissociate cells from one another. Triturate epidermal sheets and cell suspension using a 5 ml serological pipet and pass through a 40 μm cell strainer to separate cells from intact sheets. Rinse Petri dish with 5 ml PBS to collect remaining cells and pass through cell strainer as well. Transfer cell suspension to a 15 ml conical tube and pellet cells by centrifugation at 200 × g for 5 min.
7. Resuspend cell pellet in 8 ml complete M154 and seed into a 100 mm tissue culture-treated dish. Replace culture medium every 48 h.
8. Passage keratinocytes prior to achieving 80% confluency. Use 0.05% trypsin/EDTA (5 min at 37°C) for this purpose and inactivate enzyme with an equal volume of DMEM containing 10% NCS (J2-3T3 growth medium). Remove DMEM by centrifugation at 200 × g for 5 min, then re-suspend cell pellet in complete M154. Seed 5×10^5 cells per 100 mm dish.

3.2. Establishment of Organotypic Raft Cultures of Human Epidermis

Combining gene silencing approaches with an organotypic model of human epidermis permits loss-of-function analysis during morphogenetic development of a stratified epithelium. For this purpose, keratinocytes transfected with siRNA oligonucleotide duplexes (*see* **Section 3.3**) or transduced with retroviral EGFP-miRNA constructs (*see* **Section 3.5**) are seeded onto a collagen lattice infused with murine fibroblast cell lines; these cultures are raised to an air–liquid interface to allow for the formation and organization of the four distinct (i.e., basal, spinous, granular, cornified) epidermal cell layers.

3.2.1. Setting a Collagen–Fibroblast Plug

1. Expand J2-3T3 cells in growth medium. Three nearly confluent 100 mm dishes contain sufficient cells ($\sim 6 \times 10^6$) for six collagen–fibroblast plugs (*see* **Note 2**).
2. Release J2-3T3 cells from the substrate by incubating in 0.05% trypsin/1 mM EDTA for 5 min at 37°C and inactivating the enzyme in an equal volume of growth medium. Count an aliquot of the single cell suspension using a hemacytometer and centrifuge 2.5×10^5 cells per ml of collagen–fibroblast plug at $300 \times g$ for 5 min. A total of 4.5×10^6 cells will be incorporated into six collagen–fibroblast plugs.
3. Resuspend 4.5×10^6 cells in 1.8 ml ice-cold 10 × Collagen Plug Resuspension Buffer. Pipet vigorously and transfer single cell suspension to a 50 ml conical tube that has been prechilled on ice.
4. Add 1.8 ml ice-cold 10 × DMEM and mix by pipetting.
5. Add 14.4 ml ice-cold 5 mg/ml rat tail collagen I (final concentration; 4 mg/ml) and mix quickly to prevent polymerization but gently to limit excessive air bubbles from forming in the slurry. The phenol-red color indicator should be red if the pH is neutral. It may be necessary to add a few drops of 0.5 N NaOH to neutralize the pH of the collagen–fibroblast slurry if the solution appears yellow.
6. Pipet 2.5 ml of the collagen–fibroblast slurry per plug into each well of a 6-well tissue culture plate and incubate in a 37°C incubator for 20 min to allow for polymerization. Add 2 ml of E medium on top of the collagen–fibroblast plug and incubate for at least 24 h prior to seeding keratinocytes. Collagen–fibroblast plugs can be used up to a week after having been set. Re-feed the plugs with 2 ml of E medium every 2 days.

3.2.2. Seeding Keratinocytes onto Collagen–Fibroblast Plugs

1. 1×10^6 keratinocytes transfected with siRNA oligonucleotide duplexes (*see* **Section 3.3**) or transduced with EGFP-miRNA retroviruses (*see* **Section 3.5**) are used for each collagen–fibroblast plug.
2. Trypsinize keratinocytes and thoroughly resuspend 1×10^6 cells in 2 ml of E medium supplemented with 5 ng/ml EGF. Seed single cell suspensions directly onto a single collagen–fibroblast plug. Incubate for 48 h in a 37°C humidified tissue culture incubator, replacing the growth medium daily. After 2 days, the growth medium should be exhausted and the keratinocytes will have achieved confluency.
3. Transfer sterilized stainless steel mesh to a 100 mm culture dish using sterile forceps.

4. Remove the E medium from the collagen plugs, taking care not to disturb the keratinocyte sheet on the top surface. Using a sterile spatula, release the collagen plug from the sides of the 6-well plate by sliding the spatula along the perimeter of the well. Balance the collagen plug on the broad end of the spatula by gently sliding it under the surface of the plug. Transfer the collagen plug onto the top of the steel mesh surface without creating any air bubbles at the collagen–metal interface.
5. Add E medium (~12–14 ml) without EGF supplementation underneath the steel mesh so that a liquid–collagen interface is separated by the metal surface. Ensure that air bubbles are not trapped between the mesh and the collagen plug.
6. Incubate the rafts for 1–2 weeks to allow for stratification and morphogenesis, replacing the E medium every 2 days. Harvest the raft cultures for conventional biochemical and histological analysis (*see* **Note 3**). Representative examples of hematoxylin and eosin (H&E)-stained tissue sections of raft cultures at 3, 6, and 9 days after being raised to an air–liquid interface are shown in **Fig.** 10.1. Immunostaining for desmoglein 1 shows that this desmosomal cadherin is concentrated at areas of cell–cell contact in the suprabasal layers that form in raft cultures. Western blot analysis of desmoglein 1 demonstrates that the expression levels increase over time in raft cultures.

3.3. Transient Transfection of siRNA Oligonucleotide Duplexes into Primary Human Epidermal Keratinocytes

For transient gene silencing experiments and identification of candidate target sequences for efficient silencing, siRNA oligonucleotide duplexes are introduced into submerged keratinocyte cultures using conventional lipid-based transfection reagents. For this purpose:

1. Air-dried (20 nmol) of Stealth siRNAs are resuspended in 1000 µl RNase-free H_2O to generate a 20 µM stock solution. Aliquot and store at –70°C. Use a GC content-matched siRNA duplex that has minimal homology to vertebrate mRNA transcript sequences as a negative control and an siRNA duplex that targets lamin A/C as a positive control (*see* **Note 4**).
2. Seed 2×10^5 keratinocytes per well of a 6-well tissue culture-treated plate using complete M154 as described above (*see* **Note 5**).
3. A range of siRNA oligonucleotide concentrations (individual or pooled) should be tested (5–100 nM). To transfect 25 nM of siRNA oligonucleotides into keratinocytes, prepare the following oligo-duplex reaction mixture:
   a. *Tube A*: Add 2.5 µl of 20 µM siRNA oligonucleotide to 200 µl M154 SFM and mix gently.

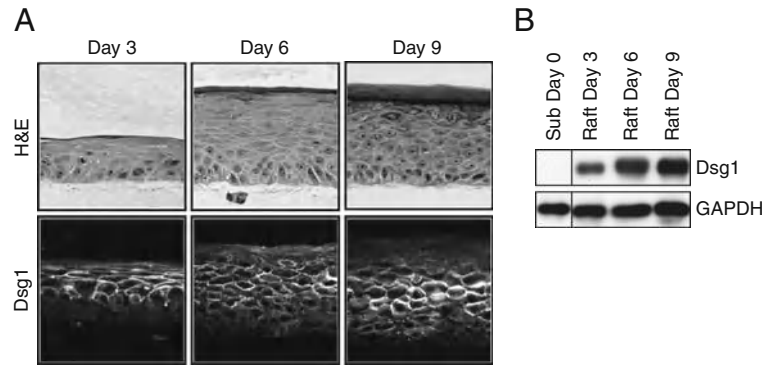

Fig. 10.1. Organotypic raft cultures model epidermal differentiation and morphogenesis. Human epidermal keratinocytes grown at an air–liquid interface form a multi-layered epithelium that differentiates over the course of 1–2 weeks; this mature tissue has the histological and biochemical markers present in normal human epidermis. **(A)** Sections of raft cultures grown for 3, 6, or 9 days were stained with H&E (upper) or immunostained for desmoglein 1 (Dsg1), a marker of keratinocyte differentiation. H&E staining demonstrates stratification by day 3, initial formation of granular and cornified layers by day 6, and fully matured epidermal morphology by day 9. Dsg1 is excluded from the basal layers but is robustly expressed in the suprabasal layers, as seen in vivo. **(B)** Lysates generated from submerged keratinocytes maintained in low calcium express low levels of the differentiation-dependent cadherin, Dsg1; however, this marker is increasingly expressed as raft cultures mature.

b. *Tube B*: Add 4.5 µl of DharmaFECT 1 to 200 µl M154 SFM and mix gently.

   Incubate for 5 min at room temperature.

c. Add contents of tube A to tube B and mix gently.

   Incubate for 20 min at room temperature.

d. Add 1.6 ml complete M154 (without antibiotics or antimycotics).

4. Replace keratinocyte culture medium with transfection reaction mixture.
5. After 4–12 h, replace transfection reaction mixture with fresh M154 growth medium. Harvest cultures for mRNA or protein analysis starting at 72 h following transfection. A representative immunoblot of total protein extracted from keratinocytes transfected with siRNA oligonucleotide duplexes specific for desmoglein 1 is shown in **Fig.** 10.3C (*see* **Note 6**).

3.4. Cloning of EGFP-miRNA into pLZRS for Retrovirus Production

In order to more stably silence gene expression in human epidermal keratinocytes, a retroviral vector was generated to allow for co-cistronic expression of EGFP with a short hairpin sequence mimicking the endogenous microRNA (miRNA) 155 (**Fig.** 10.2). In brief, EGFP cDNA was excised from

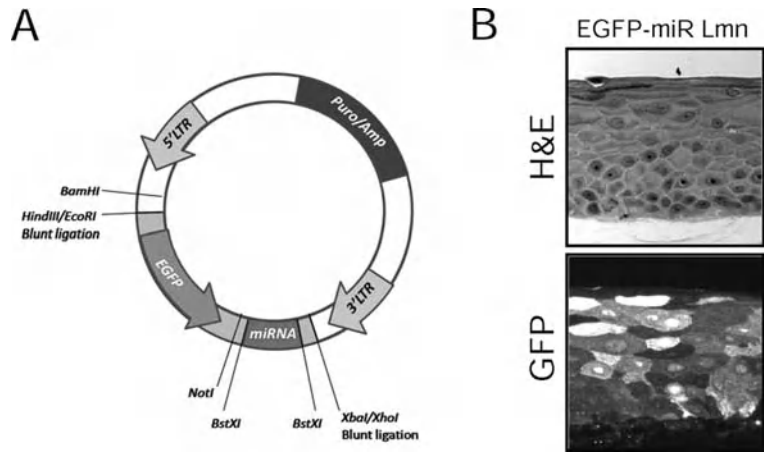

Fig. 10.2. Retroviral expression of EGFP-linked microRNA is present in all viable layers of organotypic cultures. **(A)** In the diagram of pLZRS-EGFP-miRNA, 5′ and 3′ long terminal repeats (LTR) mark the boundaries of the EGFP-miRNA sequence packaged into retroviral particles. The pLZRS vector includes ampicillin (Amp) and puromycin (Puro) resistance genes outside the LTRs, which can be used for episomal maintenance and mass selection of transduced packaging cell lines. The hairpin sequence (miRNA) targeting the gene of choice has been inserted into two *BstXI* sites downstream of EGFP, leading to co-cistronic expression of the fluorescent marker and the microRNA. **(B)** H&E staining of an organotypic raft culture grown from keratinocytes transduced with EGFP-miR Lmn (targeting lamin A/C) demonstrates normal differentiation and morphology. Immunofluorescent staining indicates that retroviral expression of EGFP-miRNA is maintained long term and persists in all viable layers of the organotypic cultures.

pEGFP-N1 (Clontech) using *EcoRI and NotI* and subcloned into pME18S. Two *BstXI* sites were inserted downstream of the 3′ UTR of EGFP to allow for insertion of a short hairpin sequence. This serves as the recipient vector for the duplex oligonucleotide targeting sequence and is termed pMIRAGE (*miR*NA for *A*ltering *G*ene *E*xpression) (12). The EGFP-miRNA fragment can be excised from pMIRAGE and inserted into pLZRS-LINKER (14–16) to generate a retroviral construct encoding a target-specific miRNA. The final construct (pLZRS-EGFP-miRNA) can then be transfected into a retroviral amphotropic packaging cell line for production of high-titer retrovirus useful for transduction of human keratinocytes.

3.4.1. Choosing a miRNA Target Sequence and Ordering Oligonucleotides for miRNA Construction

1. Select a unique 19-nucleotide miRNA target sequence within your gene of interest. This sequence is commonly derived from pre-designed siRNA oligonucleotides determined to be most efficient at transient silencing of gene expression as described above (*see* **Section 3.3**). Alternatively, several online programs are available to help select potential sequences for RNAi-based silencing (e.g., Dharmacon; http://www.dharmacon.com/DesignCenter/DesignCenter

Page.aspx). Note that the target sequences should not include restriction sites for *BamHI* (GGATCC), *EcoRI* (GAATTC), *HindIII* (AAGCTT), *NotI* (GCGGCCGC), or *XbaI* (TCTAGA).

2. Add the two bases adjacent to the 5′ end of the selected 19mer within your gene of interest for a total length of 21 nucleotides. This will serve as a template for miRNA production.
3. Four oligonucleotide primers should be designed using the sequence templates. Two sense strands (SS) and two antisense strands (AS) will be annealed, then fused to form a duplex DNA hairpin sequence. Your target sequence will be placed in the complementary region of the miRNA hairpin structure and *BstXI* overhangs will be added at the ends to allow for vector insertion.

   AS-fwd: 5′-CTTGCTGAAGGCTGTATGCTG-**Sequence 1**-GTTTTGGCCACTGA-3′

   AS-rev: 5′-CCAAAAC-**Sequence 2**-CAGCATACAGCCTT-CAGCAAGTTGC-3′

   SS-fwd: 5′-CTGAC-**Sequence 3**-CAGGACA-CAAGGCCTGTTACTAGCACTTTCG-3′

   SS-rev: 5′-AGTGCTAGTAACAGGCCTTGTGTCCTG-**Sequence 4**-GTCAGTCAGTGG-3′
4. Insert the 21-nucleotide target sequence from your gene of interest as "Sequence 2."

   Example: **5′-TTGGAGTCATGTCCTAAAGTT-3′**

   Derive "Sequence 1" by generating the complementary sequence of "Sequence 2."

   Example: **5′-AACTTTAGGACATGACTCCAA-3′**

   Generate "Sequence 3" by deleting the 9th and 10th nucleotides of "Sequence 1" to arrive at a 19-nucleotide sequence. This mismatch in length is crucial to the appropriate hairpin structure.

   Example: 5′-**TTGGAGTC–GTCCTAAAGTT-3′**

   Derive "Sequence 4" by generating the complementary sequence of "Sequence 3."

   Example: **5′-AACTTTAGGAC–GACTCCAA-3′**
5. Order 25 nmol of the four oligonucleotides (AS-fwd, AS-rev, SS-fwd, SS-rev) purified by standard desalting.

3.4.2. Annealing miRNA Sequence into pMIRAGE Recipient Vector

1. Dissolve each of the four oligonucleotides in 10 mM Tris-HCl, pH 8.0 to a final concentration of 100 μM.
2. Set up two annealing reactions of the oligonucleotide pairs:

a. Add 1 µl SS-fwd + 1 µl SS-rev to 48 µl of annealing buffer.

b. Add 1 µl AS-fwd + 1 µl AS-rev to 48 µl of annealing buffer.

3. Incubate the annealing reactions at 95°C for 4 min, 70°C for 10 min, then cool to room temperature.

4. Set up a single phosphorylation reaction by mixing the following:
   a. 1.0 µl Annealed SS oligonucleotides
   b. 1.0 µl Annealed AS oligonucleotides
   c. 1.0 µl 10 × T4 Polynucleotide Kinase Buffer
   d. 1.0 µl 1 mM ATP
   e. 0.25 µl T4 Polynucleotide Kinase (10,000 U/ml)
   f. 5.75 µl nuclease-free ddH_2O

5. To generate the final duplex miRNA sequence, incubate the phosphorylation reaction at 37°C for 30 min, de-activate the kinase at 70°C for 10 min, then cool to room temperature.

6. Prepare the miRNA recipient vector by digesting 1 µg of pMIRAGE with *BstXI* and purify the DNA using a QIAGEN Mini-Elute Reaction Clean-up Kit (*see* **Note 7**).

7. Set up a ligation reaction by mixing the following:
   a. 4 µl Phosphorylated duplex miRNA sequence
   b. 1 µl *BstXI*-digested pMIRAGE vector
   c. 1 µl 10 × T4 DNA Ligase buffer
   d. 1 µl T4 DNA ligase (400,000 U/ml)
   e. 3 µl nuclease-free ddH_2O

8. Incubate the ligation reaction mixture at 16°C overnight.

9. Transform competent bacteria (e.g., JM109) with 3 µl of the ligation reaction, then spread onto an LB plate containing 50 µg/ml Ampicillin and incubate overnight at 37°C.

10. Select and restreak individual bacterial colonies and grow up a 5 ml liquid culture in LB containing 50 µg/ml Ampicillin.

11. Purify DNA from the bacterial culture using a standard mini-prep protocol or kit.

3.4.3. Subcloning of EGFP-miRNA into pLZRS for Retrovirus Production

1. In separate reaction tubes, digest 1 µg pLZRS-LINKER with *XhoI* and 1 µg pMIRAGE containing your miRNA (pMIRAGE-EGFP-miRNA) with *XbaI* for 2 h.

2. Klenow fill the overhanging end sequences by adding the following to the two separate restriction digest reactions and incubating at room temperature for 30 min:
   a. 1 µl of 10 mM dNTP
   b. 0.5 µl Klenow Fragment (5,000 U/ml)

3. Purify both DNA products using a QIAGEN Mini-Elute Reaction Clean-up Kit (*see* **Note 7**).
4. Carry out a second digest (1 μg DNA in separate reactions) of the Klenow-filled pLZRS-LINKER with *HindIII* and pMIRAGE-EGFP-miRNA with *EcoRI*.
5. Again, Klenow fill the overhanging end sequences by adding the following to the two separate restriction digest reactions and incubating at room temperature for 30 min:
   a. 1 μl of 10 mM dNTP
   b. 0.5 μl Klenow Fragment (5,000 U/ml)
6. Treat only pLZRS-LINKER digest with 1 μl CIP for 30 minutes at 37°C then heat to 70°C for 20 minutes to partially heat-inactivate the enzyme (*see* **Note 8**).
7. Purify linearized pLZRS-LINKER (~11 kB) and EGFP-miRNA insert from pMIRAGE (~850 bp) on a 0.5% low melting point agarose gel.
8. Prepare an in-gel ligation by
   a. Melting agarose containing the vector and insert at 70°C for 10 min.
   b. Diluting the melted gel bands 1:1 (vol:vol) with nuclease-free ddH_2O.
   c. Mixing 30 μl of insert with 10 μl of vector and allowing to cool for 1 min.
   d. Adding 3 μl nuclease-free ddH_2O and 5 μl 10 × T4 DNA ligase buffer.
   e. Adding 2 μl T4 DNA ligase.

   Incubate the ligation reaction at 16°C overnight.
9. The next day, heat the ligation reaction to 50°C for 5 min to re-liquefy the agarose.
10. Transform competent bacteria (e.g., JM109) with the entire 50 μl of the in-gel ligation reaction by pipetting up and down vigorously. The gel will re-solidify after placing the bacteria back on ice, but the DNA should remain in solution.
11. Select and restreak an isolated bacterial colony and grow up a 5 ml liquid culture in LB containing 50 μg/ml Ampicillin.
12. Digest 1 μg with *BamHI* and *NotI*, then run a sample out on a 1% agarose gel to verify fragments of ~11.5 kb (pLZRS + miRNA) and ~800 bp (EGFP). Perform DNA sequence analysis to confirm the orientation and integrity of the insert.
13. The next day, purify DNA from the bacterial culture using a standard mini-prep protocol. Use this construct (pLZRS-EGFP-miRNA) to transfect the Phoenix retroviral packaging line as detailed later.

3.5. Retroviral Supernatant Production and Transduction in Human Epidermal Keratinocytes

Production of replication-deficient retroviruses for infecting human keratinocytes is achieved by transfection of Phoenix amphotropic packaging lines with the retroviral plasmid pLZRS-EGFP-miRNA. Retroviral supernatants from transfected cells can be harvested and used to stably transduce human keratinocytes with an efficiency of greater than 90%. A plasmid (pLZRS-EGFP-miRNA) containing hairpin sequences that target the nuclear filament protein, lamin A/C, is used as a positive control for gene silencing and does not grossly affect the morphology of organotypic raft cultures (**Fig.** 10.2). The epifluorescent signal from transduced keratinocytes can be employed for visual confirmation of EGFP-miRNA-positive cells and allows for flow cytometric sorting of subpopulations of cells expressing miRNAs.

3.5.1. Production of Retrovirus from Phoenix Amphotropic Packaging Lines

1. Seed 1×10^6 Phoenix cells per 60 mm tissue culture-treated dish so that cultures are approximately 75% confluent the next day. Phoenix cells attach very lightly to tissue culture dishes. Therefore, all pipetting should be done against the side of the dish and not directly onto the cells to prevent detachment. For subculturing, the cells can be readily detached from the surface of a culture dish after a 30 sec incubation in 0.05% trypsin/EDTA.
2. Replace the growth medium (4 ml) and prepare the following transfection reaction mixture (*see* **Note 9)** after all reagents have been brought to room temperature:
   a. *Tube A*: Dilute 8 μg of LZRS-EGFP-miRNA DNA in 400 μl Opti-MEM.
   b. *Tube B:* Dilute 20 μl Lipofectamine 2000 in 400 μl Opti-MEM and mix gently.

      Incubate for 5 min at room temperature.
   c. Add contents of Tube A to Tube B and mix gently.

      Incubate for 20 min at room temperature.
3. Add the transfection reaction mixture to Phoenix cell cultures in a drop-wise fashion and incubate the cells overnight at 37°C. Replacing the growth medium after 8–10 h can limit cell toxicity associated with lipid-based transfection reagents.
4. Discard the transfection medium in a 10% bleach solution and add fresh growth medium. Phoenix cells transfected with pLZRS should be treated as potentially hazardous at this stage since these replication-deficient viral particles have the potential to infect human cells. All medium and tissue culture supplies in contact with the transfected cells

should be immediately treated with 10% bleach and discarded using appropriate BSL2 measures for biohazardous waste disposal.

5. Using an inverted microscope equipped for fluorescence imaging, confirm the presence of EGFP-positive cells 24 h post-transfection. Expand transfected Phoenix cells into two 100 mm tissue culture-treated plates.
6. Retroviral supernatants can be harvested 72 h post-transfection. Phoenix cells containing high copy numbers of pLZRS-EGFP-miRNA can also be drug-selected as described below.
7. About 72 h post-transfection, replace cultures with growth medium containing 1 μg/ml puromycin (*see* **Note 10).**
8. Expand Phoenix cells in selection medium upon achieving 80% confluency. A single 100 mm dish of cells can be expanded into five 100 mm dishes. From these
   a. cryopreserve cells from two dishes in 90% FBS containing 10% DMSO;
   b. expand one dish for further propagation as described above;
   c. harvest retroviral supernatants from two dishes as described later.

3.5.2. Harvesting Retroviral Supernatants from Drug-Selected Phoenix Cell Lines

1. To harvest retroviral supernatants, first grow mass-selected Phoenix cell lines to 80% confluency.
2. Discard the selection medium, wash the cells once with PBS to remove trace amounts of puromycin, then re-feed with 5 ml of growth medium per 100 mm dish (*see* **Note 11).**
3. Incubate the cultures at 32°C for 24 h to accumulate viral particles in the supernatant.
4. Carefully remove the growth medium without disturbing the Phoenix cells and pool the supernatants into a sterile 50 ml conical tube.
5. Spin the supernatants at 500 × g for 5 minutes to pellet any Phoenix cells and/or debris. Supernatants can be used for infection immediately after spinning, stored at 4°C for 24 h, or snap-frozen in a dry-ice/ethanol bath and stored for longer time periods (up to 1 year) at –70°C (*see* **Notes 12 and 13).**
6. After the initial harvest, the same Phoenix cells can be used for a second round of retroviral production by adding new growth medium to the cells and again incubating overnight at 32°C.

3.5.3. Transduction of Keratinocytes with Phoenix-Derived Retroviral Particles

1. Seed 5×10^5 keratinocytes per 100 mm culture dish in complete M154. Cells must be sub-confluent at the time of infection since the retrovirus requires cell division for stable incorporation into the target cell genome.
2. The next day, pre-incubate keratinocytes in M154 containing 4 μg/ml polybrene for 5 min at 32°C.
3. Prepare a retroviral infection cocktail (*see* **Note 14**) by adding
   a. 2.5 ml complete M154 containing 8 μg/ml polybrene.
   b. 2.5 ml fresh retroviral supernatant.
4. Remove the medium from the target keratinocytes and replace with 5 ml of the retroviral infection cocktail.
5. Incubate the cells at 32°C undisturbed for 90 min to allow for infection (*see* **Note 15**).
6. Discard the retroviral cocktail in a 10% bleach solution and wash the infected cells twice with PBS, discarding the washes into bleach.

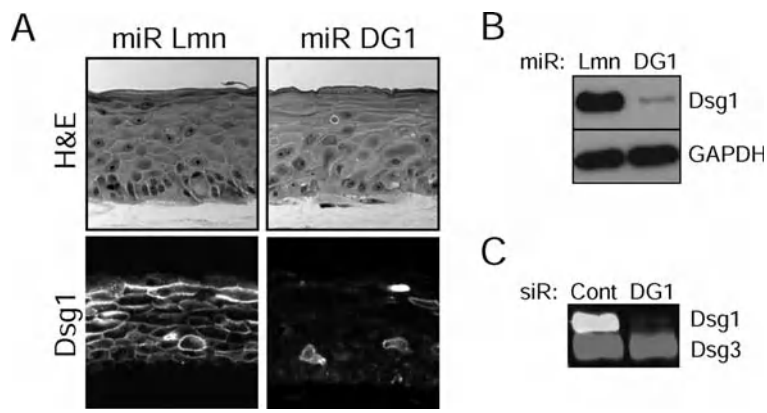

Fig. 10.3. Efficient gene silencing in organotypic cultures is achieved by introducing miRNAs or siRNAs into keratinocytes. The differentiation-dependent expression of Dsg1 was silenced in keratinocytes grown as raft cultures by retroviral expression of a miRNA or by transfection of siRNA oligonucleotides. **(A)** Keratinocytes were transduced with a miRNA targeting Dsg1 (miR DG1) or lamin A/C (miR Lmn) as a control and grown at an air–liquid interface for 6 days. Immunostaining indicates that while Dsg1 is present in control cultures, its levels are drastically reduced in cells expressing the Dsg1-specific miRNA. **(B)** Lysates from day 6 cultures expressing miR Lmn or miR DG1 were immunoblotted for Dsg1. Transduction with EGFP-miR DG1 leads to >90% reduction in the levels of Dsg1. GAPDH serves as a loading control. **(C)** Likewise, transfection of keratinocytes with siRNA oligonucleotides resulted in effective gene silencing. While cells transfected with control siRNA (siR Cont) expressed Dsg1, those transfected with a Dsg1-specific siRNA pool (siR DG1) showed greatly reduced Dsg1 levels. Expression of Dsg3, a related desmosomal cadherin, serves as a loading control. Its expression was not altered by siR DG1, indicating that siRNA-mediated silencing was specific for Dsg1.

7. Re-feed the target cells with keratinocyte medium and culture at 37°C. Keratinocytes will begin expressing EGFP-miRNA within 24 h and can be expanded or frozen for future use.
8. For enhanced knockdown in organotypic raft cultures, two sequential infections are employed with fresh supernatants of retrovirus encoding miRNA-based sequences. Expand keratinocytes following initial infection and perform a second infection as described above. A representative example of organotypic raft cultures established from keratinocytes transduced with miRNAs specific for lamin A/C or desmoglein 1 is shown in **Fig.** 10.3. Only a small percentage of cells retain the ability to express desmoglein 1 following the introduction of a miRNA targeting this desmosomal cadherin compared to lamin A/C controls. Western blot analysis demonstrating efficient knockdown of desmoglein 1 in these raft cultures is also shown.

4. Notes

1. We do not suggest pooling foreskins from different individuals for keratinocyte isolations and instead assign a specific clone I.D. # for each isolate. Whenever possible, three separate clones per experiment are utilized to better account for genetic differences of donor tissue. Several commercial sources exist for human epidermal keratinocytes isolated from neonatal foreskins. Other than M154, a variety of reduced calcium, FBS-free culture medium formulations based on the original formulation described in Boyce and Ham (17) are also available.
2. Primary human dermal fibroblasts can also be used to populate the collagen lattices for raft cultures. In other organotypic models of human epidermis, these fibroblasts are used to contract the collagen plug over the course of a week. Collagen contraction is not necessary for this raft model of human epidermis.
3. A single raft can be dissected and epidermal pieces can be processed for total RNA extraction (Tri Reagent; Molecular Research Center), total protein extraction (8 M Urea in SDS Sample Buffer), co-immunoprecipitations (RIPA buffer), histological analysis (fixed in 4% paraformaldehyde for 60 min and embedded in paraffin), frozen sections (embedded and frozen in OCT compound), or ultrastructural analysis (fixed in 2.5% glutaraldehyde in phosphate buffer supplemented with 1 mM $CaCl_2$).

4. Select three distinct target sequences for each gene of interest in order to optimize the probability of silencing using individual or pooled siRNA oligonucleotides. Unique 3′UTR of specific genes serves as an experimentally convenient target for RNA interference and facilitates the re-introduction of the wild-type cDNA in cells in order to control for potential off-target effects of siRNA oligonucleotides. Alternatively, two silent point mutations that correspond to the siRNA oligonucleotide target region can be introduced in the cDNA sequence of the gene being studied in order to render a "rescue" construct resistant to silencing. If the siRNA oligonucleotide target region is not perfectly conserved among closely related species (e.g., mouse), it might also be possible to express the wild-type homologous cDNA in order to restore expression of the target protein.
5. For certain applications (i.e., calcium-induced differentiation of submerged cultures or raft cultures), it may be necessary to seed keratinocytes to confluency. In this case, perform transfections at the time of seeding of 1×10^6 keratinocytes per well of a 6-well plate. In general one must increase the concentration of siRNA oligonucleotide two-fold and volume of transfection reagent 1.25-fold under these conditions although this will need to be optimized for each target sequence. The transfection mixture can be used to resuspend and simultaneously seed keratinocytes under these conditions.
6. Depending on the half-life of the protein of interest, longer time periods following siRNA oligo-duplex transfection might be required in order to observe efficient knockdown of the gene of interest.
7. Standard ethanol precipitation techniques can be used in place of a DNA clean-up kit. For this purpose
   a. Add 2.5–3 volumes of 95% ethanol/0.12 M sodium acetate to the DNA sample.
   b. Incubate at –70°C for >1 h.
   c. Spin tubes at >12,000 rpm for 15 min at 4°C.
   d. Wash pellet in 500 µl 70% ethanol and spin at >12,000 rpm for 5 min.
   e. Resuspend pellet in 20 µl 10 mM Tris-HCl, pH 8.0, or nuclease-free ddH_2O.
8. It is not possible to completely heat-inactivate CIP. As an alternative, Antarctic Alkaline Phosphatase (NEB) can be used to remove 5′ phosphate groups from pLZRS-LINKER in place of CIP.

9. Calcium phosphate-based transfection procedures can also be used to introduce pLZRS into Phoenix packaging lines at relatively high efficiency.
10. Although 1 μg/ml puromycin serves as a good starting point for drug selection of Phoenix cells transfected with pLZRS-EGFP-miRNA, a range of concentrations (0.5–2 μg/ml) may need to be tested for each construct.
11. If retroviral supernatants are to be used for infecting primary human keratinocytes, Phoenix cells can be temporarily grown in the harvest phase at 32°C in complete M154. This reduces exposure of target primary cells during the infection to excess serum and calcium contained in Phoenix growth medium.
12. As an alternative to centrifugation, one can pass the retroviral supernatant through a 0.45 μm sterile filter to remove contaminating Phoenix cells and debris.
13. Retroviral supernatants can be concentrated using an Amicon concentration spin column (Millipore) with a 30 kDa molecular weight cut-off. The volume of supernatants can be reduced from 10 ml to approximately 0.2 ml by centrifugation at 5,000 × g for 10 min at 4°C.
14. Frozen, concentrated viral stocks can also be used to prepare an infection cocktail. In this case, dilute 0.1 ml of frozen viral supernatant that has been rapidly thawed at 37°C into 5 ml complete M154 containing 4 μg/ml polybrene. Take into account that there is a reduction in retroviral titers for each freeze-thaw cycle.
15. For higher infection efficiency, target cells can be centrifuged at 300 × g for 60 min at 32°C during the infection period; this is often referred to as a spin infection.

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Chapter 11

Scanning for Transcription Factor Binding by a Variant EMSA

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Abstract

Detection of in vitro protein–DNA interaction is one of many investigational analyses for transcription factor regulation of gene promoters. The electrophoretic mobility shift assay (EMSA) has proven widely popular in this respect by integrating individual techniques (protein isolation, nucleic acid radiolabeling, and gel electrophoresis) into one protocol. However, relatively short DNA oligomers are often used which in many cases presupposes what one sequence out of a promoter of possibly thousands of base pairs is the candidate region interacting with a transcription factor. This can be an experimentally distressing situation when multiple putative binding sites of less than perfect consensus may be present making selection of any one or even a few potential sites uncertain or when one is seeking improved throughput as opposed to a one factor:one oligomer approach for in vitro testing of algorithm-predicted binding sites. We describe here our use and refinement of a variant EMSA that can employ multiple and relatively long (up to 1000 bp) probes of promoter sequence in one binding reaction for interaction with nuclear proteins in general and individual transcription factors in particular. We provide labeling and electrophoresis methods suitable for such probes and also highlight the mobility shift differences one can expect with the variant probe method.

Key words: Electrophoretic mobility shift assay, EMSA, Transcription factor, Gene promoter, DNA binding.

1. Introduction

A number of electrophoretic mobility shift assay (EMSA) variations have been described for detection of macromolecular interactions. While many versions of component combinations exist, e.g., DNA–RNA, RNA–protein, protein–protein, the most common involves protein–DNA complexes where a short, double-stranded DNA oligomer is radiolabeled and mixed with known or potential DNA-binding proteins derived from native or recombinant sources. Complexes are visualized

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as slower migrating complexes resolved from free, individual components following resolution by gel electrophoresis. Identification of what proteins have entered into complexes with the radiolabeled probe DNA can be queried by inclusion of antibody against the protein of interest; the interaction of the antibody–antigen probe further retards the complex migration generating a "super-shifted" band species. Specificity of the probe DNA–protein interaction is assessed by quantitative and qualitative challenge with unlabelled competitor DNA via titrations of self-sequence DNA and unrelated or mutated-sequence DNA. While this common approach is of great utility in testing potential protein–DNA interactions it is severely limited in the extent of the DNA length typically examined at any given time. For instance, promoters known to be functionally responsive to certain transcription factors may have multiple, low consensus match predicted binding sites for that given factor. This necessitates numerous trial and error oligomers being generated to "walk" through the promoter to find sequences physically interacting with the transcription factor. Longer and/or multiple fragments in the hundreds of base pairs, such as those easily generated from restriction enzyme digestion of cloned promoter sequence, would provide a more comprehensive target to survey numerous sites simultaneously. Nevertheless, use of longer or multiple probes within one reaction calls for some adaptation of the binding protocol, electrophoresis conditions, and band pattern interpretation (1). In this protocol, we provide labeling and electrophoresis methods suitable for such protein–DNA interactions. Also, we highlight the mobility shift differences one can expect with the variant probe method especially noting the differences in band patterns from when short oligomers are used.

2. Materials

2.1. Cell Culture and Transfection

1. Cell line: COS-7 (American Type Culture Collection, Manassas, VA; cat. no. CRL-1651).
2. 1 × PBS: 4.31 mM Na_2HPO_4 (S5136), 1.47 mM KH_2PO_4 (P5655), 2.68 mM KCl (P5405), 137 mM NaCl (S5886, all Sigma catalogue numbers). Prepare with pyrogen-free Nanopure-type water, aliquot and autoclave on liquid cycle for 25 min.
3. Media: DMEM:F12 (Invitrogen, cat. nos. 12100-046 and 21700-075 combined at 3:1), supplemented with 3.07 g/L $NaHCO_3$(Sigma, cat. no. S5761) and adjusted to final pH of

7.1 with HCl. Complete media to final concentrations of 5% FBS (Invitrogen, cat. no. 10437) and 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen, cat. no. 15140-122).

4. Trypsin/EDTA: 0.1% trypsin, 0.1% glucose, 0.02% EDTA with 100 U/mL penicillin, 100 µg/mL streptomycin in PBS.
5. 2 × HBS – HEPES-buffered saline: 50 mM *N*-(2-hydroxyethyl) piperazine-Ń-2-ethanesulfonic acid (H4034), 280 mM NaCl (S5886), 1.5 mM Na_2HPO_4 (S5136, all Sigma catalogue numbers). Dissolve in Nanopure-type water and adjust final solution to pH 7.10 (*see* **Note 1**). Filter sterilize and aliquot as needed for typical experiments, usually 5–10 mL. Store at –20°C. Mix thoroughly after thawing.
6. 5 × $CaCl_2$: 1.25 M calcium chloride dihydrate (Sigma, cat. no. C7902). Dissolve, filter sterilize, and aliquot as needed for typical experiments, usually 1–2 mL. Store at RT.

2.2. Nuclear Extract Preparation

1. **Buffer A:** 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl. Prepare, filter sterilize, and store at 4°C.
2. **Buffer C:** 20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA. Prepare, filter sterilize, and store at 4°C. Before use add protease inhibitors; add DTT to 0.5 mM.
3. **Buffer D:** 20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA. Prepare, filter sterilize, and store at 4°C. Before use add protease inhibitors; add DTT to 0.5 mM.
4. Micro-dialysis units (Slide-a-lyzer Thermo Scientific/Pierce Biotechnology, Rockford, IL, cat. no. 69574) or self-fabricated (2) dialysis tubes (*see* **Note 2**).
5. Protein assay reagents, e.g., Bradford (Bio-Rad, cat. no. 500-0006).

2.3. Probe DNA Preparation and Labeling

1. Plasmids and restriction fragments: HiSpeed Plasmid Midi Kit (Qiagen, cat. no. 12643), fragment purification following electrophoresis QIAquick Gel Extraction Kit (Qiagen, cat. no. 28704).
2. Probes: T4 DNA Polymerase (Promega, cat. no. M4211, 7.9 U/µL), post-labeling purification, QIAquick PCR Purification Kit (Qiagen, cat. no. 28104). ^{32}P-α-dATP (GE Healthcare, cat. no. AA0074, ~6,000 Ci/mM).
3. Optional: double-stranded oligomers for binding competition annealed in 100 mM Tris-Cl, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$.

2.4. Binding Reaction and EMSA Reagents

1. 2 × Binding Buffer: 20 mM HEPES, pH 7.9, 12% glycerol, 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA. Before use add DTT to 2 mM.
2. Binding reaction additives: Poly[d(I-C)] (Roche, cat. no. 10108812001) to bottle of 10_{A260} units add 500 µL sterile, nuclease-free H_2O. BSA (NEB, cat. no. B9001S). IGEPAL CA-630 (Sigma, cat. no. I-8896).
3. Polyacrylamide gel: Acrylamide/Bis (ratio 37.5:1.0) (Bio-Rad, cat. nos. 161-0101 and 161-0201), 10% APS (Bio-Rad, cat. no. 161-0700), TEMED (Bio-Rad, cat. no. 161-0800).
4. Running buffer (0.5 × TBE): 44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0.
5. Autoradiograph film: Hyperfilm (GE Healthcare, cat. no. RPN6L).

3. Methods

3.1. Cultured Cell Growth and Optional Transfection

1. Culture five 100 mm plates of cells for nuclear extract preparation. Recombinant proteins may be derived from calcium phosphate transfection of COS-7 cells (*see* **Note 3**) in the following steps.
2. Seed cells ~16 prior to transfection with 8×10^5 cells per 100 mm plate.
3. For each 100 mm plate, add 500 µL 2 × HBS to a Falcon 2059 tube (*see* **Note 4**), scaling up or preparing multiple transfection mixes as needed, set aside.
4. In an autoclaved 1.5 mL microfuge tube combine 10 µg plasmid DNA(s) (*see* **Note 5**) and sterile water to a total of 400 µL. Tap to mix and add 100 µL 1.25 M $CaCl_2$. Vortex on medium 3–5 sec and centrifuge briefly (5–10 sec) to bring all liquid to the bottom of tube.
5. Add DNA/water/$CaCl_2$ mixture drop-wise into 2 × HBS while shaking 2059 tube (*see* **Note 6**). Allow the precipitate to form for 20 min (*see* **Note 7**).
6. Before adding precipitate to cells, vortex the 2059 tube on medium speed for 2–5 sec to resuspend. Add precipitate drop-wise across the entire surface of plate. Swirl each plate two to three times in a figure 8 motion. Incubate plates overnight.
7. Sixteen hours post-transfection, aspirate off precipitate-containing media, rinse twice with room temperature PBS, and refeed cells with media supplemented with serum and/or growth factors as per experimental conditions.

8. Approximately 36 h after the media change in step 7, harvest cells as required for nuclear extract preparation (*see* **Note 8**).

3.2. Nuclear Extract Preparation

1. Chill all solutions, pipettes, tips, tubes, etc., to 4°C prior to starting nuclear extraction.
2. Record relevant culture information (*see* **Note 9**). Rinse cultures twice with cold PBS and scrape (*see* **Note 10**) into 15 mL conical centrifuge tube; pellet for 5 min at 4°C, 250*g*.
3. Resuspend cell pellet in 1 mL cold PBS, transfer to a microfuge tube and pellet for 2 min at 4°C, 16,000*g*. Remove supernatant, estimate and record packed cell volume (PCV) (*see* **Note 11**).
4. Resuspend pellet thoroughly in one PCV of cold Buffer A; swell cells on ice for 15 min. Keep cells suspended by tapping tube every 5 min.
5. Using cold Buffer A, pre-wet a chilled 1 mL syringe fitted with a 23-gauge needle. Slowly draw cell suspension into syringe. Quickly eject in one stroke. Do not make bubbles. Repeat four times (*see* **Note 12**).
6. Pellet in microfuge at top speed at 4°C for 5 min. Draw off supernatant as cytoplasmic fraction, discard. Resuspend pellet in two-thirds PCV Buffer C. Be sure there are no clumps.
7. Rotate, 30 min at 4°C, on end-over-end tube rotator such as LabQuake; be sure material remains evenly mixed during extraction.
8. Pellet nuclear debris for 5 min at 4°C top speed in microfuge. Retrieve supernatant, transfer to micro-dialysis unit, and dialyze (*see* **Note 2**) against Buffer D for 2 h at 4°C (*see* **Note 13**). After dialysis use 2 µl for Bradford assay. Be sure protein standards are adjusted with an equal volume of Buffer D.
9. Prepare the remaining volume as 25–50 µL aliquots and quick-freeze in liquid N_2. Store at −80°C. Avoid repeated freeze/thaws (*see* **Note 14**).

3.3. Probe DNA Preparation, Labeling, and Quantitation

1. Digest plasmid DNA (*see* **Note 15**) with appropriate restriction enzyme(s) to generate fragment(s) encompassing the candidate transcription factor binding sites and possessing at least one 5′ overhang (*see* **Note 16**) (3).
2. Purify desired DNA fragment(s) using agarose gel electrophoresis and QIAquick Gel Extraction kit.

3. Use approximately 0.2 μg DNA per fragment for T4 DNA polymerase labeling: 4μL T4 DNA polymerase 10 × reaction buffer, 2 μL 1 mM dCTP, 2 μL 1 mM dGTP, 2 μL 1 mM dTTP, 1 μL T4 DNA polymerase, 5 μL ^{32}P-α-dATP (50 μCi), plus nuclease-free water to a final 40 μL volume (*see* **Note 17**).
4. Incubate at 37°C for 30 min. Save 1 μL of this as representing CPM/μL of reaction; dilute it 10-fold in any buffer or water. Spot 1 μL on a small strip of Whatman 3MM filter paper cut to fit small scintillation vials. Prepare a blank strip.
5. Adjust remaining volume to 240 μL with PBI binding buffer, load onto QIAquick PCR column, wash and elute in 50 μL. Spot 1 μL of eluate on a small strip of filter paper.
6. Place strips into scintillation vials, do not add scintillation cocktail. Count on tritium channel. The CPMs determined are from the ^{32}P decay and are referred to as Cerenkov counts.
7. Calculate total reaction CPM as 1 μL spot from step 4 CPMs × 10 (for dilution) × μL of reaction; incorporated probe CPM as 1 μL spot CPM × μL used for post-labeling eluate; labeling efficiency = incorporated/total × 100%. Typical incorporations will range depending on the moles of recessed 3′ ends available for fill-in reaction to occur.
8. From incorporated CPMs determine volume required for 25,000–50,000 CPM (*see* **Note 18**). Dilution may be required to get easily measured volumes. Use elution buffer for dilution. Store probes at −20°C with appropriate shielding.

3.4. Binding Reaction

1. Label one microfuge tube for free probe and one for each binding combination to be tested and set on ice.
2. Prepare a Master Mix of 10 μL chilled 2 × Binding Buffer, 4 μL poly[d(I-C)], 0.5 μL 10 μg/μL BSA, 1 μL 10% IGEPAL, and 25,000–50,000 CPM-labeled probe, titrated (4) as necessary (*see* **Note 19**) for the number of total binding reactions plus two (one for free probe, one for pipetting allowance). Keep on ice.
3. Assemble individual binding reactions. Component order and handling for the example EMSA (**Fig. 11.1**) were Master Mix, nuclear extract, Buffer D (to standardize variable volumes of nuclear extract), cold competitor (and water as necessary to bring final reaction to 1 × Binding Buffer); mixed to distribute components; addition of labeled probe (*see* **Note 20**).

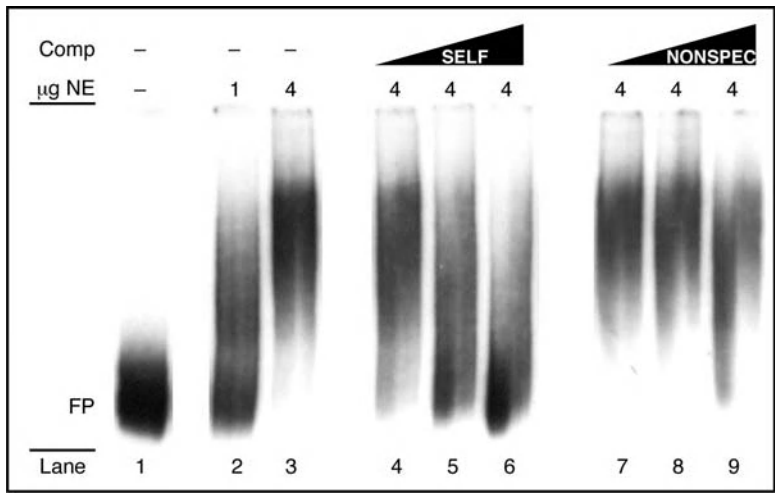

Fig. 11.1. Variant EMSA. A 702 bp example fragment containing three repeats of a peroxisome proliferator response element (PPRE) and surrounding plasmid sequences derived from pGL4 was used as probe. Triangles across top of lanes 4–6 and 7–8 represent an approximate 10-fold increase in the amount of competitor used, either unlabeled 702 bp fragment or a fragment derived from the pGL4 plasmid without the PPRE repeats. NE, nuclear extract protein. Comp, competitor. FP, free probe.

4. Tap gently to mix and incubate at RT for 20 min (*see* **Note 21**).
5. Return binding reactions to ice for 5 min.
6. Load binding reaction on gel; do not add bromophenol blue (5) to binding reactions (*see* **Note 22**).

3.5. EMSA Gel Casting, Pre-run, Run, and Gel Exposure

1. Combine gel components for a final concentration of 4% acrylamide/bis, 0.5 × TBE, 5% glycerol, 0.1% ammonium persulfate, and 0.1% TEMED; cast and allow to polymerize (*see* **Note 23**) at least 1 h before pre-running. Dilute running buffer from stock and chill to 4°C.
2. Assemble gel on electrophoresis box and mark position of wells. Remove comb and using syringe with fine gauge needle, flush out each well briefly. Pre-run at 100 V for 90 min to 2 h at 4°C (*see* **Note 24**).
3. Stop the pre-run and disconnect the gel apparatus from the power supply. Using syringe and fine gauge needle, rinse out wells (*see* **Note 25**).
4. Load gel wells with incubation mixture. Fill empty wells with 1 × Binding Buffer. Start the run immediately for 5 min at 20 mA to limit diffusion or loss of complexes (*see* **Note 26**).
5. Reduce current to 5 mA for a short overnight (~14 h) run at 4°C (*see* **Note 27**).
6. Remove gel from apparatus and carefully separate plates. Keeping track of gel orientation, transfer gel to Whatman 3MM paper.

7. Cover gel surface with plastic wrap film, avoiding wrinkling as much as possible. Dry under vacuum for approximately 60 min at 80°C.
8. Expose dried gels to film with one intensifying screen. Start with an overnight exposure at −80°C.

3.6. Interpretation of Binding Pattern(s)

Legitimate protein–DNA interactions in the variant EMSA do not necessarily produce individual, resolved bands as with short oligomer probe EMSAs (6). Instead, loss of intensity from free probe band(s) (**Fig. 11.1**, lane 1) is indicative of protein binding (lane 2) to the probe(s). As the DNA fragments under consideration may have sites for binding multiple proteins, and especially if multiple probes are used, it is more likely that a smear of probe signal will occur as opposed to a discreet band. Our sample variant EMSA used a 702 bp DNA fragment prepared by a restriction digest of a reporter plasmid and COS-7 cell nuclear extract enriched for PPARγ by transfection of an expression vector for human PPARγ cDNA. The DNA fragment contains three repeats of a peroxisome proliferator-activated receptor-binding site, bounded by plasmid backbone with no known eukaryotic protein binding sites.

Molecular weight (e.g., 433 kDa for the example 702 bp fragment) of the variant EMSA probe(s) becomes more of a determining factor in migration compared to a short probe (e.g., 15 kDa for 25 bp oligomer) because of their increased length. Thus, a definitive supershift from addition of antibody targeting one of the candidate binding proteins may not be evident even if that protein is accounting for some of the change in probe mobility. Alternatively, it may form a complex too large to enter the gel matrix.

The relevance of any change in probe migration is facilitated by a titration of nuclear extract (7). As more protein with relevant binding factors is added, the probe tends to be retarded into larger, slower but not necessarily well-resolved species (lane 3). Sufficient amounts of protein should be titrated into the binding reaction so as to shift all of the signal from the free probe position (lane 3); however, excess nuclear extract protein can result in most of the probe signal being trapped at the well's edge. In this case, the number of CPM should instead be decreased.

Specificity of the change in probe mobility in the presence of nuclear extract protein can still be addressed by competition with a titration of unlabeled probe (lanes 4–6) versus DNA fragments without relevant binding sites (lanes 7–9). Across increasing amounts of competitor, even the lowest amount (lane 4) of self-competitor (unlabeled 702 bp fragment) caused a redistribution of signal, much of which is returned to the position of the free probe by the highest amount of self-competitor (lane 6). The highest amount (lane 9) of non-specific competitor had a minimal effect

on bound-probe migration and may reflect that excess non-specific competitor can disrupt even specific protein–DNA complexes. Nevertheless, lanes 4–9 illustrate the value of including a range of competitor(s) in determining specificity in the variant EMSA.

4. Notes

1. Final pH is critical; assess with a reliably calibrated meter. In order to confirm batch-to-batch consistency of 2 × HBS it is important to use a new batch alongside the previous batch in a transfection with a reporter plasmid of choice, e.g., β-galactosidase. If both batches give similar results in a reporter assay, the new batch can be released for use.
2. Microfuge tube dialysis unit. Heat large end of a Pasteur pipette with a Bunsen burner and use it to melt out a hole in central surface of a flat cap microfuge tube. Prepare one tube for each nuclear extract. Transfer nuclear extract to bottom of pierced-cap tube and cover mouth of tube with a small square of dialysis membrane (approx. 2 × 2 cm). Gently close tube so that lip of cap holds membrane in place at mouth of tube. Invert with a sharp snap to deliver nuclear extract against membrane. Insert tube in a floating rack from underside of rack and float on dialysis buffer. Be sure no air bubbles are trapped between outside surface of the membrane and dialysis buffer.
3. Depending on the transcription factor of interest there may be sufficient amounts present in the cells of choice under native conditions. Otherwise, mammalian expression constructs may be transfected to enrich for the particular factor and facilitate its downstream detection. We have used SV40 origin-based constructs to take advantage of their replication in T-antigen-expressing cells, e.g., COS-7, thus increasing the ultimate expression of the construct transgene.
4. Round-bottomed tubes facilitate better reagent mixing.
5. Total DNA amount may be increased to 20 μg per 100 mm plate with the addition of carrier DNA or additional expression construct. We have not found significant increases in the production of recombinant proteins with expression vector in excess of 10 μg per 100 mm plate.
6. It should take about 45 sec to drip 0.5 mL of DNA/water/$CaCl_2$ mixture into the 2 × HBS. If multiple mixtures are being made, mark the time the addition was completed on the 2059 tube to assure all tubes have a consistent precipitation time.

7. The precipitate should be very faint and white. If coarse, readily visible precipitate is formed the transfection efficiency will be lower; consider discarding the tube and starting over.
8. Optimal time post-transfection for maximum yield of recombinant protein will need to be empirically determined.
9. The degree of confluence may affect expression level of the nuclear protein of interest. For an uncharacterized protein a pilot study at 50%, 100% and 2d post-confluent may be advisable.
10. For cells with strong cell–cell adherence, trypsinization may be required to disaggregate cell sheets for efficient downstream extraction. Be sure to remove all trypsin residues by serum neutralization and re-pelleting.
11. For pellet volume estimation, mark a dummy tube that holds a measured 250 μL and 500 μL as a visual reference.
12. Additional repeats may be necessary for dense cell suspensions or resistant cell sheets. If uncertain of cell lysis, examine pre- and post-lysis samples on hemacytometer.
13. Dialysis buffer volume of 200–250 mL is more than enough and is a convenient volume for handling micro-dialysis units.
14. Immunoblot analysis for specific DNA-binding proteins may be useful if changes in amounts of these proteins from untransfected versus transfected or control versus treated cells is considered important to degree of mobility shift.
15. Plasmid DNA derived from *E. coli* (Dam^-/Dcm^-) stains can extend the range of restriction enzymes used for promoter digestion.
16. Fill-in labeling as opposed to kinase end-labeling offers the potential advantage of protecting the radiolabel from phosphatases present in nuclear extracts. Fragments with one blunt end may be used but will have lower labeling activity.
17. Radioactive samples and wastes should be handled as per institutional policies.
18. The amount of probe will have to be determined for each experimental combination. Unlike short-probe EMSAs, probe is usually in limiting amounts so that distinct changes in intensity of free probe band(s) can be used as an indication of successful protein–DNA binding.
19. Component amounts, nuclear extract protein, poly[d(I-C)], competitor, etc., timing of their addition, and total binding reaction incubation time must be individually optimized by titration for various transcription factors and target binding sites.

20. The order of addition of reagents to the binding reaction is important. Usually, to maximize its effectiveness, the competitor DNA must be added to the reaction along with the extract prior to the labeled DNA target, thus favoring protein binding to unlabeled probe (as opposed to direct competition with the labeled probe during the binding reaction). A 200-fold molar excess of unlabeled target is usually sufficient to outcompete any specific interactions.
21. For supershifting, add antibody against candidate transcription factor after initial binding reaction time and incubate for an additional 10 min. Antibody effect may range from supershift of the complex to band loss, depending on epitope location and time of addition. The range of antibody effect must be determined empirically from pre-incubation with nuclear extract before introduction of probe, to simultaneous incubation with binding mix, to post protein binding. Parallel reactions with control immunoglobulin should be conducted.
22. Charged dye molecules may disrupt protein–DNA binding. Add 0.2 μL 1% bromophenol blue as a migration marker to free probe only. Optional: add 3 μL of 25% Ficoll 400 to a 20 μL binding reaction to aid delivery of the sample to the well bottom.
23. Be sure no detergent residue remains if plates have been previously used for protein SDS-PAGE. Plate separation after electrophoresis can be facilitated by pre-treating one plate with a silicone-based water repellant, e.g., Sigmacote, Sigma cat. no. SL2. Establish a convention as to which one of a set of plates is treated, such as the notched back plate. Gels will tend to remain on the untreated plate. To store gels, saturate a paper towel with running buffer, drape over wells, and cover wells and plates with plastic wrap. Store at 4°C for up to 1 week. Typical percentages are 4–5% for the variant EMSA and 6–7% for oligomer-based probes. Standard sized gels of 165 mm × 220 mm × 1 mm (width × length × thickness) typically provide better resolution than minigels.
24. This time is typically used for preparation of the binding reaction. Coordinate the end of the binding reaction incubation with the end of the pre-run.
25. Glycerol can leach out of the gel and into the well during the pre-run, prevent the loaded binding reaction from evenly sinking into the well thus distorting the migration pattern. Flush wells with running buffer from top chamber of gel box until no Schlieren lines are evident in the buffer in the well.
26. Other protocols perform entire run at constant voltage, e.g., 180 V for 90 min at 4°C.

27. Depending on band pattern, bromophenol blue migration 10–14 cm from bottom of well is a good marker for a sufficiently long run on 5–6% gel. Time the run length to keep the free probe on the gel. There are two advantages to this: (1) position of the free probe is a necessary landmark for determining actual shift and (2) this reduces radioactive contamination of bottom chamber and buffer. All used running buffers, filter papers for drying, etc., should be disposed of as per institutional provisions.

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Chapter 12

Chromatin Immunoprecipitation for Identifying Transcription Factor Targets in Keratinocytes

Kori Ortt and Satrajit Sinha

Abstract

Protein–DNA interactions, such as those that are necessary for transcription, are critical in regulating cellular function and behavior. The identification of DNA sequences that interact with transcriptional regulatory proteins is an important step necessary to better understand the molecular mechanisms regulating gene expression. Chromatin immunoprecipitation (ChIP) is one such procedure that provides a snapshot of which transcription factors are occupying specific DNA sequences. This method allows one not only to determine whether a particular genomic region is occupied by transcription factors but also to identify specific regulatory sequences that potentially control expression of their target genes. Recently, ChIP has been combined with both microarray analysis and a new generation of sequencing allowing a true genome-wide examination of transcription factor binding. Identifying the exact DNA sequence that a transcriptional regulatory protein binds, the precise timing of this association, and what other factors are involved in these interactions are important steps that will shed light on the transcriptional control mechanisms that dictate the biology of all cells, including keratinocytes.

Key words: Chromatin, Immunoprecipitation, Keratinocyte, Transcription factor, Genomic, Antibodies.

1. Introduction

The coordinated expression of genes is critical to all biological processes. To understand the complexity of development and disease it is essential that we decipher the underlying principles that control gene expression, a quite complex process in higher eukaryotes. Transcription factors, which usually bind to specific DNA sequences, play a central role in regulating the well-coordinated expression of genes in a temporal–spatial fashion. Hence,

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deciphering the precise genomic-binding location of transcription factors is likely to reveal the identity of the gene regulatory sequences as well as lead to clear understanding of the transcriptional networks in cells. Over the past several years, in vitro experiments such as gel shifts and in vivo DNase footprinting studies along with other genetic, molecular, and biochemical approaches have provided a glimpse into the transcriptional networks that regulate the function and behavior of cells. However, technical challenges and persistent questions about the biological relevance of the data obtained from such experiments have limited their usefulness and unconditional adoption by investigators.

ChIP, on the other hand, reveals an in vivo snapshot of the direct binding of transcription factors to DNA-binding sites in the context of chromatin. ChIP strategies have been successfully utilized to not only determine whether a candidate genomic site is occupied by a specific transcription factor but also identify transcription factor target genes. This method involves cross-linking living cells or tissues, usually with formaldehyde, which fixes proteins to their DNA substrates inside cells. Next, chromosomes are extracted and fragmented by physical shearing or enzymatic digestion. Utilizing a specific antibody against a protein of interest, specific DNA sequences that are associated with this protein can be isolated by immuno-affinity purification. These purified DNA fragments can then be assayed by a variety of molecular techniques, such as Southern blot or polymerase chain reactions, to determine association of a particular DNA sequence with the protein.

There are a number of different techniques that allow for large-scale identification of transcription factor binding sites in the genome following ChIP (**Fig. 12.1**) (1). Once the DNA fragments are isolated they can be cloned and subsequently sequenced to identify both transcription factor target genes and binding sites. Recently, a new generation of sequencing technology has emerged that combines with chromatin immunoprecipitation to provide a genome-wide look at transcription factor binding (ChIP-seq) (2, 3). In this protocol, after size selection, all the resulting ChIPed DNA fragments are sequenced simultaneously using a Genome Sequencer. Alternatively, the DNA fragments isolated from ChIP can be visualized by hybridization to a microarray of genomic sequences (ChIP-on-chip) revealing a genome-wide catalog of transcription factor target genes (4). With next-generation technologies we are beginning to better understand the molecular mechanism of transcription that regulate the function and behavior of cells.

While the ChIP assay is an important tool to probe a wide range of biological questions, it is undoubtedly technically challenging. Here, we provide a protocol for ChIP using keratinocytes

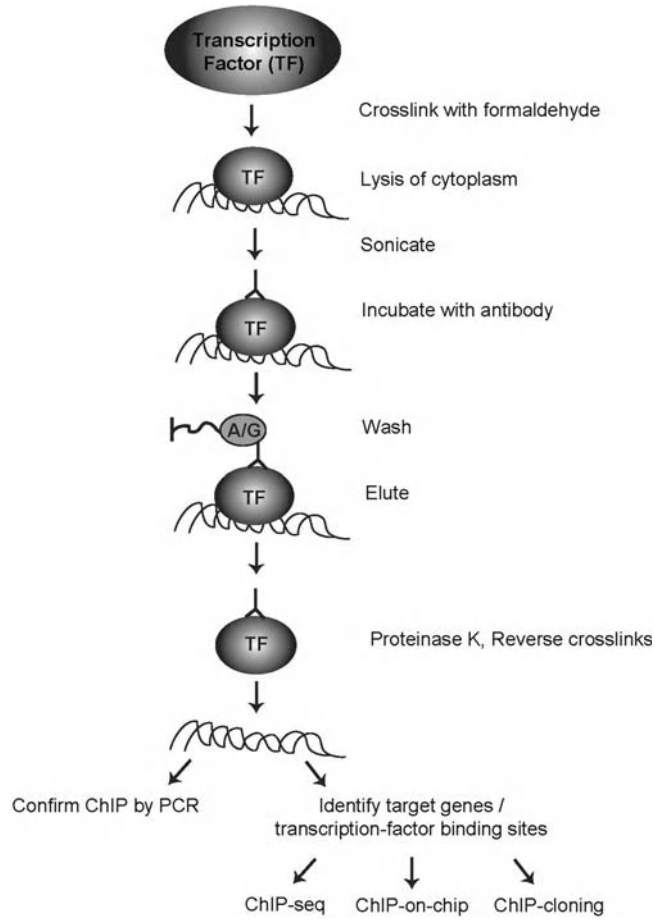

Fig. 12.1. A schematic outlining the chromatin immunoprecipitation (ChIP) process in keratinoyctes. Following ChIP, identification of target gene sequences can be accomplished through various techniques as indicated.

followed by cloning of the ChIPed DNA fragments (**Fig. 12.1**). The reader is directed to several useful reviews for detailed protocols on ChIP-on-chip (4–6).

2. Materials

2.1. Cross-Linking Keratinocytes

1. 0.5×10^7 cells per antibody
2. 37% formaldehyde
3. $1\times$ Phosphate Buffered Saline

2.2. Lysis of Keratinocytes

1. Lysis buffer: 50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA
2. Protease inhibitors: 0.1 mM PMSF, 0.1 mM benzamidine

2.3. Immuno precipitation

1. Chromatin immunoprecipitation (ChIP) dilution buffer: 20 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS, 1.2 mM EDTA, protease inhibitors (*see* **Note 1**)
2. Salmon sperm DNA/protein A agarose slurry: ChIP dilution buffer, 200 μg sonicated salmon sperm, 500 μg BSA, 1.5 mg recombinant protein A beads
3. Antibody of choice
4. Low-salt wash buffer: 20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA
5. High-salt wash buffer: 20 mM Tris-HCl, pH 8.1, 500 mM NaCl, 0.1%, 1% Triton X-100, 2 mM EDTA
6. LiCl wash buffer: 10 mM Tris-HCl, pH 8.1, 250 mM LiCl, 1% NP-40, 1% deoxycholate sodium salt, 1 mM EDTA
7. 1 × Tris-EDTA (TE) buffer: 10 mM Tris pH 8.0, 1 mM EDTA

2.4. Elution and Purification of DNA

1. Elution buffer: 1% SDS, 100 mM $NaHCO_3$
2. 5 M NaCl
3. RNAse A: 20 mg/ml (Invitrogen, Cat #12091-039)
4. 10% SDS
5. Proteinase K (Invitrogen, Cat #25530-031)
6. Qiagen PCR purification kit (Qiagen, Cat #28104)

2.5. Cloning of Immunoprecipitated DNA

1. Linkers (can be ordered through any company that synthesizes oligonucleotides)
2. 2 × Ligation buffer (Promega, Cat #C6711)
3. T4 DNA ligase (Invitrogen, Cat #15224-017)
4. 5 × T4 DNA polymerase buffer (Invitrogen, Cat #18005-025)
5. T4 DNA Polymerase (Invitrogen, Cat #18005-025)
6. Glycogen (Fermentas, Cat #R0561)
7. Standard PCR reagents

3. Methods

3.1. Cross-Linking Keratinocytes

1. Add formaldehyde to a final concentration of 1% directly to media in which keratinocytes ($0.5–1 \times 10^7$ cells) are growing and incubate at 37°C for 10 minutes (*see* **Notes 2 and 3**). Methods to grow keratinocytes have been well described in relevant chapters of this book.
2. Pour off the media and wash the cells five times with cold 1 × PBS.

3.2. Lysis and Sonication of Keratinocytes

1. Scrape the cells into lysis buffer with appropriate protease inhibitors.
2. Sonicate chromatin to an average length of 400–500 bp. Be sure to keep the samples cold during the sonication procedure. The sonication time and number of pulses will vary depending on sonicator, cell type, and extent of cross-linking. As a starting point, we have performed the sonication step using 10 pulses of 8 seconds each at setting 2 on a Branson 250 sonifier. Between pulses, samples should be allowed to cool on ice for at least 1 minute (*see* **Notes 4– 6**).
3. Centrifuge for 10 minutes at 12,100 × g at 4°C.
4. Measure DNA concentration of the supernatant and continue with immunoprecipitation. The sonicated DNA can also be stored at this stage for future use.

3.3. Immunoprecipitation

1. Transfer the supernatant to a new tube and dilute 5-fold with ChIP dilution buffer.
2. To reduce nonspecific background, pre-clear the samples with 40 µl of salmon sperm DNA/protein A agarose slurry for 1 hour at 4°C with agitation (*see* **Note 7**).
3. Pellet agarose beads by centrifugation at 1000 × g for 3 minutes and collect supernatant fraction.
4. Save 1% of total supernatant as a total input control. This will be your input fraction.
5. The remaining supernatant is incubated with a least 5 µg of antibody overnight at 4°C with moderate rotation (*see* **Notes 8–10**).
6. Collect the immune complexes by adding 60 µl of the salmon sperm DNA/protein A agarose slurry for 1 hour at 4°C with rotation. Pellet beads by a centrifugation at 2000 rpm for 3 minutes. Supernatant fraction will be unbound fraction.
7. Agarose beads are then washed consecutively for 3–5 minutes with rotation in 1 ml of each solution as described (*see* **Note 11**):
   a. Low-salt wash buffer
   b. High-salt wash buffer
   c. LiCl wash buffer
   d. Twice in 1 × TE

3.4. Elution and Purification of DNA

1. Elute the immune complex by adding 250 µl of elution buffer to pelleted beads. Prepare elution buffer fresh each time. Mix gently for 15 minutes at room temperature or rotate.
2. Spin beads and repeat elution with another 250 µl of elution buffer (optional). Combine two eluants. This is your bound fraction.

3. Reverse formaldehyde cross-links by adding NaCl to a final concentration of 0.3 M to the eluants and incubate at 65°C for 4–6 hours. Alternatively, incubate overnight at 65°C without any addition of NaCl. Make sure to also incubate the input fraction.
4. To recover DNA add 1/10 volume of 3 M sodium acetate pH 5.2 and at least 3 volumes of cold ethanol. Add glycogen (0.05–1 μg/μl final concentration) to increase the recovery of DNA and to better visualize the pellet. Precipitate for 2 hours at –20°C.
5. Spin at 12,100 × g for 30 minutes, discard the supernatant, and air-dry the DNA pellet.
6. Resuspend in 100 μl ddH_2O. Add 1 μl (20 mg/ml) RNase A and incubate for 1 hour at room temperature.
7. Add 2 μl 10% SDS and 1 μl of proteinase K (10 mg/ml) and incubate overnight at 37°C.
8. Recover DNA with Qiagen PCR purification kit. Elute in 50 μl of ddH_2O.
9. Use 5-10 μl of immunoprecipitated DNA for PCR.
10. Analyze the efficiency of the immunoprecipitation by PCR (*see* **Note 12**) and then proceed to cloning for identification of target genes. Alternatively, proceed to ChIP-sequence or ChIP-on-chip (*see* **Notes 13 and 14**).

    a. Design of primers for validating ChIP (*see* **Note 15**):

        i. 20–24 mer's
        ii. Tm should be 58–60°C
        iii. G/C content should be 40–70%
        iv. Avoid runs of an identical nucleotide (e.g., CCCC) especially G's
        v. Keep amplified fragments between 150 and 250 bp
        vi. Test primers on genomic DNA

3.5. Cloning of Immunoprecipitated DNA

1. Combine the DNA from at least two (~100 μl total) immunoprecipitated samples into one tube (*see* **Note 16**).
2. Blunt end the immunoprecipitated DNA (at this stage you should typically have between 1 and 10 ng of DNA) by adding 30μl of 5 × T4 DNA polymerase buffer, 0.5 μl BSA (10 mg/ml), dNTP's (100 μM final concentration), 1 μl T4 DNA polymerase (3U/μl), and ddH_2O to a total volume of 150 μl. Incubate for 20 minutes at 12°C. Stop reaction by adding EDTA to a final concentration of 10 mM and heating the sample to 75°C for 20 minutes. Next precipitate the DNA by adding 1/10 volume of 3 M sodium acetate (pH 5.2),

glycogen (0.05–1 μg/μl final concentration) and at least 3 volumes of cold ethanol. After ethanol precipitation, the DNA is dissolved in 25 μl of ddH_2O (*see* **Note 17**).

3. To amplify immunoprecipitated DNA use two linkers. Please note that one linker should be phosphorylated at the 5′ end. Also, linkers should contain a restriction enzyme site to be used for cloning (*see* **Note 18**). Here is an example:
   a. Linker #1 (phosphoprimer):
      5′-/5Phosp/AGAAGCTTGAATTCGAGCAGTCAG-3′
   b. Linker #2 (primer)
      5′–CTGCTCGAATTCAAGCTTCT-3′
   c. Anneal the oligonucleotides to generate the linker
   d. Mix 100 μl of 10 μM phosphoprimer and 100 μl of 10 μM primer
   e. Boil 5 minutes to remove secondary structures
   f. Cool slowly at room temperature to anneal oligonucleotides. You now have 200 μl of 5 μM blunt PCR linker
   g. Dilute linker to 1 μM, aliquot and store at –22°C
4. Ligation
   a. Ligate the linker to 7 μl of immunoprecipitated DNA sample with 10 μl of 2 × ligation buffer, 1 μM linker, and 4 U T4 DNA ligase. Add ddH_2O to a total volume of 20 μl
   b. Incubate at 15°C or room temperature for a few hours or overnight
5. PCR
   a. Sample reaction

5–10 μl	of immunoprecipitated DNA
10 μl	10 × Taq polymerase buffer
10 μl	10 mM dNTPs
1 μl	100 μM primer (oligonucleotide #2)
0.5 μl	Taq polymerase
to 100 μl ddH_2O	

   b. Sample PCR conditions

1 cycle	94°C – 2 minutes
35 cycles	94°C – 1 minute
	55°C – 1 minute
	72°C – 3 minutes
1 cycle	94°C – 1 minute
	55°C – 1 minute
	72°C – 1 minute

Product should appear as a smear ranging between 200 and 500 bp.

6. Purify amplified DNA by phenol-chloroform extraction.
7. Remove linker DNA by digestion with 10 U of appropriate restriction enzyme (depends on which site you designed in your linker).
8. Purify DNA from linker using Qiagen PCR kit.
9. Clone the purified DNA into vector of choice by standard methods.
10. Perform restriction enzyme digestion to check for inserts and then sequence clones.
11. Following sequencing, Perform BLAST search against the genome database to identify target genes and the genomic location of the transcription factor binding sites.

4. Notes

1. For immunoprecipitation we have obtained consistent results with the Chromatin Immunoprecipitation (ChIP) Assay kit [Catalogue #17-295 from Millipore (formerly Upstate)]. Kits with similar capabilities are available from other companies including Active Motif (Catalogue #53018) and Research and Development Systems (Catalogue #ECP001).
2. The protocol described here is designed for performing ChIP in keratinocytes. However, ChIP can also be performed with mouse skin epidermis. For this, skin samples are isolated and treated with Dispase II (Roche Catalogue #04942078001) overnight at 4°C. The epidermis is then separated, washed five times with PBS, and finely chopped. Next, the epidermis is cross-linked with 1% formaldehyde in PBS at room temperature for 15 minutes. About 0.125 M glycine is then added for 3–5 minutes to stop cross-linking followed by five washes with PBS and subsequent steps for standard ChIP procedures are followed.
3. Formaldehyde is the most common cross-linking agent used for ChIP. To capture indirect associations between DNA and transcriptional cofactors you can use other cross-linking agents such as dimethyl apidimidate (DMA) and disuccinimidyl glutarate (DSG) in conjunction with formaldehyde (7).
4. To capture weak or transient protein–DNA interactions a longer incubation time with formaldehyde may be required. However, increasing the cross-linking time can make fragmentation of the chromatin more difficult.

5. Sonication is a critical step because long chromatin fragments are not as efficiently immunoprecipitated. Sonication times will vary depending on cell and tissue type. Do not sonicate for too long and be sure that all sonication is performed on ice. Sonicating more than 30 seconds could cause sample overheating and denaturation. Another method that can be used to shear DNA is MNase digestion (micrococcal digestion). However, this method of DNA shearing results in fragments that are smaller than the optimum ~500 bp size, which may be too small for ChIP. Also, be cautious of using a water bath sonicator as it has been noted that the energy produced by this method is not concentrated enough to provide necessary shearing within a reasonable time period.
6. To obtain a good resolution for ChIP, it is important that the DNA fragments be between 400 and 500 bp. If the average sonicated DNA fragment is greater than 1000 bp, then it becomes difficult to distinguish between DNA–protein interactions in a specific genomic location and DNA segments that are inadvertently immunoprecipitated by interaction of transcription factor to regions several hundred base pairs away from the area of interest.
7. Choice of Protein A or Protein G beads depends on the source of the antibody. For example, if antibody has been raised in rabbit, use Protein A agarose. On the other hand, if the origin of the antibody is goat, Protein G agarose is the optimum choice.
8. It is important that the epitope recognized by the antibody be available after cross-linking and not buried in the protein complex to work successfully in ChIP. Antibodies also have to be of very high avidity so that the interaction with the protein will survive the washing steps. It is also important that the protein you are trying to immunoprecipitate cross-links efficiently to the chromatin. Thus, it is not surprising that the success of the ChIP experiment is very much dependent on the availability of ChIP-grade antibodies. Although for some transcription factors, ChIP-grade antibodies are available from commercial companies, this might not be the case for antibodies that are developed in the laboratory. In such cases, there is a convenient method that can be used to determine the efficiency of antibodies for ChIP using a specific cell line (8). This is a human cell line, HEK293 UAS-TK-Luc, with a Gal4-responsive luciferase gene integrated in the genome. Your protein of interest needs to be cloned in-frame with Gal4 DNA-binding domain into a mammalian expression vector. After transfection, the protein of interest is expressed as a fusion to the Gal4 DNA-binding domain in the reporter cell line. Next, ChIP is performed utilizing

ChIP-grade antibodies against Gal4 (Santa Cruz, Cat # Sc-577X) and in parallel for comparison, with the sera directed against your protein of interest. We have successfully utilized this method to validate our homemade antibodies.

9. In such cases where a ChIP-grade antibody is unavailable, Promega offers a new option that bypasses the need for antibodies. The HaloCHIP System (Catalogue #G9410) is unique in that it covalently captures cross-linked protein–DNA from cells without the addition of antibodies. The system utilizes the HaloTag which is fused to a DNA-binding protein of interest. After transient transfection, the cells are cross-linked, lysed, and sonicated. The complexes are captured from the lysate through covalent binding between the HaloTag moiety of the fusion protein and a special resin provided in the system. One limitation is that this method is not applicable for ChIP with endogenous proteins.
10. In addition to performing ChIP with the antibody against the protein of interest, one should also perform ChIP utilizing an isotype control as well as a ChIP with no antibody. These will serve as controls.
11. In order to prevent chromatin from interacting with the beads, the ChIP assay utilizes both low and high salt buffers. ChIP differs from DNA hybridization assays such as Southern blot, in that higher salt leads to higher stringency washing.
12. To determine the efficiency of the ChIP, one should utilize PCR to amplify a genomic region known to be bound by the protein of interest (positive control) as well as a random genomic area that should not be bound by the protein (negative control).
13. ChIP-on-chip combines chromatin immunoprecipitation (ChIP) with microarray technology (chip) (4–6). ChIP-on-chip is similar to ChIP in that it investigates the in vivo interactions between transcription factors and DNA. More importantly, Chip-on-chip efficiently utilizes a scalable way to identify binding sites of DNA-binding proteins.
14. A new sequencing technology known as ChIP-seq provides a genome-wide coverage of transcription factor binding (2, 3). This technique has many advantages over microarray hybridization: lower cost, minimal hands-on processing, and a requirement for fewer replicate experiments as well as less input material (2). It has been shown that ChIP-seq data have a high degree of similarity to results obtained by ChIP-on-chip for the same type of experiment. All the resulting ChIP–DNA fragments are sequenced simultaneously using a Genome Sequencer. In contrast to large sets of tiling arrays

required for lower resolution ChIP-on-chip, ChIP-seq can scan for genome-wide associations with high resolution in a single sequencing run.

15. The following programs available on the Internet are very useful when designing primers: Primer 3 (http://frodo.wi.mit.edu/), OligoCalc (www.basic.northwestern.edu/biotools/oligocalc.html), and BLAT Search Genome available from UCSC Genome Bioinformatics (http://genome.ucsc.edu/cgi-bin/hgBlat).
16. ChIP can often be challenging due to the overwhelming excess of non-specifically precipitated DNA. To overcome this, before cloning ChIPed DNA fragments, you can purify the PCR-amplified fragments by incubating them with your recombinant protein of interest (e.g., GST-fusion proteins) (9). This will enrich for DNA fragments obtained from ChIP that are more likely to contain binding sites for your protein of interest.
17. It is advisable to treat the immunoprecipitated DNA with T4 DNA polymerase because the physical shearing by sonication generates DNA with recessed and heterogeneous ends, which could hinder the ligation of the linkers.
18. Linkers should be designed to contain restriction enzymes that are compatible with the vector that the DNA fragments are to be cloned into. For our experiments, we have used the pBluescript vector (Stratagene).

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Chapter 13

Gene Expression Profiling of Mouse Epidermal Keratinocytes

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Abstract

DNA microarray technology is a powerful tool for genome-wide gene expression analysis of biological samples including skin and epidermal keratinocytes. Here we review the methodology for expression profiling analysis of skin tissue or purified keratinocytes from mice. We explained the methodology and protocols for RNA preservation and purification, RNA quality and integrity tests, and DNA microarray technology types that can be used. Furthermore, using a data set of mice samples, we explained how to perform chip raw data preprocessing and normalization, differential expression analysis, gene-clustering and functional analysis of gene deregulation.

Key words: Microarray, Profiling, Clustering, Differential expression, RNA purification, Transgenic mouse, Skin, Primary keratinocytes, Enrichment, Functional analysis, Gene ontology.

1. Introduction

Analysis of gene expression profiling is a useful tool for basic and applied research in all areas of biology. After the sequentiation of the human genome and other model organisms was performed, the possibility to analyze gene expression changes in a genome-wide scale became feasible. Since the first experiments performed in the late 1990s until now, all related technological areas have been improved in order to optimize and standardize protocols, augment the probe density of microarray technology, or develop new software and statistical algorithms. All these advances would allow gene expression microarray analysis to be used in a wider scale and to get more reproducible results, independent of chip platform technology or laboratory.

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Here we present methods and protocols for gene expression microarray analysis of skin tissue of primary keratinocytes from mice, either obtained from newborn or adult animals. Although multiple recent studies focusing on epidermal or skin biology used the gene expression technology (1, 2), a systematic comparison using different tissue sources has not been reported. Consequently, we performed a comparison between tissue and cultured primary cells, extracting the genes and gene family functions that characterize the differences between both types of samples. Similarly, we compared gene expression patterns that distinguish samples from newborn mice relative to adult animals.

2. Materials

2.1. RNA Extraction

2.1.1. From Mouse Tissue

1. Dissecting tools: Fine-tipped forceps, and straight fine Scissors 10.5 cm.
2. 1.5 ml or 2 ml plastic tubes, nuclease free.
3. RNAlater from Applied Biosystems (cat. no. AM7020), which is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA (*see* **Note 1**).
4. Liquid nitrogen.

2.1.2. From Cultured Cells

1. 1 × PBS.
2. 1.5 ml or 2 ml plastic tubes, nuclease free.
3. RNAlater.
4. Lysis reagent from RNeasy Plus Mini Kit (Qiagen, cat. no. 74134).
5. 14.3 M β-mercaptoethanol (β-ME).

2.2. RNA Preservation and Purification

1. 14.3 M β-ME.
2. Ethanol (96–100%).
3. Sterile, RNase-free pipet tips.
4. Water bath or heating block capable of reaching 55°C.
5. 1.5 ml or 2 ml microcentrifuge tubes.
6. Microcentrifuge (with rotor for 2 ml tubes).

2.3. Kits and Reagents

1. RNeasy Plus Mini Kit is recommended for cultured cells.
2. RNeasy Fibrous Tissue Kit (Qiagen, cat. no. 74704) in the case of purification from skin.
3. Homogenizer Mixer Mill 400 device from Restch, with 1.5 mm diameter-grinding balls.

4. RNAlater.
5. Double-distilled sterile water, RNase-free.

3. Methods

As a high-throughput technique, a major pitfall of microarray analysis is the dependency of expensive and specialized technology to perform it, so researchers must have access to Genomic Facilities. There are two different approaches: (i) the "in-house" microarray analysis, by which the facilities make the chips by themselves, from particular or commercial collections of DNA probes; and (ii) the commercial approach, by which the researcher client buys the chips from specialized companies and used the recommended technology (kits, solutions, devices, etc.) to perform the assays.

On the other hand, researchers have to decide whether to use cDNA or oligonucleotides as probes for the profiling analysis. Furthermore, there are two types of microarray experiments: (i) two color, by which two different samples can be hybridized to one single array and (ii) one color, by which only one sample can be analyzed in a single array.

Here we explained the procedures for experiments performed using a commercial technology (Affymetrix), based on oligonucleotide probes and one-color option. We have selected this methodology for several reasons: (i) very high density of DNA probes (up to 55 k for gene expression chips); (ii) high reproducibility; (iii) widely extended in the scientific community, allowing direct comparison with published results.

3.1. Total RNA Purification

1. Primary keratinocytes can be obtained from newborn mice following different protocols. We usually follow the protocol described in Santos et al. (3).

3.1.1. Purification of Total RNA from Primary Keratinocytes

2. Remove culture media and wash cells three times with 1 × PBS. Lyse cells in the tissue culture with lysis solution contained into the RNeasy Plus Mini Kit (Qiagen) (*see* **Note 2**) and follow the manufacturer protocol and recommendations.

3.1.2. Purification of Total RNA from Mice Skin

1. Shave fur from the selected area of the skin using an electric razor.
2. Euthanize the mouse per your animal care regulations.
3. Harvest the tissue quickly from the animal. The tissue piece should be approx. 100–200 mg and cut up into two to three pieces.

4. Total RNA purification can be performed preferentially soon after tissue collection. Alternatively, skin pieces collected must be preserved in RNAlater or flash-frozen in liquid nitrogen.
5. For RNA purification purposes, 30 mg tissue pieces can be processed per column of RNeasy Fibrous Tissue Mini Kit (Qiagen) minipreparation.

3.2. RNA Quality Control Analysis

1. Genomic DNA removal from samples. Both RNeasy Plus and RNeasy Fibrous Tissue Mini Kits (Qiagen) include on-column DNase treatment, such that the DNA removal is performed during RNA purification.
2. Quantification of total RNA quantity. Quantify RNA yield by spectrophotometer analysis using the convention that 1 absorbance unit at 260 nm equals 40 μg/mL RNA. The absorbance should be checked at 260 and 280 nm for determination of sample concentration and purity. The A260/A280 ratio should be close to 2.0 for pure RNA. However, smaller values can also be acceptable as it may change from tissue to tissue or between cell type origin.
3. Integrity of total RNA samples. Integrity of total RNA samples can be assessed qualitatively on an Agilent 2100 Bioanalyzer (*see* **Note 3**).

3.3. Overview of Target Labeling and Hybridization/Staining

Affymetrix GeneChip probe arrays are manufactured using technology that combines photolithography and combinatorial chemistry. Up to 1.3 million different oligonucleotide probes are synthesized on each array. Each oligonucleotide is located in a specific area on the array called a probe cell. Each probe cell contains hundreds of thousands to millions of copies of a given oligonucleotide. We give a short explanation of the GeneChip technology (*see* **Note 4**):

1. Target preparation. Double-stranded cDNA is synthesized from total RNA or purified poly-A messenger RNA isolated from tissue or cells. An in vitro transcription (IVT) reaction is then done to produce biotin-labeled cRNA from the cDNA. The cRNA is fragmented before hybridization.
2. Target hybridization. A hybridization cocktail is prepared, including the fragmented target and probe array controls. It is then hybridized to the probe array during 16-hour incubation.
3. Probe array washing and staining. Immediately following hybridization, the probe array undergoes an automated washing and staining protocol on the fluidics station.
4. Probe array scan. Once the probe array has been hybridized, washed, and stained, it is scanned. Each workstation running Affymetrix Microarray Suite or GCOS can control one

scanner. The software defines the probe cells and computes intensity for each cell. Each complete probe array image is stored in a separate data file identified by the experiment name and is saved with a data image file (.dat) extension.

3.4. Data Analysis

1. Computer Operating System. Most of the software can be used in Windows, MacOS, and Linux operating systems. However, we encouraged authors to check the compatibilities from the specific software sources.
2. Chip normalization. We recommend the Robust Multichip Average method or RMA, which is widely used, easy to manipulate, and is also implemented into most of the microarray software tools available. RMAExpress is a free software which implements RMA and can be used in Windows and Linux systems. RMAExpress can be directly downloaded from http://rmaexpress.bmbolstad.com. Briefly, it consists of three steps: a background adjustment, quantile normalization, and finally summarization (4, 5).
3. Unsupervised clustering analysis (*see* **Note 5**). Living cells regulate gene expression in clusters of genes/proteins which share common functions or features. Expression co-regulation has allowed researchers to develop bioinformatic tools for cluster analysis to search for genes sharing similar expression patterns between different samples/conditions: hierarchical clustering (HCL) (6), k-nearest neighbor (KNN), self-organizing tree algorithm (SOTA) (7), and others. These methods apply mathematical models in order to extract genes or samples that share expression patterns, giving measures of association/dissociation between them (8). An example of sample clustering using HCL is showed in **Fig. 13.1A**. Gene chip microarrays were done from skin tissue or primary keratinocytes, either obtained from newborn or 30-day old mice. Observe that samples are clustered according to whether the sample was primary tissue or cells, revealing that major gene expression differences between the samples are due to the type of biological sample instead of the age of the animals.
4. Differential expression analysis. Supervised method to extract genes which display differences in expression levels between groups of samples. Student T-test and Significance Analysis of Microarrays (SAM) can be used to compare expression between two groups of samples. Analysis of Variance (ANOVA) and SAM allow to compare more than two groups of samples. All these methods apply filtering probabilistic models for differential expression comparing mean and standard deviation of expression values. T-test analysis was done to extract the genes that can define the differences between

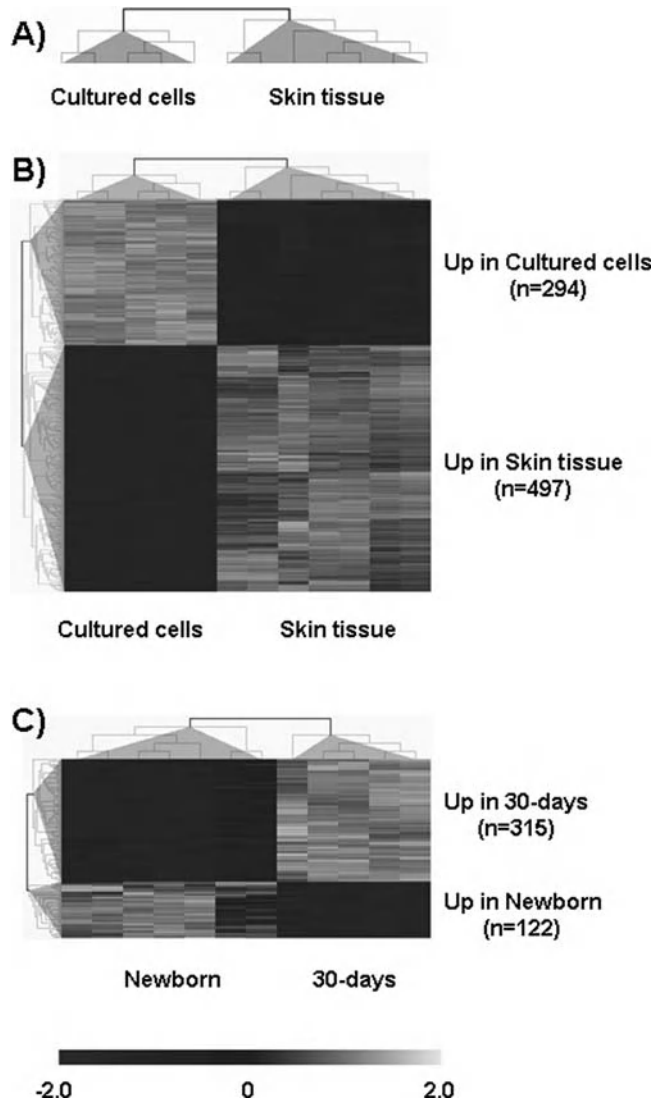

Fig. 13.1. Hierarchical clustering (HCL) of mice samples. Gene expression values are represented in log2 scale (standardized, i.e., mean = 0, st dev = 1), and within the interval from –2 to 2. **(A)** Gene chip microarrays were done from skin tissue or primary keratinocytes, either obtained from newborn or 30-day-old mice. Sample clustering showed that major gene expression differences are those that exist between cultured cells and skin tissue. Note that HCL was done with all the probe sets of the Gene Chip.**(B)** HCL of genes and sample genes differentially expressed between skin tissue and cultured cells, using T-test. Shown are the number of probe sets overexpressed in either sample group. **(C)** HCL of genes and sample genes differentially expressed between newborn animals and 30-day-old animals, using T-test. Shown are the number of probe sets overexpressed in either sample group. Raw and processed data of mouse microarray can be accessed from Gene Expression Omnibus database site (http://www.ncbi.nlm.nih.gov/geo/) with the identifier GSE11381.

skin tissue and epidermal cells in culture. We found a total number of 791 Affymetrix probe sets that are differentially expressed between both samples groups (p-val<0.00001, fold change≥1) (**Fig. 13.1B**). Furthermore, although major differences between analyzed samples are due to the type of biological sample, using T-test we have also extracted those genes that differentiated newborn from 30-day-old mice samples (p-val<0.001, fold change≥1) (**Fig. 13.1C**).

5. Functional analysis. Clustered genes or genes displaying similar differential expression between groups of samples normally share similar features, such as biological functions, molecular activities, subcellular localizations, signaling pathways. Extracting functional information from groups of genes is a fundamental question that must be answered in order to understand the biological, physiological, or phenotypic differences between samples. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp) contains multiple utilities to perform functional analysis from selected lists of genes. DAVID analysis is based on algorithms of annotation enrichment using distinct databases such as Gene Ontology (GO), KEGG, Biocarta. Using DAVID, we have extracted enriched GO Biological Processes (GOBP) from the genes that distinguish skin tissue–epidermal cultured cells. Briefly, upregulated genes in either tissue (497 gene probe sets) or cultured cells (294 gene probe sets) were uploaded to DAVID web Functional Annotation tool. After selecting the appropriate GeneChip used as a background (MOE430A), the GOBP enrichment result can be visualized and downloaded from the Annotation Summary Results, together with other annotation analysis from different databases. **Table 13.1** summarizes the enrichment result of differentially expressed genes from T-test analyses from **Fig. 13.1**. A similar analysis was done for genes that distinguish newborn from 30-day-old animal samples.
6. Clustering, differential expression, and functional analysis can be performed with most microarray analysis software. However, we recommend the Multiexperiment Viewer software (MeV), freely available from http://www.tm4.org/ as a Java running application. Furthermore, these analyses and others can be performed in Gene Expression Pathway Analysis Suite (GEPAS) (http://www.gepas.org/), a freeware web-tool package of bioinformatic applications intended to be used for microarray experiments. Both software systems contain detailed supporting information about the use of different tests for microarray analysis (either supervised or unsupervised) that may help scientists to correctly use the algorithms.

Table 13.1
GOBP enrichment of genes differentially expressed by T-test

Term	Count[1]	p-value	Gene cluster
Phosphate transport	14	8.25E-07	Up in tissue
Lipid metabolic process	41	1.27E-06	Up in tissue
Cellular lipid metabolic process	36	5.30E-06	Up in tissue
Muscle system process	14	6.05E-06	Up in tissue
Muscle contraction	14	6.05E-06	Up in tissue
Monocarboxylic acid metabolic process	21	9.67E-06	Up in tissue
Striated muscle contraction	8	3.23E-05	Up in tissue
Peptide cross-linking	5	3.70E-05	Up in tissue
Biological adhesion	39	4.73E-05	Up in tissue
Cell adhesion	39	4.73E-05	Up in tissue
Cytoskeleton organization and biogenesis	18	3.96E-03	Up in cells
Epidermis development	7	8.41E-03	Up in cells
Ectoderm development	7	1.09E-02	Up in cells
Regulation of cell motility	6	1.65E-02	Up in cells
Actin cytoskeleton organization and biogenesis	9	1.76E-02	Up in cells
Regulation of locomotion	6	2.02E-02	Up in cells
Regulation of developmental process	12	2.28E-02	Up in cells
Actin filament-based process	9	2.41E-02	Up in cells
Apoptotic program	5	2.48E-02	Up in cells
Locomotion	6	2.92E-02	Up in cells
Immune response	13	1.25E-04	Up to 30 days
Response to stimulus	27	2.05E-04	Up to 30 days
Antigen processing and presentation of exogenous peptide antigen via MHC class II	4	3.71E-04	Up to 30 days
Antigen processing and presentation of peptide antigen via MHC class II	4	3.71E-04	Up to 30 days

Table 13.1 (continued)

Term	Count[1]	p-value	Gene cluster
Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	4	4.38E-04	Up to 30 days
Antigen processing and presentation of exogenous peptide antigen	4	5.13E-04	Up to 30 days
Antigen processing and presentation of exogenous antigen	4	1.00E-03	Up to 30 days
Immune system process	15	1.01E-03	Up to 30 days
Antigen processing and presentation	5	2.88E-03	Up to 30 days
Antigen processing and presentation of peptide antigen	4	2.93E-03	Up to 30 days
Biological adhesion	7	2.56E-02	Up to newborn
Cell adhesion	7	2.56E-02	Up to newborn
Positive regulation of calcium-mediated signaling	2	4.22E-02	Up to newborn
Regulation of calcium-mediated signaling	2	4.97E-02	Up to newborn
Cellular process	39	5.48E-02	Up to newborn
Organ development	10	6.24E-02	Up to newborn
Negative regulation of neuron differentiation	2	6.45E-02	Up to newborn
Heterophilic cell adhesion	2	6.81E-02	Up to newborn
System development	11	8.00E-02	Up to newborn
Calcium-mediated signaling	2	8.26E-02	Up to newborn

[1]Number of genes from the gene cluster that belong to a specific GOBP term.

4. Notes

1. We recommend purifying total RNA immediately after cell growth has finished. If nucleic acid purification cannot be performed at this time, cells must be preserved in conditions whereby RNA stabilization is assured, such as flash-freeze cells in liquid nitrogen or, preferably, by cellular resuspension in a RNA Stabilization Reagent such as RNAlater. Immediate stabilization of RNA in biological materials is necessary

because, directly after harvesting the biological sample, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Samples are harvested and immediately submerged in RNAlater for storage (up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or at –20°C or –80°C for archival storage). For total RNA purification, it is recommended to use up to 1×10^6 cells maximum per RNeasy Plus Mini Kit (Qiagen) minipreparation column. The yield that can be obtained varies from 10 to 35 μg of total RNA.

2. We recommended Qiagen mini kits, as they fulfill all the quality control requirements for high-throughput analysis of RNA. However, kits from other manufacturers designed to purify RNA using affinity columns can be used (Ambion, Amersham, etc). For skin tissue, it is preferable to use a kit optimized for nucleotide extraction of fiber-rich tissues. Total RNA is eluted in about 50 μl of RNase-free water.
3. Bioanalyzer 2100 from Agilent is a widely used system for RNA quality assessment in most Genomic facilities. It performs an electrophoresis of small samples in a chip format. Furthermore, the system determines (for total RNA assays) the ribosomal 28S/18S ratio, giving an indication of the integrity of the sample. Additionally, the RNA integrity number (RIN) can be utilized to estimate the integrity of total RNA samples based on the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled, and repeatability of experiments is ensured. The assigned RIN is independent of sample concentration, instrument and analyst therefore becoming a de facto standard for RNA integrity.
4. For Affymetrix GeneChip Expression Analysis, we recommended to perform the next steps in a Genomic facility with the Affymetrix platform implemented: (i) target preparation; (ii) target hybridization; (iii) hybridization; (iv) probe array washing and staining; and (vi) probe array scan.
5. *Unsupervised analysis* is a statistical method for microarrays that does not used information derived about the data (samples or genes) to be analyzed. The outputs are simply a description of the relationships among the samples or genes based on gene expression patterns. Hierarchical clustering is a type of unsupervised approach, whereby genes or samples are successively grouped to form nodes that form a single hierarchical tree. The method is *supervised* when external information is used to group the samples (tumor grade, gender,

response to therapy) or the genes (functional class, chromosomal location) and relate the grouping with gene expression data.

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Chapter 14

Analysis of Tissue-Specific Gene Expression Using Laser Capture Microdissection

Martin Ruetze, Katrin Lochner, Stefan Gallinat, and Anja Knott

Abstract

Epithelial tissues exhibit optimal conditions for studying cellular differentiation since the differentiation status of a single cell can be determined by its distance to the basal membrane. For that reason Laser Capture Microdissection (LCM) may serve as a perfect tool to compare the characteristics of cells that have been collected from different *strata* of the epithelium. However, as cell boundaries are not visible in untreated tissue sections, samples have to be stained to allow for sufficient structural orientation. This usually results in a considerable reduction of RNA content in the dissected specimen. To circumvent this problem, we have established a modified hematoxylin/eosin staining protocol that concurrently allows visualization of important structures and the subsequent isolation of sufficient RNA amounts to be used for linear amplification and quantitative analyses.

Key words: Laser capture microdissection, LCM, mRNA amplification, Differentiation, Epidermis, Gene expression profiling.

1. Introduction

Laser capture microdissection (LCM) is a powerful tool to collect cells directly from histological sections. In contrast to other cell sorting methods such as FACS or adherence to coated magnetic beads, LCM is independent of the preparation of cell suspensions and does not sort on the basis of the expression of individual marker proteins. For this reason, LCM enables to directly analyze cell populations that do not show sufficiently unique cell surface marker expression profiles to apply one of the methods above. Additionally, the mode of LCM always guarantees a pure

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population of the cells from the individual structural area, whereas sorted cells may contain false positives. With respect to the epidermis, LCM for instance allows to collect keratinocytes from the basal, spinous, or granular layer. In one of the few skin-specific LCM studies, Ohyama et al. have shown that it is also possible to excise keratinocytes from the hair follicle bulge and the outer root sheath with subsequent microarray analysis (1). In another approach, Asplund et al. have collected numerous adjacent epidermal pieces of about 35 basal cells in length and analyzed them for clonogenicity by the determination of X-chromosome inactivation patterns (2).

Besides the costs for the acquisition of a LCM microscope, the main disadvantage of the method is that it only yields very small numbers of cells. Assuming that a usual laser dissected specimen contains about 20 cells, one can imagine that it would be an inappropriate deal of work to collect cell numbers that allow for analysis of protein expression. Nevertheless, LCM studies with subsequent proteomic analyses after collection of large cell numbers from tissues other than skin have been reported (3, 4). Alternatively, mRNA expression analysis is much more feasible since a very small amount of RNA can be amplified to tolerable quantities. However, a major drawback of the LCM–RNA analysis strategy is that skin sections have to be stained to allow optimal orientation on the specimen. Standard dyes as well as immunohistochemical stainings require the incubation with aqueous solutions that cause a partial elution of nucleic acids and RNA degradation by reactivation of endogenous nucleases. Depending on the experimental requirements, this elution and degradation of nucleic acids may result in insufficient RNA yield for further analyses. Ohyama et al. have overcome this problem by serial sectioning, thereby staining every fifth section for orientation and using the intervening four sections for LCM (1). Kube et al. recommend the addition of RNase inhibitors to all aqueous staining reagents (5). Alternatively, the collection of larger tissue samples with at least 500 cells was reported to enable the isolation of RNA from immunohistochemically stained tissue sections (6). As the low cellular density of the skin (especially the dermis) does not facilitate the collection of that many cells, we have improved the classical H&E staining protocol by minimizing incubation times with aqueous solutions and the usage of ethanol as solvent for eosin. The following protocol comprises this H&E staining method as well as information on the preparation of cryosections, laser microdissection, RNA isolation and amplification. The conditions described should yield RNA of sufficient quality and quantity to be used for microarray analyses of as few as 50 cells (7).

2. Material

2.1. Preparation of Skin Cryosections

2.1.1. Preparation of 5 mm Samples

1. Petri dishes
2. D-PBS
3. Tissue-Tek Cryomold (Sakura Cat. Nr. 4565)
4. Tissue-Tek O.C.T. compound (Sakura Cat. Nr. 4583)
5. Dry ice

2.1.2. Preparation of Skin Cryosections

1. PET membrane slides (Leica Cat. Nr. 11505151)
2. Cryofect Reagent (Leica Cat. Nr. 038742801)
3. Scalpel, brush, and cellulose wipes
4. Tissue-Tek O.C.T. compound (Sakura Cat. Nr. 4583)
5. Cryostat with specimen discs (e.g., Leica CM Series)

2.2. RNA Preserving H&E Staining/ Dehydration

1. Solutions of 25%, 50%, and 75% ethanol
2. Xylol
3. Aqua dest.
4. D-PBS
5. DEPC-treated water (FLUKA)
6. Mayers hematoxylin solution (Sigma Aldrich Cat. Nr. MHS80)
7. Eosin Y solution: 0.3 g eosin Y is dissolved in 100 ml ethanol
8. At least five staining troughs with one slide holder

2.3. Laser Capture Microdissection (LCM)

1. Thin-walled, Frosted Lid, RNase-free PCR tubes 0.2 ml (Ambion Cat. Nr. 12225)
2. RNase away (Molecular Bio Products Cat. Nr. 7002)
3. XB Buffer from PicoPure RNA Isolation Kit (*see* **Section 2.4**)
4. Laser microdissection system (e.g., Leica AS LMD)

2.4. RNA Isolation

1. PicoPure RNA isolation kit (Molecular Devices Cat. Nr. KIT0204)
2. 2 ml lidless tubes (PGC Scientific, Cat. Nr. 16-8101-06)
3. Thin-walled, Frosted Lid, RNase-free PCR tubes 0.5 ml (Ambion Cat. Nr. 12275)
4. RNase-free DNase (Qiagen Cat. Nr. 79254)
5. Microcentrifuge

2.5. RNA Amplification

1. RiboAmp RNA Amplification Kit (Molecular Devices Cat. Nr. KIT0521)
2. Thermocycler

2.6. RNA Gel

1. Agarose (Electrophoresis grade, e.g., Invitrogen Cat. Nr. 15510-019).
2. 10 × MOPS Buffer: 0.4 M MOPS, 0.01 M EDTA, 0.1 M sodium acetate, adjust pH 7.
3. Formaldehyde solution, 37% in aqua dest.
4. Loading buffer: 42% formaldehyde, 42% formamide, 0.8 mM ethidium bromide, 1.4 mM EDTA, 18 mM MOPS (pH 7), 4.6 μM sodium acetate, 1 μM Bromophenol blue.
5. 0.1–2 kb RNA ladder (Invitrogen, Cat. Nr. 15623-100).
6. Any horizontal gel electrophoresis system with power supply.
7. UV transilluminator (e.g., PeqLab E-Box System).

3. Methods

3.1. Preparation of Skin Cryosections

3.1.1. Preparation of 5 mm Samples

1. Always use freshly prepared skin samples and keep the tissue on ice until dehydration (*see* **Note 1**).
2. Cut skin samples into pieces of 5 mm × 5 mm and briefly wash the pieces in PBS.
3. Dehydrate the tissues by incubation in 2.3 M sucrose/PBS for 2.5 h at 4°C.
4. Place a cryomold on a piece of dry ice and cover the bottom of the well with a few drops of OCT medium (*see* **Note 2**).
5. Wait until the medium begins to freeze.
6. Place a skin sample with the epidermis facing up (*see* **Note 3**) into the cryomold and completely cover the tissue with OCT medium.
7. Wait until the whole medium is frozen and store the samples at –80°C.

3.1.2. Frozen Sectioning

1. Inactivate RNases on LCM membrane slides by UV irradiation for 1 h in a laminar flow hood. This will also result in a reduction of the electrostatical charge of the foil.
2. Thoroughly disinfect the cryostat with Cryofect reagent.
3. Adjust object and chamber temperature and place fixed tissue samples and specimen discs into the chamber for 1 h to equilibrate.
4. Fix the samples on the specimen disc with a drop of OCT medium.
5. Make 5 μM sections and transfer up to five slices on one membrane slide (*see* **Note 4**)
6. Slides can be stored at –80°C (*see* **Note 5**).

3.2. RNA Preserving H&E Staining

3.2.1. Preparations

1. All material should be RNase inactivated prior to use. Staining troughs, slide holders, forceps, etc., can either be heated to 150°C over night in a drying oven or UV irradiated for 3 h in a laminar flow hood.

3.2.2. Fixation and Rehydration

1. Prepare three staining troughs with 50%, 75%, and 100% ethanol (*see* **Note 6**).
2. Subsequently incubate the membrane slides in
   - 50% EtOH for 30 s
   - 75% EtOH for 90 s
   - 100% EtOH for 90 s
   - 75% EtOH for 90 s
   - 50% EtOH for 90 s
3. Keep the staining troughs for the next steps.

3.2.3. H&E staining

1. Prepare four additional staining troughs with 25% EtOH, PBS, DEPC-H_2O, hematoxylin solution, and eosin Y solution (*see* **Notes 7 and 8**).
2. Subsequently incubate the slides in
   - 25% EtOH for 90 s
   - PBS for 90 s
   - 25% EtOH for 90 s
   - Hematoxylin solution for 2 min
   - DEPC-H_2O for 2 $\times$ 2 min
   - 50 % EtOH for 90 s
   - 75% EtOH for 90 s
   - 100% EtOH for 90 s
   - Eosin solution for 45 s
3. Keep the 50% EtOH, 75% EtOH, and 100% EtOH troughs.

3.2.4. Dehydration

1. Prepare two additional staining troughs with 75% EtOH and Xylol
2. Subsequently incubate the slides in
   - 50% EtOH for 2 min
   - 75% EtOH for 2 min
   - 75% EtOH (second trough) for 2 min
   - 100% EtOH for 2 min
   - Xylol 2 $\times$ 5 min
3. Slides can temporarily be stored at –80°C. However, we recommend to directly proceeding to LCM for optimal RNA yield and quality.

3.3. Laser Capture Microdissection (LCM)

1. Clean LCM tube and slide holder with RNase Away (*see* **Note 9**).
2. Insert collection tubes for the dissected specimens and an additional empty tube for a negative control (*see* **Note 10**).
3. Load the caps of the collection tubes (including control) with 20 µl extraction buffer (XB) from the PicoPure RNA isolation kit (*see* **Note 11**).
4. Insert a LCM membrane slide and focus the region of interest (*see* **Note 12**).
5. Choose a medium (20 × –40 ×) magnification for optimal working conditions (*see* **Note 13**).
6. Collect at least 100 cells per tube (*see* **Note 14**, **Fig. 14.1**).
7. Directly proceed to RNA isolation.

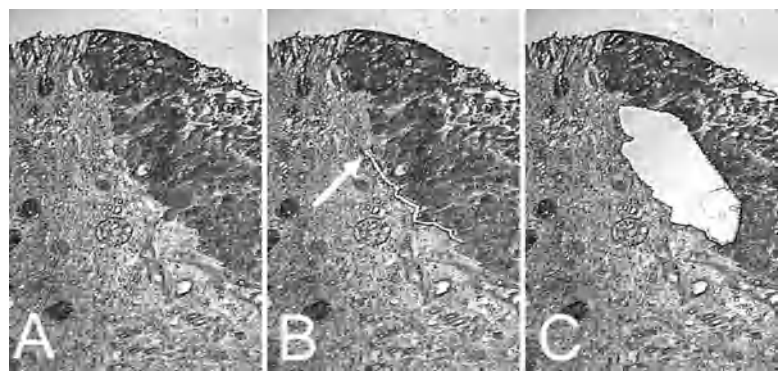

Fig. 14.1. Collection of epidermal cells by laser capture microdissection. A 5 µM skin cryosection was stained with a modified hematoxylin/eosin staining. Cells of the basal and spinous epidermal layer were captured for RNA isolation and analysis. **(A)** HE-stained cryosection before LCM. **(B)** Navigated laser cut along the basal layer. The actual laser position is indicated by the arrow. **(C)** Specimen after excision of an epidermal tissue sample.

3.4. RNA Isolation

1. LCM collection tubes can directly be used for RNA isolation.
2. If possible, carry out all pipetting steps under a sterile laminar flow hood that has previously been UV sterilized and RNase Away treated. Use filtertips for all pipetting steps.
3. Follow the instruction manual of the PicoPure RNA isolation kit. This kit is designed for the isolation of very small RNA amounts from as few as a single cell and especially recommended for LCM-collected samples.
4. Include the DNase treatment step using the RNase-Free DNase kit (Qiagen) as described in the PicoPure protocol (*see* **Note 15**).

3.5. RNA Amplification

1. Maintain the RNase-free working conditions as described above (see **Section 3.4**).

2. Use the RiboAmp Kit for RNA amplification. The protocol includes an mRNA reverse transcription step with the introduction of a T7 promoter. This promoter is then used for in vitro transcription of amplified RNA by T7 RNA polymerase.
3. For LCM samples we recommend to perform two rounds of amplification. According to the manufacturer's manual, this will yield up to a million-fold amplification of the initial mRNA amount. As described, the usage of poly dI-dC should be omitted if RNA was isolated with the PicoPure Kit.
4. Aqueous solutions of amplified RNA can be stored at –80°C.

3.6. Quality Control

1. Amounts and quality of amplified RNA can be determined by analysis in a nanodrop spectrophotometer. For a more precise analysis, the aRNA length distribution can be determined on a denaturing agarose gel.
2. Solve 1 g agarose in 87.5 ml distilled water by heating in a microwave oven.
3. Add 5 ml 10× MOPS buffer and 7.5 ml formaldehyde (37%).
4. Pour the fluid in a gel chamber and wait until the gel is solid.
5. Transfer 250–300 ng aRNA to 10 µl loading buffer and denature the RNA for 10 min at 70°C.
6. Cool the tubes on ice, briefly spin down condensed drops, and load the gel with the whole 10 µl per well. Also load 3 µl of RNA ladder solution into another well.
7. Run the gel at 60 V for 1 h.
8. Visualize the bands in a UV transilluminator.
9. After two rounds of amplification aRNAs are significantly shorter than the corresponding mRNAs and should appear as a smear with the highest intensity between 200 and 600 base pairs.

4. Notes

1. We strongly recommend the usage of cryosections for mRNA expression analyses of LCM samples. Fixation of the tissue with formalin will result in covalent cross-linking of nucleic acids to proteins and will dramatically affect the RNA recovery. If the usage of formalin-fixed paraffin-embedded (FFPE) tissues can absolutely not be avoided, RNA preservatives may be used to maintain a sufficient RNA quality, as reported by Kihara et al. (8).

2. The embedding of tissue samples in OCT medium will facilitate the preparation of cryosections without a significant loss of important morphological features. However, we observed that OCT medium partially absorbs energy of the LCM laser and results in a reduced microdissection performance. We therefore recommend to check if cryosections of unembedded frozen tissue are of sufficient quality to be used for the next steps.
3. Any orientation of the tissue may be used but as the specimen is no longer visible after OCT embedding it is important to keep in mind to which side the epidermis is facing.
4. In our hands, 5 μM cryosections were most suitable for LCM. Preparation of thicker sections is connected with the problem that the laser focus in *z*-direction does not strike the whole specimen. This will eventually result in a broader laser track with irregular borders.
5. We have not observed a considerable reduction of RNA amount and quality after freezing OCT-embedded sections at –80°C for a few weeks. However, if the storage of samples is not absolutely necessary, we recommend to directly proceed with staining and LCM.
6. After cryoembedding, the samples should never be thawed until the first fixation step. Dry ice should be used for the transportation of the slides from the cryostat or freezer. For the initial fixation step, 50% EtOH can be directly applied to the frozen tissue.
7. The intensity of eosin staining of skin sections on PET membrane slides will be much higher than on usual microscopic slides with coverslips. The preparation of thin (5 μM) cryosections, the utilization of ethanol-solved eosin, and the short incubation time will result in a reduction of the staining intensity. If LCM of 10 μM sections (e.g., for the collection of more material) is intended, a further reduction of eosin concentration and incubation time may be useful to maintain a satisfactory visualization of the tissue structure.
8. Compared to water, the solubility of eosin Y is considerably lower in ethanol. In contrast to the dark red, aqueous solutions, eosin Y will turn to bright orange when solved in ethanol.
9. We use the Leica AS LMD system for our microdissection studies. As various LCM devices are available, we will not give a detailed description of the microdissection procedure with our particular system.
10. We recommend performing the complete RNA isolation and amplification procedure with a collection tube that has not been loaded with LCM samples. The subsequent spectrophotometric determination of this negative control will show to which extent nucleic acids were non-specifically amplified or derived from contaminations.

11. RNA of LCM-collected cells will be stabilized in the extraction buffer. As the microdissection procedure may take several minutes, it should be kept in mind that the buffer continuously evaporates. Therefore, LCM tubes should be refilled with extraction buffer when necessary.
12. Depending on the experimental settings, the described H&E staining protocol may still not be sufficient for the visualization of the structures of interest. For an additional improvement of morphology, the usage of a liquid cover medium has been reported (9).
13. Note that the diameter of the laser spot decreases with higher magnification. If working with high magnification objectives ($60\times$, $100\times$) is planned, the thickness of the tissue sections should be as far reduced as possible. Otherwise, the laser will only cut the upper or lower part (z-direction) of the specimen. On the other hand, dissection at low magnification ($5\times$, $10\times$) leads to a wider laser focus that results in a broader laser track and irregular edges due to more heat generation.
14. We often encountered the problem that microdissected pieces do not fall into the collection tubes but stick to membrane slide due to electrostatical charging. This can be avoided by grounding the collection tube holder.
15. Note that the purification columns contain poly-dI-dC oligonucleotides that are not completely removed during the DNase digestion step. Remnants may interfere with spectrophotometric RNA determination, measured quantities can therefore only be regarded as approximate values. A more accurate determination of RNA quality can be performed by Q-PCR after the reverse transcription step in the RNA amplification protocol (see RiboAmp manual).

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Chapter 15

Comprehensive Transcriptional Profiling of Human Epidermis, Reconstituted Epidermal Equivalents, and Cultured Keratinocytes Using DNA Microarray Chips

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Abstract

Because of its accessibility, skin has been among the first organs analyzed using DNA microarrays; psoriasis, melanomas, carcinomas, chronic wound biopsies, and epidermal keratinocytes in culture have been intensely investigated. Skin has everything: stem cells, differentiation, signaling, inflammation, diseases, cancer, etc. Here we provide step-by-step instructions for bioinformatics analysis of transcriptional profiling of skin. Specifically, we describe the use of GCOS and RMA programs for initial normalization and selection of differentially expressed genes, DAVID and LOLA programs for annotation of genes, and statistically relevant identification of over- and under-represented functional and biological categories in identified gene sets, L2L and Venn diagrams for comparing multiple lists of genes, and oPOSSUM for identification of statistically over-represented transcription factor binding sites in the promoter regions of gene sets. The work can be a primer for researchers embarking on skinomics, the comprehensive analysis of transcriptional changes in the skin.

Key words: Affymetrix, Annotation, Clustering, Epidermal differentiation, Gene sets, Ontological categories, Skinomics, Transcriptome.

1. Introduction

The advent of genomics made possible comprehensive and efficient analysis of gene expression. DNA microarrays are an ideal approach for such systematic comparisons because they can simultaneously measure the expression of a large number of genes, potentially the entire genome (1). The microarray methodology is still very new, developing codified approaches, controls, data

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depositories, analytical methods, and even language. Its acceptance is slow and the massive amount of data may cause the inherent experimental noise to obscure the real results (2).

A microarray is an ordered arrangement of nucleic acids, proteins, tissue samples, etc., on solid supports, which enables large-scale analysis of complex biochemical samples. DNA chip is a microarray that consists of sequences from thousands of different genes arrayed at fixed locations on supports, usually silicon chips or glass microscope slides. There are five steps in a gene profiling microarray experiment: (1) Obtain DNA chips with the target genes. (2) Generate a biological sample containing a mixture of fluorescently labeled cDNAs or cRNAs. (3) Hybridize the mixture of labeled nucleic acids with the DNA on the chips. (4) Detect and measure the bound labeled nucleic acids; store data in a computer. (5) Analyze data using computational methods.

Microarrays or DNA chips that can probe complete transcriptomes come in two varieties: printed cDNA and synthetic oligonucleotide. The first, originated by P. Brown at Stanford (3), is often home-made, inexpensive, and two-color, i.e., treated and control samples can be hybridized and compared on the same chip. They are easy to customize for a specific application. Synthetic oligonucleotide microarrays are commercially available, at a price, but because each gene is probed with multiple oligonucleotides, they tend to be more reliable and require less redundancy. They are one-color, i.e., they probe only one sample per array, but the newer ones contain all the known human genes and are able to identify the splicing variants as well (4).

To address the differences in technique, data quality, etc., the microarray community has promulgated a set of guidelines known as "MIAMI" rules (*m*inimal *i*nformation *a*bout *mi*croarrays), which must be complied with if a manuscript is to be accepted in a growing list of journals. Currently, the functional annotation of many genes is inadequate, which is addressed by concerted international efforts at several genomics centers (5).

Epidermis presents the most accessible target for testing not only topical medications treating local cutaneous symptoms but also patches that treat systemic problems, and even for gene therapy. Consequently, skin was among the earliest targets of DNA microarray studies (6). The RNA from the skin surface can even be recovered using an easy, non-invasive procedure, tape stripping (7). The presence of many different cell types in skin, unfortunately, creates difficulties. Potentially very informative, skin samples taken directly from patients differ in proportions of various cell types, sample age and body sites, history of sun exposure, etc. Melanomas, basal and squamous cell carcinomas have been intensely investigated using DNA microarrays and so was psoriasis, one of the most common human inflammatory diseases (8, 9). In

addition, genomics analyses of chronic, non-healing wounds yielded critical information about pathogenesis that is revising clinical approaches to treatment (10–13).

The analysis of the transcriptional responses of dermal fibroblasts to the addition of serum was one of the first dermatologically relevant and most exciting studies using DNA microarrays (14). This model causes synchronous cell division and serves as a paradigm for cell cycle studies. In addition to the expected induction of the cell cycle regulators, the authors, unexpectedly, found that the fibroblasts initiated the expression of wound healing response proteins. Only DNA microarray-based transcriptional profiling could provide the global overview that made it possible to gain comprehensive insight into the new and exciting role of fibroblasts in wound repair.

Many subsequent DNA microarray studies focused on epidermal differentiation, skin cancers, inflammatory diseases, wound healing, ageing, stem cells, etc. Specific for skin has been a series of studies on the effects of UV light (15–19). The results from different laboratories were amazingly congruent, given the differences in experimental approaches. Like in the parable of the blind men and the elephant, Li et al.'s group focused on the metabolic and differentiation effects, Sesto et al. were interested in DNA repair, Murakami et al. in oncogenes, Howell et al. in angiogenesis, etc. The large amounts of data allow researchers to demonstrate their individual interests and inclinations. A skin-specific cDNA array, DermArray, containing >4000 gene probes, is commercially available (20). Keratinocyte-specific house-keeping genes have been characterized (21, 22). Recent reviews describe various aspects of the use of transcriptional profiling in dermatology and skin biology (23, 24). The large volume of bioinformatics data relevant to skin led to the coinage of the term skinomics (25).

Keratinocytes, the main constituent of the epidermis, have been a target of extensive basic and applied research efforts because they respond to a rich variety of inflammatory and immunomodulating cytokines, hormones, vitamins, UV light, toxins, and physical injury. Because epidermal keratinocytes perform many different functions, e.g., differentiate through a multistage process to create a protective mechanical barrier, and respond to physical and microbial damage, initiate inflammation as well as react to extracellular signals from nearby cells, keratinocytes have a relatively large "transcriptome" i.e., express many genes.

Omics studies are characterized by massive amounts of data that require bioinformatics, a new field of specialized computer-based analytical approaches, which include large databases and various analysis tools and integrate distinct and diverse disciplines

that were independent in the past (26). The data analysis algorithms are often custom-designed for a specific application and have become a wholly new field of creative research that bridges, sometimes uneasily, biology and computer sciences. However, many of these programs are easily available online, and we have used them extensively in our studies of gene expression in the epidermis (27–31). A summary of our approaches is presented here.

However, we must note that this is a very fast-moving field and new and improved algorithms are constantly being developed. It parallels the rapid expansion and development of the systems biology and informatics. New and improved approaches, types of chips, hardware, software, and data repositories are constantly being developed. To keep up with the field, we find the following two resources extremely valuable: (1) The Nucleic Acids Research publishes every January a special "Database issue" http://nar.oxfordjournals.org/content/vol36/suppl_1/index.dtl. This edition describes the function and role of all molecular biology data repositories, including those that collect microarray data. (2) Health Sciences Library System of the University of Pittsburgh maintains and keeps regularly updated links to microarray and other gene expression data analysis tools http://www.hsls.pitt.edu/guides/genetics/obrc/gene_expression/gene_expression_tools. We found these invaluable for keeping in touch with the latest developments in this field.

Transcriptional profiling specifically in skin is rapidly expanding. Unavoidably, some of the recommendations in this manuscript will be outdated even by the time it reaches print. Virtually every analysis tool mentioned is associated with a dedicated team of developers and programmers devoted to making them better, more versatile, and user friendly. Therefore, dear readers, if you see an innovation or a new useful approach, please contact us at blumem01@nyumc.org, and I will keep an updated running manual of the described procedures. Conversely, if you embark on transcriptional profiling in skin and would like to find out about new developments, or just need some hand-holding, do not hesitate to get in touch.

2. Materials

2.1. Growth and Isolation of Keratinocytes

1. Normal epidermal keratinocytes or skin samples from surgery. Human skin samples are obtained from patients undergoing elective breast reduction surgery, usually within 2 to 6 h after surgery.

2. Serum-free keratinocyte growth medium supplemented with 0.05 mg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, and 1% penicillin/streptomycin (KGM from Gibco-BRL).
3. Trypsin, adjusted to 0.025% (Gibco-BRL).
4. Trypsin inhibitor, 0.5 mg/ml in PBS (Sigma).
5. Reconstituted human epidermis (SkinEthic Laboratory, Nice, France).
6. Dispase (2.4 U/ml, Roche).
7. RNAse inhibitor (4 U/ml Roche).
8. RNA*later* (Ambion).

2.2. Separation of Epidermal Layers and Isolation of Basal Layer Keratinocytes

1. PBS, phosphate-buffered saline.
2. Solution of 0.05% trypsin, 0.02% EDTA (Gibco-BRL).
3. Solution of 0.5 mg/ml trypsin inhibitor (Sigma).
4. Cell Strainer tissue filters (Falcon).
5. Magnetic beads, M-450 Rat anti-Mouse-IgG1, prepared as suggested by the manufacturer (Dynal).
6. Antibody 3E1 clone against integrin β4 from (Gibco-BRL).

2.3. Isolation of Total RNA from Preparation of Labeled Probes and Hybridization

1. Qiashredders and on-column RNAses-free DNAse Set (Qiagen).
2. RNeasy kits (Qiagen).
3. Trizol (Invitrogen).
4. RNAlater (Ambion).
5. Affymetrix microarrays.

2.4. URL Sites Listed

1. http://www.affymetrix.com
2. http://rmaexpress.bmbolstad.com/
3. http://david.abcc.ncifcrf.gov/
4. http://depts.washington.edu/l2l/
5. http://www.lola.gwu.edu/
6. http://rana.stanford.edu/software
7. http://www.tm4.org/
8. http://www.pangloss.com/seidel/Protocols/venn.cgi
9. http://www.cisreg.ca/cgi-bin/oPOSSUM/opossum
10. http://nar.oxfordjournals.org/content/vol36/suppl_1/index.dtl
11. http://www.hsls.pitt.edu/guides/genetics/obrc/gene_expression/gene_expression_tools

3. Methods

3.1. Provenance and Maintenance of Samples

1. Normal epidermal keratinocytes from human foreskin were initiated using 3T3 feeder layers as described (32, 33) and then frozen in liquid N_2 until used. Once thawed, the keratinocytes are grown without feeder cells in defined serum-free keratinocyte growth medium supplemented with 0.05 mg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, and 1% penicillin/streptomycin (KGM from Gibco-BRL) at 37°C, in 5% CO_2. The medium is replaced every 2 days (**Note 1**).
2. The reconstituted human epidermis consists of a three-dimensional multilayered keratinocyte structure grown on air–liquid interface, without any other cell type (SkinEthic Laboratory, Nice, France). All media for cell culture were prepared without antibiotics and antimycotic agents (34, 35).
3. Human skin samples are obtained from patients undergoing elective breast reduction surgery, usually within 2–6 h after surgery (**Note 2**). The fat layer and most of the dermis are removed using surgical scissors and by gentle scrapping with a scalpel, leaving the epidermis as the predominant cellular structure (~0.2 mm deep). Samples are then cut into strips of approximately 0.5 × 3 cm and stored in RNA*later (Ambion)*overnight at 4°C.

3.2. Isolation of β4+ and β– Keratinocytes from Skin

1. Skin, discarded after reduction mammoplasty, as described above, is first washed six times with PBS and excess liquid drained. Using scissors and a scalpel, as much fat and dermis as possible are removed. The tissue is cut into 3 mm strips and incubated with dispase (2.4 U/ml, Roche) and RNAse inhibitor (4 U/ml Roche) at 4° overnight.
2. Next day, the epidermis is gently separated from the dermis using forceps and incubated in 0.05% trypsin, 0.02% EDTA (Gibco-BRL) at 37°. After 10 min, 2 volumes of 0.5 mg/ml trypsin inhibitor (Sigma) are added and the tissue filtered through Cell Strainer (Falcon). The trypsinization of the tissue is repeated twice more. The cells are collected by centrifugation, examined using trypan-blue, counted, and, if appropriate, the isolates combined. This represented the unfractionated, total epidermal cell population.
3. Magnetic beads, M-450 Rat anti-Mouse-IgG1, are prepared as suggested by the manufacturer (Dynal). The cells are incubated with the beads in the following ratio: 100 µl beads: 10–20 µg β4 antibody: 4×10^6 cells (exactly!) in 1 × PBS, 0.1% BSA, at 4° for 1–2 h. We used M-450 Rat anti-Mouse-IgG1 beads and the 3E1 clone β4 antibody from Gibco-BRL (30). The beads are

separated on a magnetic separator for 2–3 min, washed three to four times with PBS, collecting and combining the non-adherent, β4– cells as the suprabasal cell population. The beads bound to the β4+ basal cells are used in RNA isolation without removing the cells from the beads.

3.3. Isolation of Total RNA from Human Epidermis

To obtain RNA of appropriate quality for chip analysis from in vivo epidermis, we have tested several purification methods. After extensive experimentation, we settled on the following approach:

1. First, the epidermal cells are disrupted and the RNA is isolated using Trizol (Gibco).
2. This is followed by the use of Qiashredders to homogenize cell extracts with centrifugation at 1,800 × g for 2 min.
3. DNA is removed with on-column DNAse digestion using RNAses-free DNAse set (Qiagen). RNeasy kits from Qiagen are used to prepare the RNA according to the manufacturer's protocols (**Note 3**). If the sample is not immediately processed for RNA isolation, it is cut into 3 mm wide strips and stored in RNAlater overnight at 4°C, then at –20°C. With this procedure, we routinely prepare RNA of high quality (**Note 4**).
4. From the reconstituted epidermis and cultured keratinocytes, total RNA is isolated using Qiashredders to homogenize cell extracts and RNeasy kits procedure. The RNA samples are stored in water at –80°C until hybridization.
5. To ensure good RNA quality, 28S and 18S ribosomal bands are visualized on a non-denaturing agarose gel and $OD_{260/280}$ spectrophotometric ratio of at least 1.8 is ascertained. Five micrograms of total RNA is reverse transcribed, amplified, and labeled as described (21).
6. Approximately 5–8 μg of total RNA is reverse transcribed, amplified, and labeled as described (15, 36). Labeled cRNA is hybridized to the arrays (Affymetrix), which are washed, stained with anti-biotin streptavidin–phycoerythrin-labeled antibody using Affymetrix fluidics station and then scanned using the Agilent GeneArray Scanner system (Hewlett-Packard).
7. Labeled cRNA is hybridized to oligonucleotide microarrays from Affymetrix. Arrays are washed, stained with anti-biotin streptavidin–phycoerythrin-labeled antibody and scanned using the GeneChip system (Hewlett-Packard) and GeneChip 3.0 software to determine the expression of each gene.

RT-PCR, Northern and Western blot analyses that confirm microarray data will not be described here, any standard molecular biology protocol compilation can be consulted for this purpose.

3.4. Choices of Chips

As noted above, there are two varieties of microarrays that can probe complete transcriptomes come in: printed cDNA and synthetic oligonucleotide. The cDNA microarrays are often homemade, customized for a specific application, inexpensive and two-color, i.e., treated and control samples can be hybridized and compared on the same chip (3). Commercially available oligonucleotide microarrays are more expensive, one-color, i.e., they probe only one sample per array, but they tend to be more reliable and consistent in performance, require fewer replicates, contain all the known human genes and can identify the splicing variants (4). If a fully functional and reliable microarray production core facility is accessible, then the cDNA microarrays, being much cheaper, are preferable. However, the costs and efforts associated with acquisition, maintenance, and quality control of the cDNA bank, the robotics, printing, and quality control of the produced microarrays, as well as development of customized software for data analysis precludes individual groups from producing the microarrays. Commercial products, which come already quality-controlled, with equipment and software already developed allow immediate experimentation with very short set-up times. We have chosen Affymetrix microarrays for our studies, the manufacturer being the leader of the field.

3.5. Evaluating the Quality of Hybridization

1. The scanning of an Affymetrix chip generates a .DAT file, i.e., a picture representation of the chip and an .RPT file, which contains many of the quality control metrics. The first and obvious step is to look at the chip (**Fig. 15.1**). The hybridization should be uniform throughout the chip surface, without major blotches or scratches. In addition, the background frame, i.e., the peripheral area without the DNA, should appear dark; this indicates a strong, undegraded fluorescently labeled probe.
2. We find the .RPT files also very useful (**Fig. 15.2**). The value for noise (RawQ) should be less than 5 and the scaling factor close to 1. The number of genes present should approach 50%; values in the 10–20 range indicate inadequate labeling of the probe. The Sig $\acute{3}/\acute{5}$ ratio for the internal controls, actin and GAPDH, should be close to 1; values above 2 indicate degradation of the mRNA, values above 4 should not be used. The spike-in controls of *Escherichia coli* biotin gene DNA are added at different levels; the detection for BIOC and BIOD must be "P" (present), while BIOB, spiked in at very low levels, could be "M" (marginal), none should be "A" (absent) (**Note 5**).
3. A very useful quality control is provided by the scatter correlation graph (**Fig. 15.3**). While commonly used to identify differentially expressed genes, the scatterplots can identify

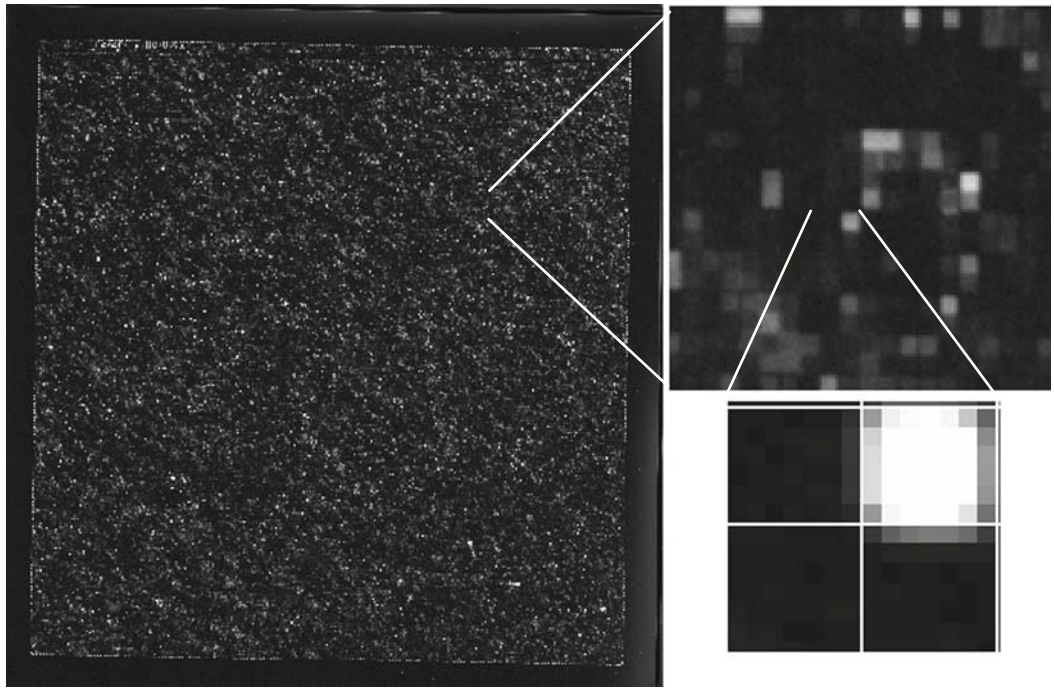
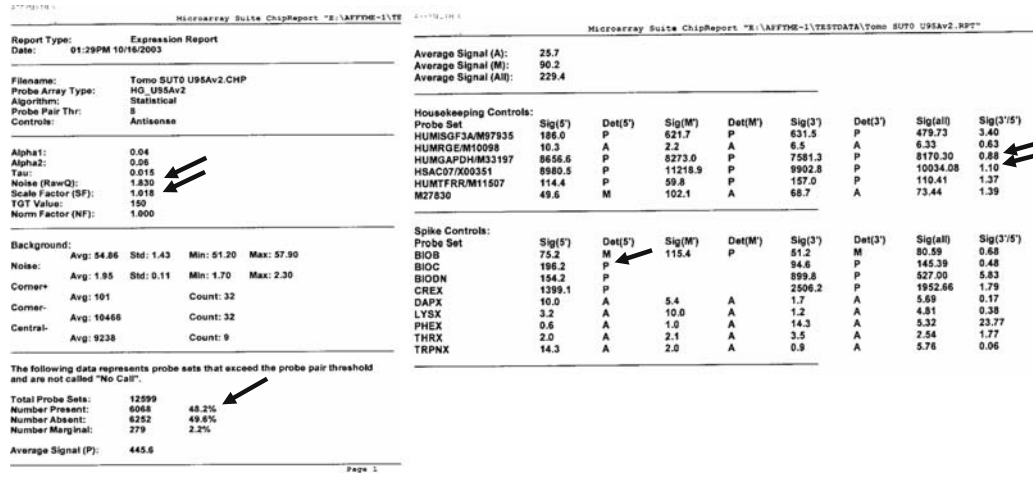


Fig. 15.1. Affymetrix microarray chip. The left side shows the entire chip, the right two zoom in to show details of pairs of perfect match and mismatch hybridization, top, and actual pixels, bottom. The picture on the bottom right shows the grid, thin white lines separating the chip features. Note that the rim pixels are discarded and only the 4×5 array of pixels in the middle is averaged to determine the level of hybridization.



Microarray Suite ChipReport "E:\AFFYME-1\TE

Report Type: Expression Report
Date: 01:29PM 10/16/2003

Filename: Tomo SUT0 U95Av2.CHP
Probe Array Type: HG_U95Av2
Algorithm: Statistical
Probe Pair Thr: 8
Controls: Antisense

Alpha1: 0.04
Alpha2: 0.06
Tau: 0.015
Noise (RawQ): 1.830
Scale Factor (SF): 1.018
TGT Value: 150
Norm Factor (NF): 1.000

Background:
Avg: 54.86 Std: 1.43 Min: 51.20 Max: 57.90
Noise:
Avg: 1.95 Std: 0.11 Min: 1.70 Max: 2.30
Corner+
Avg: 101 Count: 32
Corner-
Avg: 10466 Count: 32
Central-
Avg: 9238 Count: 9

The following data represents probe sets that exceed the probe pair threshold and are not called "No Call".

Total Probe Sets: 12599
Number Present: 6068 48.2%
Number Absent: 6252 49.6%
Number Marginal: 279 2.2%

Average Signal (P): 445.6

Page 1

Microarray Suite ChipReport "E:\AFFYME-1\TESTDATA\Tomo SUT0 U95Av2.RPT"

Average Signal (A): 25.7
Average Signal (M): 90.2
Average Signal (All): 229.4

Housekeeping Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
HUMISGF3A/M97935	186.0	P	621.7	P	631.5	P	479.73	3.40
HUMRGE/M10098	10.3	A	2.2	A	6.5	A	6.33	0.63
HUMGAPDH/M33197	8656.6	P	8273.0	P	7581.3	P	8170.30	0.88
HSAC07/X00351	8980.5	P	11218.9	P	9902.8	P	10034.08	1.10
HUMTFRR/M11507	114.4	P	59.8	P	157.0	P	110.41	1.37
M27830	49.6	M	102.1	A	68.7	A	73.44	1.39

Spike Controls:

Probe Set	Sig(5')	Det(5')	Sig(M')	Det(M')	Sig(3')	Det(3')	Sig(all)	Sig(3'/5')
BIOB	75.2	M	115.4	P	51.2	M	80.59	0.68
BIOC	196.2	P			94.6	P	145.39	0.48
BIODN	154.2	P			899.8	P	527.00	5.83
CREX	1399.1	P			2506.2	P	1952.66	1.79
DAPX	10.0	A	5.4	A	1.7	A	5.69	0.17
LYSX	3.2	A	10.0	A	1.2	A	4.81	0.38
PHEX	0.6	A	1.0	A	14.3	A	5.32	23.77
THRX	2.0	A	2.1	A	3.5	A	2.54	1.77
TRPNX	14.3	A	2.0	A	0.9	A	5.76	0.06

Fig. 15.2. Affymetrix chip report. The arrows point to the values important for evaluating the quality of the hybridization, as described in the text.

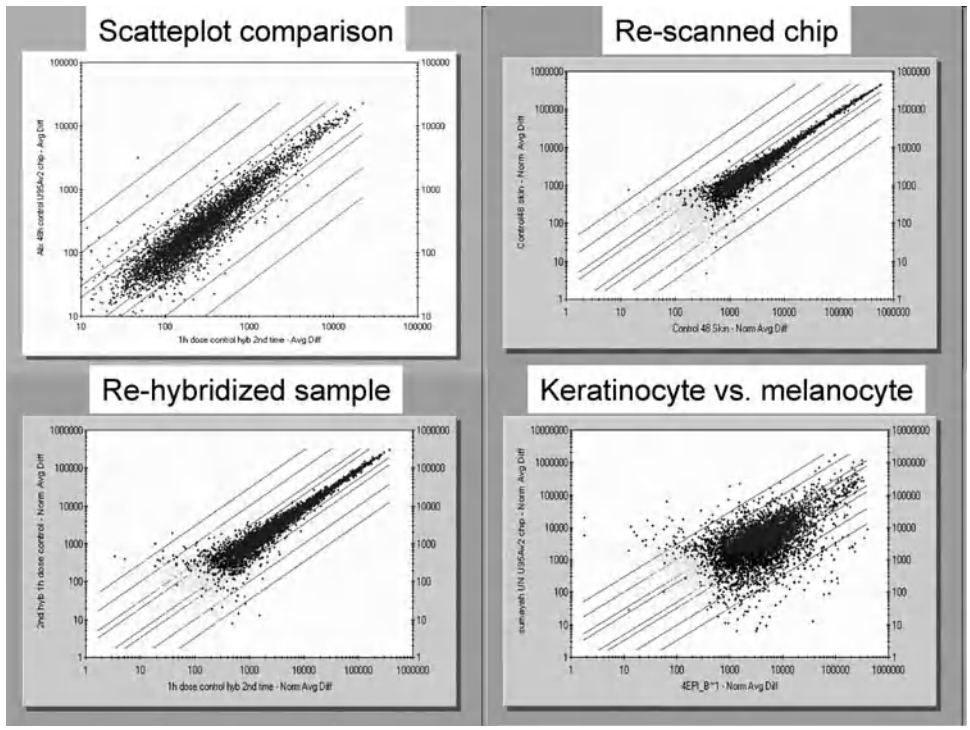


Fig. 15.3. Scatterplots. The hybridization values from two microarrays are plotted on each axis. Top left represents a comparison of two similar samples; top right re-scanning of the same chip, while bottom right duplicate hybridizations of the same sample to two chips. Note that even in the re-scanning the very low numbers do not perfectly repeat. Bottom right compares two very different samples, keratinocytes and melanocytes, where even the diagonal representing the similarly expressed genes is difficult to discern.

an outlying sample, as well as indicate the source of the discrepancy. Samples in **Fig. 15.4** demonstrate a poorly labeled sample and a misaligned scanner.

4. We would like to draw attention to another important and sometimes overlooked aspect of microarray analysis. Scanning a microarray has an inherent measurement inaccuracy, as do

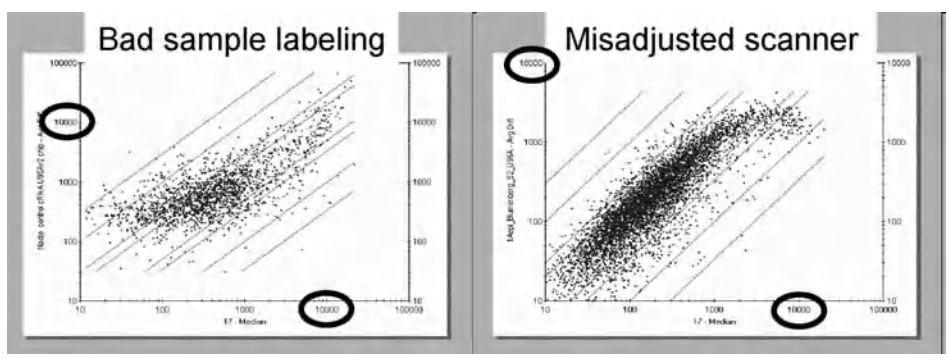


Fig. 15.4. Scatterplots of things gone wrong. Note the scale difference in the right panel.

all scientific measurements. But, while most of us are comfortable in deciding that a 10% difference in measuring DNA concentration (OD_{260} 1.00 vs. 1.10) is not meaningful, such differences are sometimes reported in microarray experiments as relevant. More importantly, we know that both readings of $OD_{260} = 0.002$ and $OD_{260} = 0.004$ mean that the DNA was lost somewhere during the preparation; however, such low values are accepted sometimes in microarray results and even interpreted as a 2-fold difference. In **Fig. 15.3** we demonstrate that even a re-scan of the very same microarray will show some deviation, especially at the low fluorescence values. Replicate hybridizations of the identical sample to two chips in parallel also show some difference at the low expression values, generally not much worse than the re-scan of the same chip. The attempts to compare very dissimilar samples, in this case keratinocytes vs. melanocytes, can also produce unacceptable scatterplots. These arguments indicate the importance of data normalization and selection, before sophisticated meta-analyses.

3.6. Array Data Analysis; GCOS

GeneChip GCOS v.-1.2 software (Affymetrix) is used for the first steps of data extraction and analysis (27, 37). In the following step-by-step protocol, first start the GCOS software from Affymetrix (**Fig. 15.5**).

1. In the left-side box chose "*Data Source: Local*". Double click "*Image Data*," choose the image file (.DAT) you want to see and analyze. When it loads, click "*Grid*" at the bottom to remove the white mask, then you can see the scanned image itself. Is the picture uniform or are there areas that are too dark or too bright? You may zoom in a small area with the mouse and the "*In*" button to see the details: Are there many dust spots or smudges? Check if the border frame of the image is appropriately dark. You should then restore the *grid* and check all four corners; make sure the grid is appropriately placed (**Fig. 15.1**, bottom right) and adjust if necessary.
2. If the quality of the image is OK, push the "*Analyze*" button above the image. You are then asked to enter a chip data name (.CHP). Then there comes the "*Expression Analysis Settings*" box. The signal of each probe array is scaled to the same target intensity value of, e.g., 500 arbitrary units. It is important to set the same value for all chips so that all can be compared to each other and with the subsequent experiments. Make sure to use the same default settings for Scaling, Normalization, etc. First analyze the 0-h control chip; in the subsequent analyses you can select "Baseline" and check the box of "*Use Baseline Comparison Data*," click "*Browse*," then you can pick up the .CHP file to be used as a control or for comparison

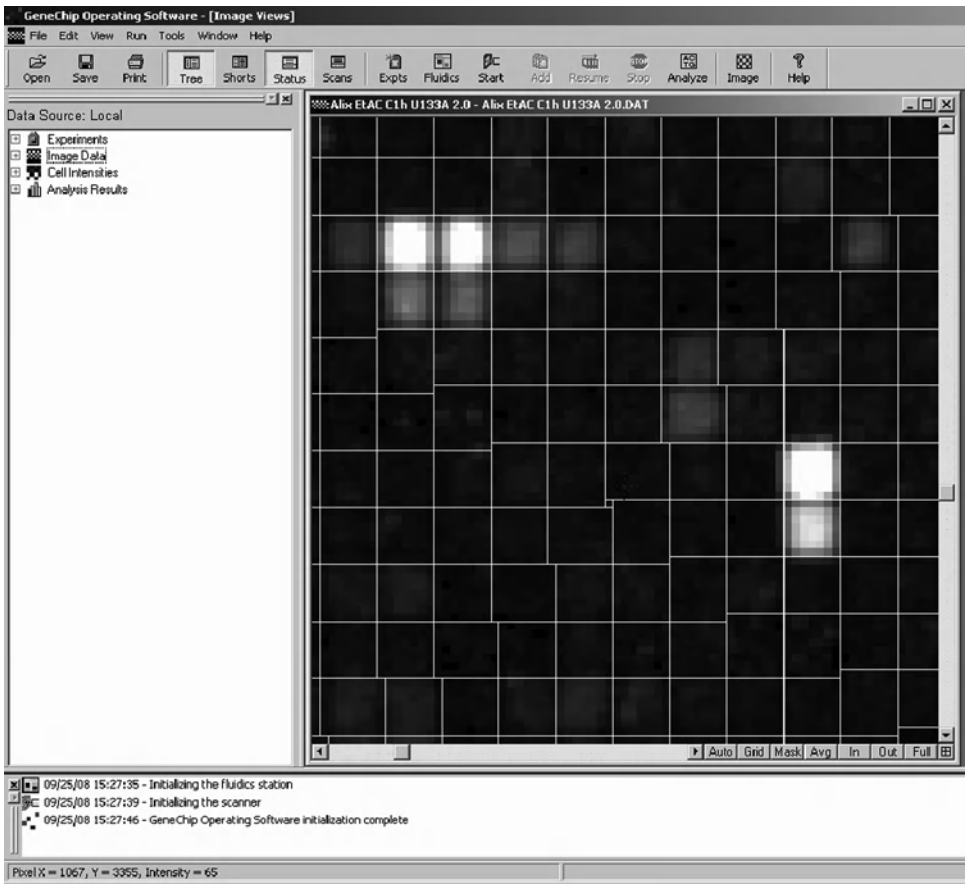
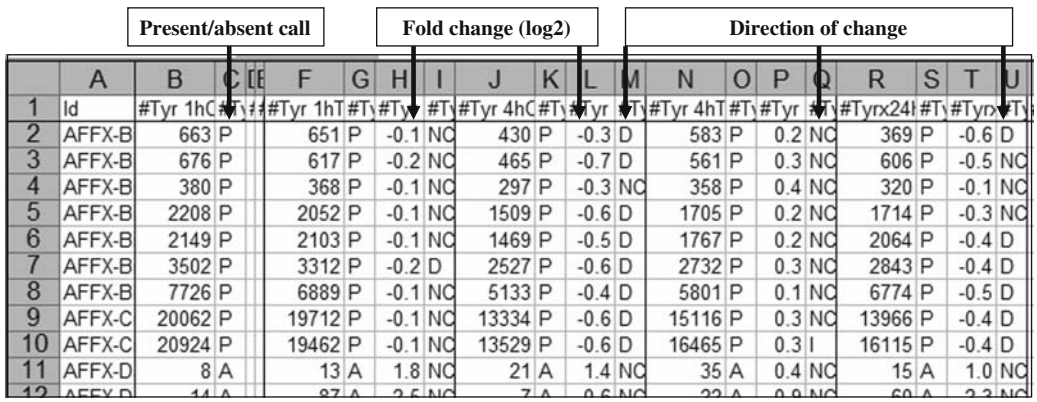


Fig. 15.5. Screenshot of the GCOS analysis result. The RPT, DAT, CEL, and CHP files are all accessible by opening the menus of Experiments, Image Data, Cell Intensities or analysis results, top left. Note that the picture of the chip is from an Image Data file and that the grid is present in the picture indicated by thin white lines.

with the image you are currently analyzing (i.e., comparing the current image to the already analyzed 0-h control image). Click "*OK*." Repeat for all the images you want to review and analyze.

3. Once you have finished viewing all the image data, double click "*Analysis Results*" in the left-side box and you will see all the results. You may re-format the result table by clicking "*Pivot*" at the bottom of the table and "*Options*" above the table. Here comes "*Analysis Options*," choose "*Pivot.*" In "*Statistical Absolute Results*," check the boxes of *Signal*, *Detection*, and *Detection p-value*. If you have chosen "Use Baseline Comparison Data" in the previous step, in "Statistical Comparison Results," check the boxes of *Signal Log Ratio* (The change in expression level for a transcript between a baseline and an experiment array. This change is expressed as the $\log_2$ ratio.), *Change*, and *Change p-value*.

4. In the result table, "*Signal*" means the absolute value of the expression level of the gene. For those with Detection p-value <0.04, they are considered "*present*," i.e., "*P*" in the Detection column, for those p≥0.06, absent, "*A*," and others, marginal, "*M*." For those compared with a baseline, in the column of "*Change*," *NC* means no change, *D* decrease, and *I* increase (**Fig. 15.6**).



	A	B	C	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
1	Id	#Tyr 1hC		#Tyr 1hT				#Tyr 4hC		#Tyr		#Tyr 4hT		#Tyr		#Tyrx24h		#Tyrx	
2	AFFX-B	663	P	651	P	-0.1	NC	430	P	-0.3	D	583	P	0.2	NC	369	P	-0.6	D
3	AFFX-B	676	P	617	P	-0.2	NC	465	P	-0.7	D	561	P	0.3	NC	606	P	-0.5	NC
4	AFFX-B	380	P	368	P	-0.1	NC	297	P	-0.3	NC	358	P	0.4	NC	320	P	-0.1	NC
5	AFFX-B	2208	P	2052	P	-0.1	NC	1509	P	-0.6	D	1705	P	0.2	NC	1714	P	-0.3	NC
6	AFFX-B	2149	P	2103	P	-0.1	NC	1469	P	-0.5	D	1767	P	0.2	NC	2064	P	-0.4	D
7	AFFX-B	3502	P	3312	P	-0.2	D	2527	P	-0.6	D	2732	P	0.3	NC	2843	P	-0.4	D
8	AFFX-B	7726	P	6889	P	-0.1	NC	5133	P	-0.4	D	5801	P	0.1	NC	6774	P	-0.5	D
9	AFFX-C	20062	P	19712	P	-0.1	NC	13334	P	-0.6	D	15116	P	0.3	NC	13966	P	-0.4	D
10	AFFX-C	20924	P	19462	P	-0.1	NC	13529	P	-0.6	D	16465	P	0.3	I	16115	P	-0.4	D
11	AFFX-D	8	A	13	A	1.8	NC	21	A	1.4	NC	35	A	0.4	NC	15	A	1.0	NC
12	AFFX-D	14	A	87	A	2.6	NC	7	A	0.6	NC	22	A	0.9	NC	60	A	2.3	NC

Fig. 15.6. Spreadsheet of GCOS analysis results. The present/absent/marginal calls are indicated. Note that the fold regulation is given in $\log_2$ units (1 = 2-fold increase, –2 = 4-fold decrease, etc.). The direction of change is indicated as no change, "NC," increase, "I,, decrease "D" or marginal increase and marginal decrease, "MI" and "MD."

5. Save the result of the GCOS analysis as .txt file. You will later open it with Excel. GeneChip data mining software, Affymetrix-DMT, version 3.0, is used to derive scatterplots (**Fig. 15.3**), Self-Organizing Maps, and Correlation Coefficient Clusters of the genes.
6. Absent Genes marked by the program as absent from all chips are discarded. We also calculate the median and the standard deviation of all absent genes. Then, to eliminate genes exhibiting potentially false differential expression, we select only those genes that possessed relative signal intensity greater than one standard deviation above average for all genes scored as "absent" in the samples. All those genes with expression values below the median + standard deviation in all microarrays are discarded, as these values are considered to be insufficiently reliable. In practice, if a chip is scaled to 500, values below 100 are discarded. Save this spreadsheet as a new file. If you do not plan to use RMAExpress (see below), you can use the data from this file to annotate the genes, find overrepresented ontological categories, promoter analyses, etc.
7. We usually examine the absolute expression levels individually, calculate fold changes against controls, and performed the *t*-test and the Mann–Whitney test against controls using GCOS programs. In a time course experiment (27–29, 40),

genes are considered regulated if both of the following criteria were met in at least one time point: (1) the GCOS statistical test must find the gene regulated (induced or suppressed) and (2) the expression level in the treated sample must be more than 2-fold induced or suppressed (>1 or <–1 on $\log_2$ scale) relative to the control. We do not further analyze genes whose function is unknown or hypothetical, according to our annotation table, based primarily on the Gene Ontology Consortium data (27, 38).

3.7. RMA Express

RMAExpress can be downloaded from http://rmaexpress.bmbolstad.com/. It is a stand-alone program for computing gene expression values for Affymetrix chip data using the Robust Multi-chip Average expression summary. RMA can also perform chip quality assessments. It consists of three steps: background adjustment, quintile normalization, and summarization (41). To run RMA, you will need to download from the Affymetrix site www.affymetrix.com the appropriate *CDF* file for your chip type, e.g., HG-U133A_2.cdf. RMAExpress uses the *.CEL* files from your data set.

In most cases you do not need to use the RMADataConv program. You should just read in your data directly into RMAExpress using the "Read Unprocessed files" option. It will ask you for the locations of your CDF and CEL files, and then it will read in the data. Next, select "Compute RMA measure" to actually compute the expression measure. It will bring a dialog box up asking you to select specific options (just leave the defaults), press ok and it will compute RMA expression summaries. Then choose "Write results to file" to output the expression summaries to a text file.

1. Once you have downloaded and installed RMAExpress and the CDF file, invoke the program (**Fig. 15.7**). Click on "File" -> "Read unprocessed file." There comes a dialog box, "Please select your CDF file," select the data file corresponding to the type of chip you are using, e.g., HG-U133A_2.cdf. Then another dialog box appears, "Please select your CEL file," select the files you want to analyze. Then you may use the menu bar "File"–"Add new CEL files" to add additional CEL files for the current analysis. After it shows "Done reading in datafiles," you may select from the "File" menu – "Compute RMA measure." A dialog box "Select preprocessing steps" appears; for "Background Adjust" chose "Yes," for Normalization, "Quantile" (sic), and check the box of "Store Residuals,"
2. After it is done, select from the menu bar Show-Residual Images. Now you can check the quality of the chips. For example, chips that are consistently and evenly pink are very good, while a chip that shows blue smudges or a red border probably is not a good chip (**Fig. 15.8**).

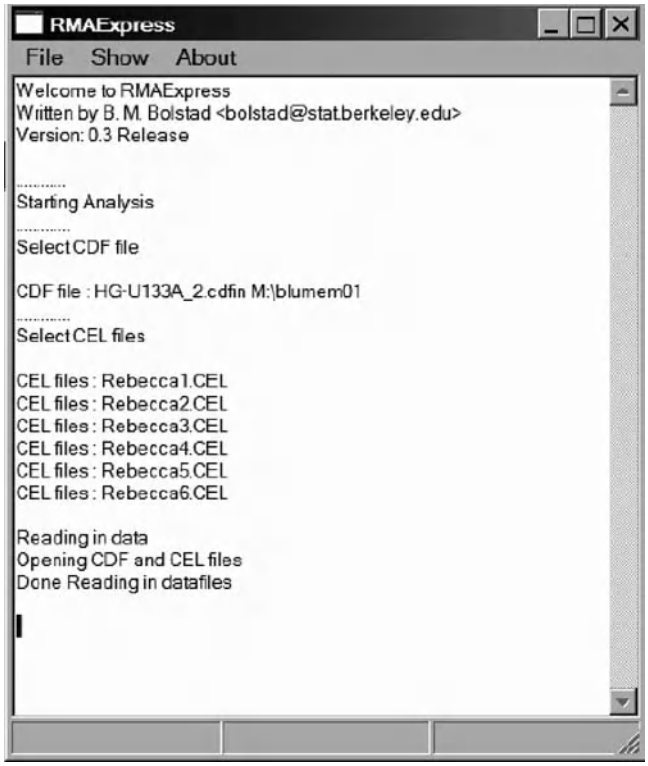


Fig. 15.7. Screenshot of RMA express.

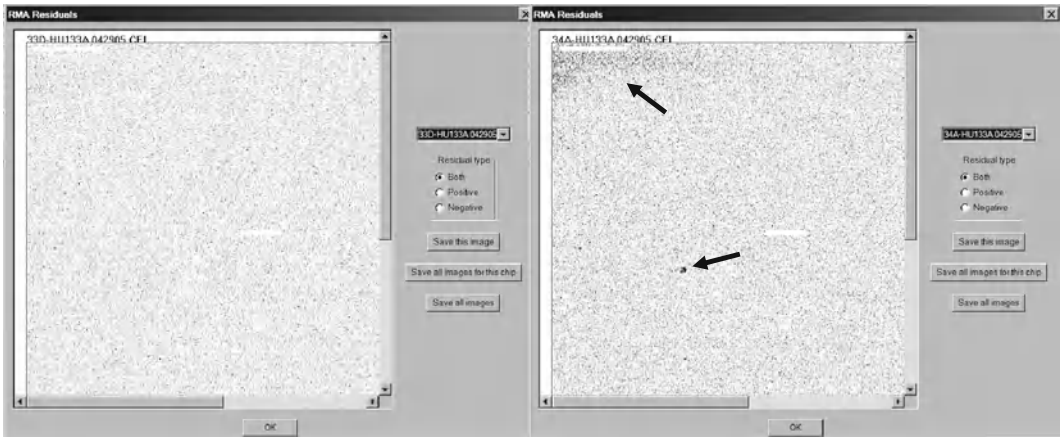


Fig. 15.8. RMAExpress "residuals." A near-perfect microarray is shown on the left. The one on the right shows uneven hybridization and a smudge, arrows.

3. Save the results as text file: "File"–"Write results to file (log scale)," and "Write results to file (natural scale)." You will later open it with Excel (**Fig. 15.9**).

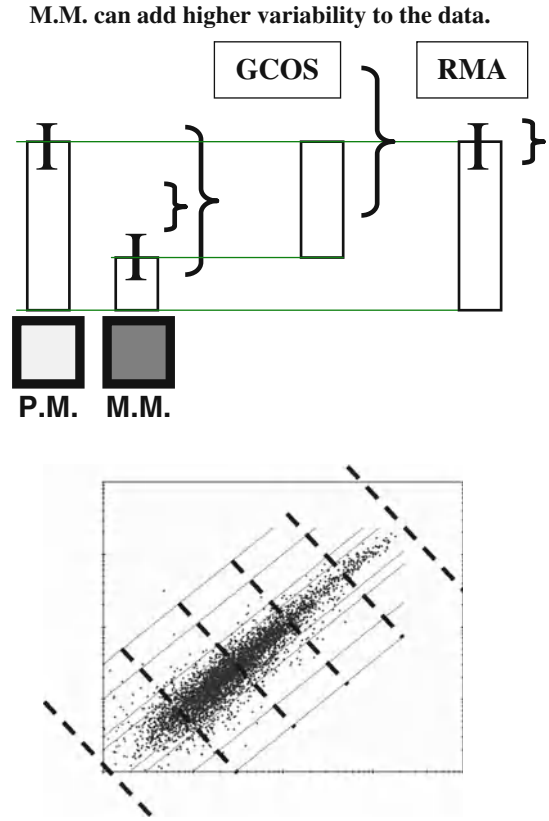


Fig. 15.9. Differences between the GCOS and RMA approaches. The mismatch, MM, values can add to the data variability, top. RMAExpress uses perfect match values only and normalizes the expression values in quintiles, across all the chips, bottom.

3.8. Integrating the Results of GCOS and RMAExpress

1. Use Excel to open two .txt result tables, the RMAExpress table with log scale and the GCOS results table. Merge the two files by using copy and paste functions (alternatively, use the dataloader function of the add-ins suite of programs, www.add-ins.com, which we find to be inexpensive but very useful). *Make sure the order of the genes of GCOS, RMA, and annotation table are the same before you copy and paste!* This is easily accomplished by sorting the two files by their Affymetrix IDs in the first column. Also copy and paste part of the functional annotation table, e.g., HG-U133A_2_annot, into the above merged table, including at least the columns of gene title, gene symbol, function, and Entrez Gene id. Now you have a table showing results of GCOS, RMAExpress, and functional annotation together. Make sure to save this original version of the data, you may have to return to it if you make a mistake later.

2. First, filter out all those genes that are not expressed in any of your samples. Use the results of GCOS to select those genes at least "present" (P) once in all the experiments analyzed. Select the first row of the table and from the menu bar click on "Data," "Filter," and select "Autofilter." Now each cell in the first row has a downward arrowhead. For *all* those columns showing the results of Detection from GCOS (i.e., columns with P, A, or M), click the arrowheads, choose "custom," then "does not equal to," then type "p." Delete all the rows that remain. Now from the menu bar click on "Data," "Filter," and select "Show All." What remains are only those genes that are present at least once. Sort the spreadsheet again to remove the empty rows. Save as a new file, e.g., "Present." Since we have finished using GCOS data at this time, you might want to delete the columns derived from the GCOS file retaining the RMA data, to make the table smaller and easier to manipulate. (Alternatively, you may want to keep the "Signal Log Ratio" and "Change" columns, to compare the GCOS and RMA results.)
3. Next, select those genes that are regulated at least once in all the samples compared. First, subtract the expression level of the control/background chip from the level on the experimental chip to obtain the fold change of expression in experimental compared with the control samples. Insert a new blank column; this will be the fold change column. Find the two columns of RMA expression level (note the data are in $\log_2$ scale) for both the experiment and the control in the table, say column B and column C (**Fig. 15.10**). In the uppermost cell corresponding to the first gene of the blank column, type "=B2-C2," then enter. You get the difference between the values in B2 and C2 cells. Since it is in $\log_2$ scale, "1" represents a 2-fold upregulation and "–1" a 2-fold downregulation. You will notice there is a small "+" on the right lower corner of that cell. Just hold the mouse and drag all the way down to the last cell corresponding to the last gene of that

	A	B	C	D	E	F	G	H
1	Probesets	Control	Chip 1	Chip 2	Chip 3	C - B	D - B	E - B
2	212143_s_at	11.292	11.336	11.288	11.331	**0.04**	**0.00**	**0.04**
3	210095_s_at	7.547	9.900	9.586	9.388	**2.35**	**2.04**	**1.84**
4	210096_at	6.637	5.534	5.591	5.719	**-1.10**	**-1.05**	**-0.92**
5	205363_at	8.380	8.406	8.462	8.417	**0.03**	**0.08**	**0.04**
6	207324_s_at	3.641	4.804	4.480	4.712	**1.16**	**0.84**	**1.07**
7	213240_s_at	8.598	7.017	6.617	6.546	**-1.58**	**-1.98**	**-2.05**

Fig. 15.10. Spreadsheet of RMAExpress analysis results**.** The data are already $\log_2$ transformed; to get fold regulation, the values are simply subtracted.

blank column. In the same way, you can obtain the differences between any two pairs of chips you want to compare. Save this spreadsheet.

4. You may use the same method as above to exclude those genes not regulated even once. For example, if you set 2-fold as the threshold of regulation, from the downward arrowheads of those columns you just obtained, choose "custom," then choose "is less than" and type "1" in the right box, click "and," on the lower box, choose "is greater than," type "–1" in the right box. After you have done so for all the columns of results of subtraction between the experiment and the control, delete all the rows that remain in the table. This will delete all rows where expression levels are different – less than 2-fold. From the menu use "Data,""Filter,""Show All," re-sort. Save as a new file, which contains all the genes at least regulated once. Sort this table by Gene Symbol, eliminate those redundant, duplicated rows to make all genes with the same gene symbol appear only once in this table. Save this file. Use this file as a basic work-horse table to select those genes you want to analyze with the following programs.

3.9. Annotation of Gene Lists

We developed an extensive gene annotation table describing the molecular function and biological category of the genes present on the chip (T. Banno, and M. B., unpublished). The table is based on the data by J.M. Rouillard (42) and the Gene Ontology Consortium http://cgap.nci.nih.gov/Genes/GOBrowser http://dot.ped.med.umich.edu:2000/ourimage/pub/shared/JMR_pub_affyannot.html. The regulated genes were functionally classified according to this table, which is available from the authors.

1. For annotation we find the DAVID program extremely useful and convenient http://david.abcc.ncifcrf.gov/. Once on this site (**Fig. 15.11**), choose "Start Analysis" in the upper bar. In the left blue box, choose "Upload" and under Enter Gene List field, paste a list of the genes you want to analyze from work-horse Excel file. This is done most conveniently by sorting the spreadsheet by descending fold change values, selecting the IDs from the first column for all values >1 (i.e., 2-fold upregulated) and pasting directly into the DAVID field. Select Identifier: Affy_ID; choose List Type: Gene List, and click Submit List.
2. After a few seconds the "Gene List Manager" appears in the left blue box. In "Select to limit annotations by one or more species" choose "HOMO SAPIENS" if you only want to analyze human genes (of course, not if you work with murine samples). For "Background," usually you do not have to do anything. Now you have "successfully submitted gene list," Step 1. To Analyze above gene list with one of DAVID tools choose "Functional Annotation Tool." Now appears the "Annotation Summary Results" screen.

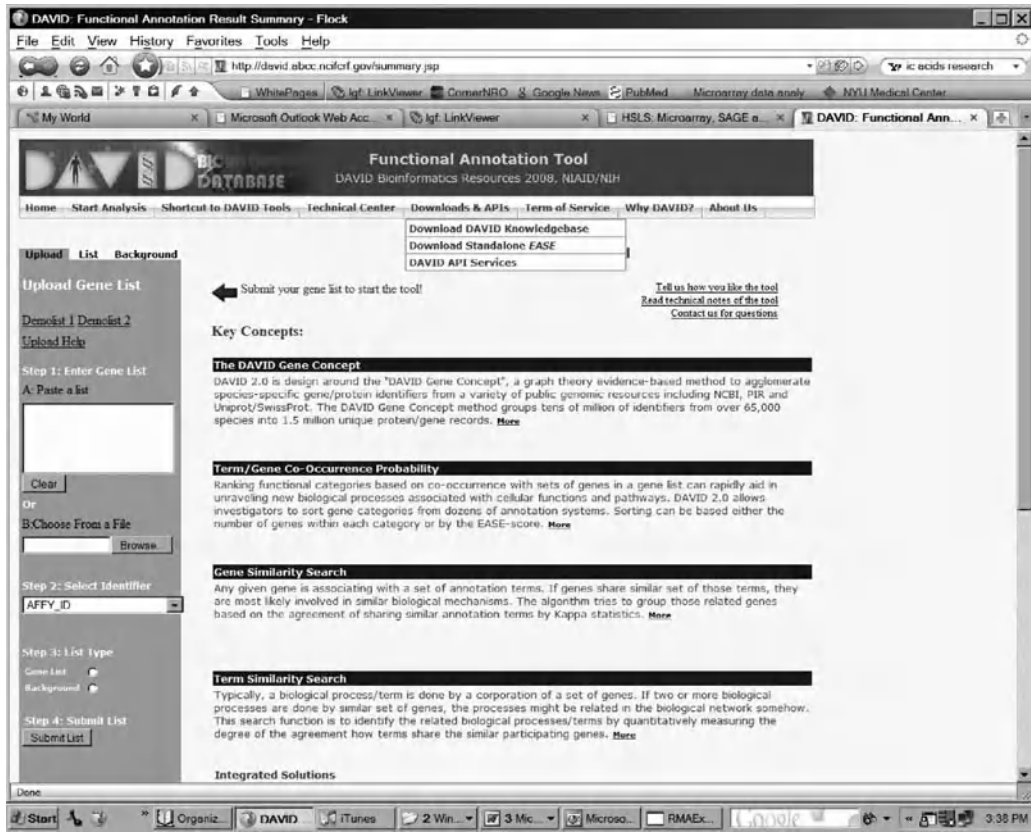


Fig. 15.11. Screenshot of the DAVID analysis portal. The list of regulated genes, as Affymetrix IDs, can simply be pasted in the field A.

3. In the set of menus we find the following most useful:
   In"Main Accessions": AFFY_ID, ENTREZ_GENE_ID, and GENBANK_ACCESSION (**Fig. 15.12**).

   In "Other Accessions": none.

   In "Gene Ontology": GOTERM_BP_ALL, GOTERM_CC_ALL, and GOTERM_MF_ALL, and somewhat redundant PANTHER_BP_ALL and PANTHER_MF_ALL.

   In "Protein Domains: none.

   In "Pathways": BIOCARTA and KEGG_PATHWAY.

   In "General annotations": CYTOBAND, GENE_NAME, and GENE_SYMBOL.

   In "Functional Categories": SP_PIR_KEYWORDS.

   In "Protein Interactions" and "Literature": none.

   In "Disease": OMIM_DISEASE.

   In "Tissue Expression": none.

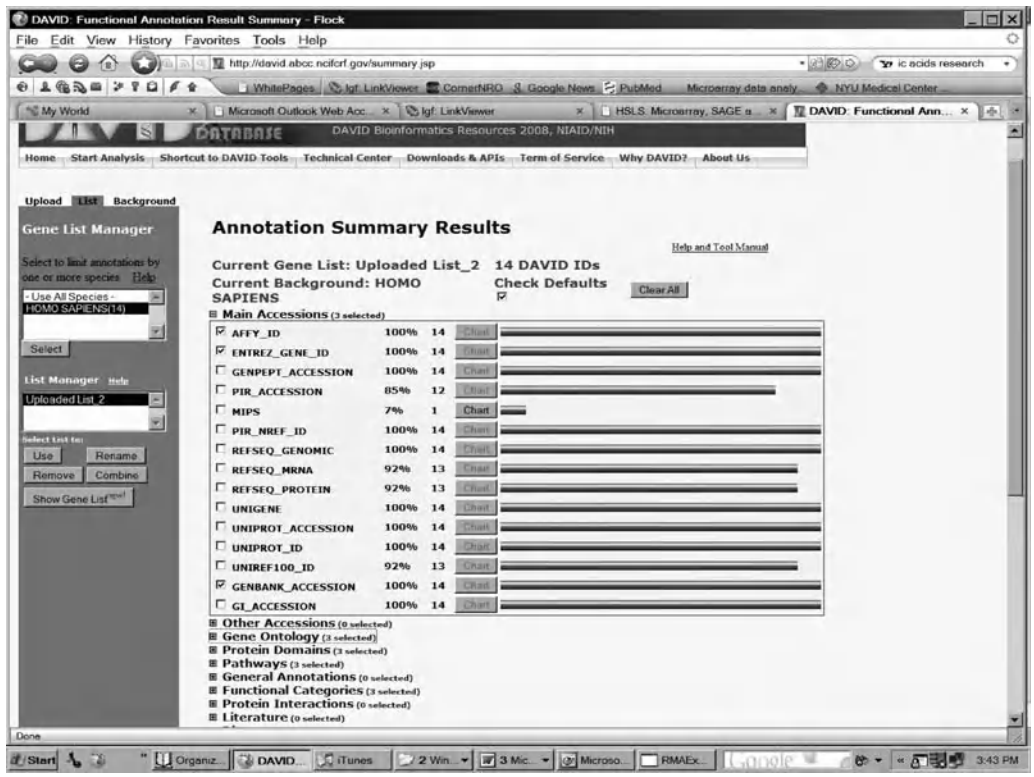


Fig. 15.12. Choosing the annotation fields in DAVID. The chosen annotations will be displayed for all submitted genes in an easy to download spreadsheet.

4. You should experiment with these and see which ones are useful to you, which are not. You may want more detailed descriptions or you may not be interested in chromosomal location, etc. However, it is important to choose a consistent set of annotations and use the same set in future analyses, which will make all the spreadsheets have a consistent structure.
5. Choose "Functional Annotation Table." Here come the results. Choose "download file" near the right upper corner and save it as a text file. Click once (not twice) the file, use the right button of mouse to choose "open with" and select "Microsoft Office Excel." In the Excel file, you get many annotation columns; save this file as "Microsoft Office Excel Worksheet" file type. This is a very important file because it contains all the annotations and identifiers for all the genes. Note, however, that it does not have the results of your microarrays: the chip data are not in it. If you want, you can insert them using the DataLoader function of the Add-ins (see above).

6. Return to DAVID and click "Functional Annotation Chart." Here come the results. Choose "download file" near the right upper corner and save it as text file. Click the file once (not twice), use the right button of mouse to choose "open with" and select "Microsoft Office Excel." In the Excel file, the ontological categories are sorted by the p-value. In general, p-values below 10^{-5} are highly significant! These ontological categories are statistically over-represented in the set of genes you submitted. Save this file as "Microsoft Office Excel Worksheet" file type. Back in David, you can click on "Term" to have it described, "RT" for related terms, and "Genes" to get the list of genes that belong to this ontological term. You can play with the options; we sometimes find it useful to restrict the categories to those with four or more genes.
7. Return to DAVID and click "Functional Annotation Cluster." Here come the results. Choose "download file" near the right upper corner and save it as text file. Click the file once, use the right button of mouse to choose "open with" and select "Microsoft Office Excel." Save as "Microsoft Office Excel Worksheet" file type. Back in David, you can click on "Term" to have it described, "RT" for related terms, and the horizontal bar to get the list of genes that belong to this *cluster of ontological terms*. You can play with the options; we did not find it useful. The important value in this view is "Enrichment Score." While cut-off depends on the number of genes submitted, for 3–400 genes, scores >4 are very significant.

3.10. L2L Microarray Analysis Tool

A simpler way to analyze quickly the biological processes, molecular functions, and cellular components in your list can be found in the L2L set of programs http://depts.washington.edu/l2l/ (**Fig. 15.13**).

1. Name your data. Be sure to use a new file name whenever you do a new analysis.
2. Upload your data. The file should be a text file with Affy_ID of all the genes you want to analyze. You cannot copy and paste directly from the work-horse spreadsheet, you have to create a new spreadsheet with only the Affy_IDs and save it as a .txt file.
3. Select your array type, e.g., HG-U133A 2.0.
4. Select a database for comparison. There are seven databases to be chosen, see below. Each one is a totally different database and you should use a new file name for each in step 1. Submit for processing. You may save the result by choosing one of the saving options at the bottom, but the "printer friendly" version is not, and the "Raw data" version gives you a long table of all results. We find it much more convenient to select all the

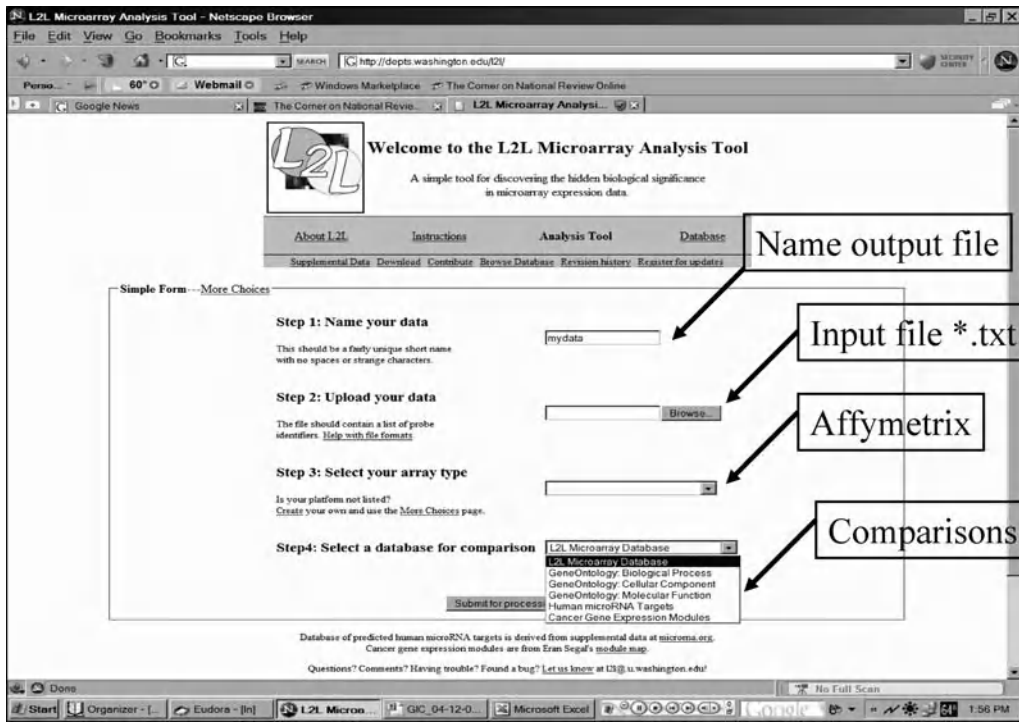


Fig. 15.13. Screenshot of the L2L analysis portal. The needed inputs are marked with arrows.

data in the spreadsheet, then right-click and use the copy function, switch to a new excel file, and right-click the first cell and choose the "paste special" with "text" to download the data. In the Excel worksheet you can edit the data easily. This way, only the results with a significant p-value, e.g., $<10^{-3}$, will be kept. Once you get the result, you may click the number in "actual matches" to see the genes in your list that were selected.

Of the seven databases to be chosen, Gene Ontology, Biological Process, Molecular function, and Cellular Component are the most useful. These will tell you a lot about functions of the genes in your list. The Reactome Protein–protein interactions will suggest which signaling pathways are over-represented in your list (note these are not the signaling pathways that induce your genes; these are the pathways that the induced genes belong to). The L2L microarray database will compare your list with those uploaded by the L2L managers; we did not find this informative: all our lists significantly overlapped all uploaded lists of keratinocyte genes. The Human microRNA targets can suggest which miRNAs the genes in your list may have in common. Be very careful with the results: algorithms predicting miRNA targets are notoriously unreliable at the moment. Use this list only as a hypothetical, preliminary, initial direction-suggesting step. The seventh

database, Cancer Gene Expression Modules, can be useful, but it leads to complex data analysis approaches, which are beyond the scope of this chapter.

3.11. LOLA: List of Lists Annotated

Once you start generating lists of genes from multiple experiments or downloaded from publications, you will want to compare lists to see whether they have similarities, intersect, or not. A convenient tool for this can be found at LOLA http://www.lola.gwu.edu/ (**Fig. 15.14**).

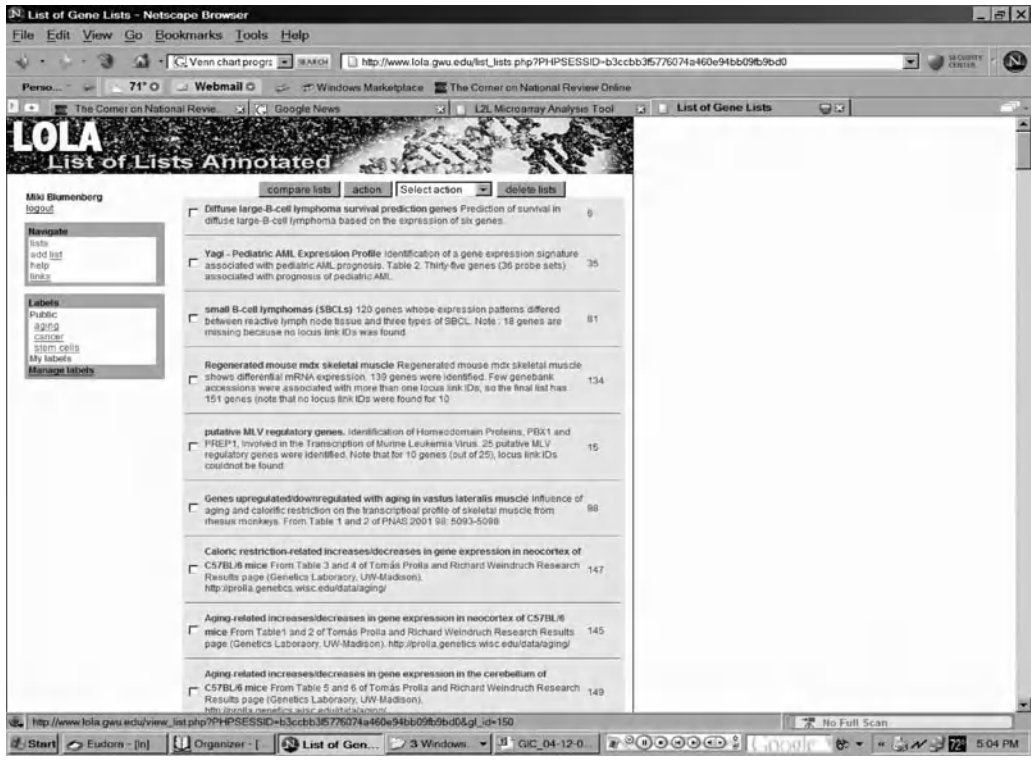


Fig. 15.14. Screenshot of the LOLA analysis result. The site provides several sets of genes, but more useful is to build a collection of your own gene sets.

1. You should set up an account for your group of collaborators. LOLA will continue to remember all your lists. Go to the LOLA site and sign in before you begin. Once you sign in successfully, there comes a list of all gene lists you have added to the database before, if any. If you want to add a new list, choose "add list" in a left-side box. Fill in a gene list name, choose the species and the gene identifier type. You may upload a text file with the gene identifiers or just paste gene list. Then click on "create gene list." LOLA will look for the genes and return the found and the not-found genes. It will ask you whether you want to submit the list, if yes, click the

submit gene list button near the bottom of the page. Now the newly added list should be within the lists (usually added to the bottom).

2. For "compare lists," just choose those lists you want to compare. You may choose as many lists as you want at a time. LOLA will compare all possible pairs. Or you may just choose two lists, compare, and then do it again with the other two. After you have picked those lists you want to compare, choose "compare lists." Once you get the result, you may copy and paste the result to an Excel worksheet for subsequent editing. Usually those pairwise comparisons with a p-value $<10^{-4}$ will be kept.
3. If you are interested in the genes common to two lists, you can click on the number in the intersection column and the intersect pops up. You can save it by clicking the "save" button at the bottom. In the next screen remember to indicate that you are submitting Entrez Gene identifiers, not Affymetrix. In the next screen click the "submit gene list" button and it will be saved as "Intersection of your-name-of-the-first-list and your-name-of-the-second-list."

3.12. Clustering and Comparison of Lists

There are several additional tools available for comparisons and clustering of lists. Commonly, microarray studies include hierarchical trees of genes, self-organizing maps, and principal component analyses.

1. The data set containing the expression patterns of the regulated genes can also be clustered and visualized using Cluster and Tree View software available at http://rana.stanford.edu/software (43). First, the data are imported into the Cluster and Tree View software in a tab-delimited format. A data set containing the expression patterns of the regulated genes can be clustered based on the similarity of gene expression and based on the similarity between different samples (i.e., clustering genes or clustering chips, respectively). The clusters are observed using the TreeView program (44).
2. Many of clustering programs are available from http://www.tm4.org/; these are very easy to implement and apply. They could be useful, in theory. However, in practice, we generally did not find these very informative. They will cluster genes into induced and suppressed, highly induced, early induced, or similar. Individual clusters can, then, be analyzed using DAVID or L2L. In studies using many chips with many patient samples, clustering of the samples (as opposed to genes) can be extremely interesting, pointing out to a specific subset of, e.g., patients. We had no opportunity to use clustering programs this way.

3. We do find simple Venn diagrams also very useful. The site http://www.pangloss.com/seidel/Protocols/venn.cgi is very easy and convenient to use. You upload three or four lists of genes, Affymetrix or any other identifying symbol, and it will generate the diagram as well as provide the lists of all intersect and unique genes, as in **Fig. 15.15**.

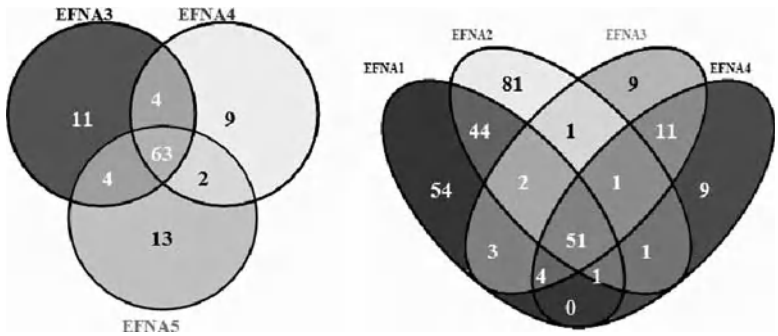


Fig. 15.15. Venn diagrams. The overlaps among three or four lists of genes can be easily visualized.

3.13. Promoter Analysis

We find the oPOSSUM set of programs for promoter analysis, http://www.cisreg.ca/cgi-bin/oPOSSUM/opossum, very convenient and easy to use (45, 46). For "Human Single Site Analysis," we prefer to use "Custom Analysis," button near top right, which allows better-tailored analysis (**Fig. 15.16**).

1. Choose species (human), gene ID type (usually we use Entrez gene, or RefSeq; you cannot use the Affymetrix IDs), and enter a list of co-expressed genes; it is easiest to copy and paste gene IDs. The list will be compared to a list of 15,000 random genes.
2. Select transcription factor binding site matrices; we usually select by taxonomic supergroup, choose "vertebrate."
3. Select parameters. You can calibrate the following parameters to obtain the optimal statistical p-value. (**a**) Level of conservation: Top 10%, 20%, or 30% of conserved regions; this depends on the % homology between the human and murine promoter sequences that will be analyzed. We use 30%, the least restrictive cut-off. (**b**) Matrix match threshold: 75%, 80%, or 85%; this is the % match between the "optimal" canonical transcription factor binding site and the similar site in the promoter DNA. We use 80%, which gave us the best p-value for the NF-kB-dependent genes (29). (**c**) Amount of upstream/downstream sequence. We usually start with the promoter-proximal sequences, e.g., 250/0 and repeat in 250–500 bp increments (*see* **Fig. 15.17**). Once you have decided the parameters to be used, you have to decide "Number of results to display," choose "**OR** only results with Z-score $>= 10$ and Fisher score $<=$ 0.001." Finally, "sort results by Fisher score." Then submit.

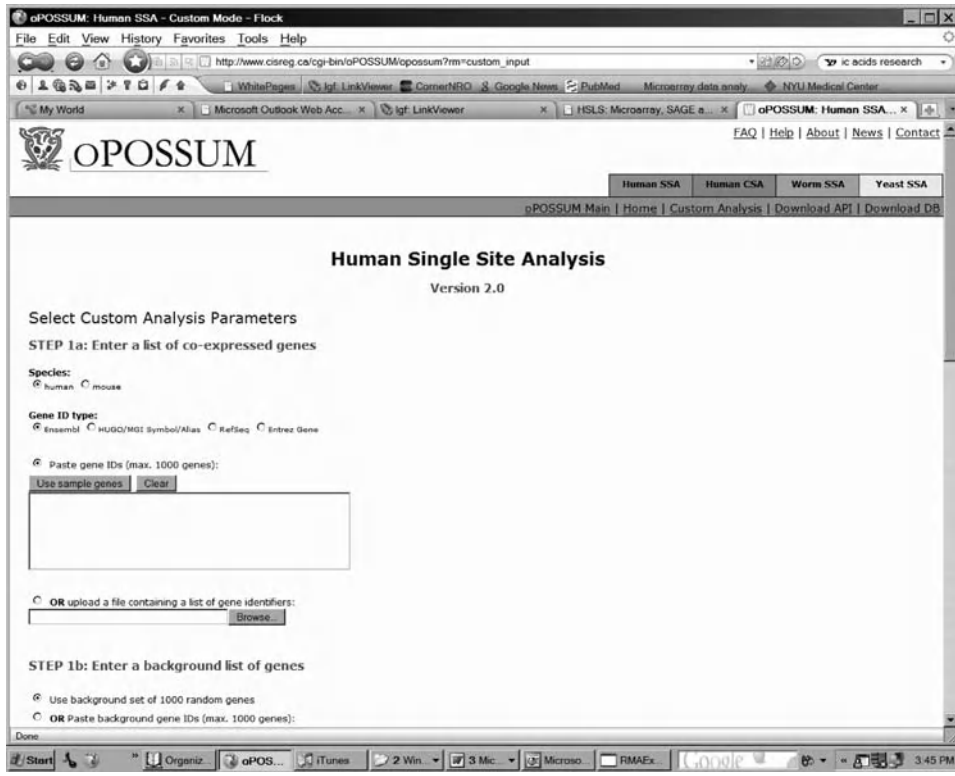


Fig. 15.16. Screenshot of the oPOSSUM analysis result. Remember to click on the custom analysis button, which will give you more options for analysis.

4. The next screen will mirror the parameters you choose, on top, which is followed by the lists of included and excluded genes. The included ones are those for which human/murine sequences were found and are among the, say, top 30% of all comparisons; the excluded did not make this cut.
5. Below this is the data file (**Fig. 15.18**). The first three columns give the transcription factors, their classes, and their information contents, "IC." This last value is related to the size of the binding site: small values, 8–10, are usually 4–5 bp long and occur very frequently in all sequences; large values, >14, are larger and rarer sites. "Target gene hits" are different from "Target TFBS hits," because some genes have multiple transcription factor binding sites, TFBSs. The most important columns are the "Z-score" and "Fisher score," which are calculated from the target TFBS hits and the target gene hits, respectively. Fisher score better than 10^{-5} is usually highly significant.
6. The analysis result can be downloaded as text file and then be opened with Excel to edit.

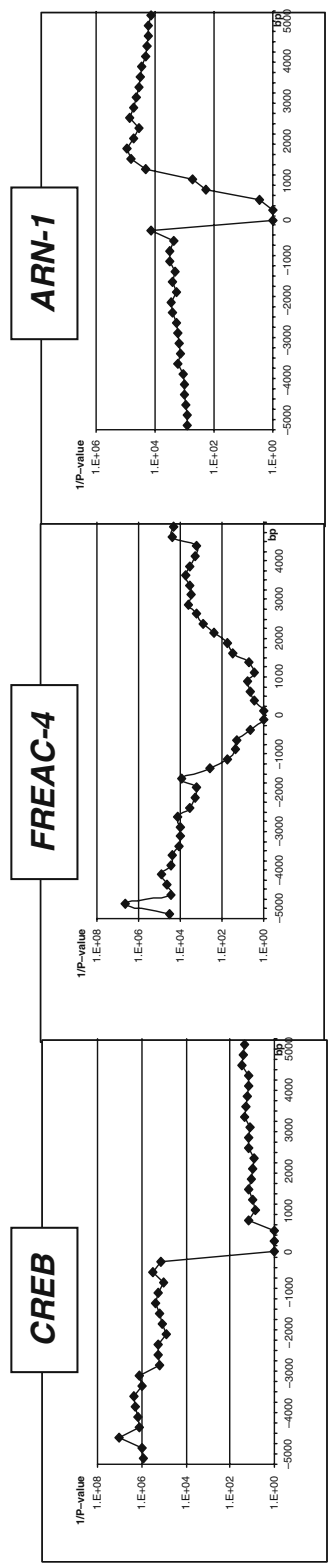


Fig. 15.17. Results of an oPOSSUM analysis. The binding sites for different transcription factors can aggregate close upstream from the transcription start site or be found at significant lengths, upstream or downstream. Data from (41).

Analysis Results

Selected Parameters

Conservation level: Top 10% of conserved regions (min. conservation 70%)
Matrix match score: 80%
Upstream sequence length: 5000
Downstream sequence length: 5000
Number of genes submitted: 59
Number of genes included: 30
Number of genes excluded: 29

Target Genes

Analyzed: NM_003380 NM_080927 NM_005010 NM_006158 NM_001175 NM_004723 NM_004811 NM_000888 NM_001255 NM_006290 NM_015714 NM_001005619 NM_005556 NM_052815 NM_007350 NM_007308 NM_001813 NM_001274 NM_022549 NM_001007231 NM_001786 NM_138578 NM_003633 NM_004083 NM_004336 NM_018351 NM_016522 NM_006640 NM_000268 NM_001003828

Excluded: --- NM_002859 NM_000213 /// NM_001005731 NM_001191 NM_003897 NM_000345 NM_033379 NM_016343 NM_001449 NM_000498 NM_005103 NM_013327 NM_000224 NM_199187 NM_016418 NM_181825 NM_181826 NM_181827 NM_181828 NM_181829 NM_181830 NM_181831 NM_181832 NM_181833 NM_181834 NM_181835 NM_014882

oPOSSUM Analysis

TF	TF Class	TF Supergroup	IC	Background gene hits	Background gene non-hits	Target gene hits	Target gene non-hits	Background TFBS hits	Background TFBS rate	Target TFBS hits	Target TFBS rate	Z-score	Fisher score
RELA	REL	vertebrate	14.757	4846	10304	15	15	8017	0.0035	40	0.0064	12.2	3.056e-02
REL	REL	vertebrate	10.515	7798	7352	22	8	18677	0.0081	74	0.0117	10.17	1.242e-02
ELK1	ETS	vertebrate	8.812	10697	4453	24	6	41413	0.0180	142	0.0225	8.618	1.778e-01
SPIB	ETS	vertebrate	9.060	13162	1988	28	2	132552	0.0403	416	0.0462	7.599	2.275e-01
NF-kappaB	REL	vertebrate	13.345	5960	9190	15	15	11447	0.0050	44	0.0070	7.173	1.570e-01
SP1	ZN-FINGER, C2H2	vertebrate	9.719	9192	5958	22	8	36361	0.0158	121	0.0192	6.889	1.074e-01
MZF1_5-13	ZN-FINGER, C2H2	vertebrate	9.400	9979	5171	25	5	42890	0.0186	140	0.0222	6.692	2.919e-02
NFYA	CAAT-BOX	vertebrate	12.925	4824	10326	16	14	8190	0.0057	30	0.0076	6.425	1.196e-02
ESR1	NUCLEAR RECEPTOR	vertebrate	17.683	439	14711	2	28	465	0.0004	3	0.0009	6.402	2.163e-01
STAT1	Stat	vertebrate	18.431	2165	12985	7	23	2693	0.0016	12	0.0027	6.352	1.269e-01

Fig. 15.18. Results of an oPOSSUM analysis in a spreadsheet form**.** Additional information can be obtained by clicking on the blue values.

4. Notes

1. The cells are expanded through three passages for the experiments, trypsinized with 0.025% trypsin, which was neutralized with 0.5 mg/ml of trypsin inhibitor. We avoid using serum to neutralize the trypsin because it can promote certain aspects of keratinocyte differentiation. For most of our experiments, we use third-passage keratinocytes 1 day after reaching confluence.
2. The advantages of these samples include the relatively homogenous age group, 20–35-year-olds, sun-protected area, and rather large yields.
3. Trizol gives good yields and effectively disrupts the epidermis, but the purity of the RNA is inadequate for the subsequent steps; the RNA isolation kit gives adequate purity, but inefficiently disrupts the tissue, which is why the two are used in series.
4. All solutions contain RNAse inhibitors. Skin is particularly rich in RNAses, necessitating their inhibition.
5. Please note that these are good rules of thumb. If these diagnostic markers are less that optimal, but *similar in all chips*, they can be used and will give acceptable results. Much more problematic is the situation where one of the chips is very different from the others. Such chips should not be compared to others in the series.

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Chapter 16

Molecular Profiling of the Epidermis: A Proteomics Approach

Jianjun Shen and Susan M. Fischer

Abstract

Mouse skin has been used extensively as a model system to study the development of cancer. Recent emphasis has been focused on elucidating the molecular mechanisms by which chemical carcinogens or tumor promoters cause the growth of cutaneous malignancies. In this regard, many transgenic or knockout mouse models have been generated in order to have a particular gene of interest overexpressed or knocked out in the epidermis of the skin**.** We have used a proteomics approach of protein separation using 2D gel electrophoresis and protein identification using mass spectrometry to study the molecular profiles of the epidermis in several transgenic mouse skin models. Identification of altered expression of proteins can shed light on the molecular processes that have been perturbed in these mouse models.

Key words: Skin, Epidermis, Protein profiling, Proteomics, 2D gel, Sample preparation.

1. Introduction

The regulation of gene expression in eukaryotes is a tightly controlled process involving multiple levels of regulation, including transcriptional control, RNA processing and transport, stability, and translational controls. These various levels of control, and particularly those associated with mRNA stability and turnover, can result in a lack of correlation between mRNA levels and corresponding protein levels. Because biological processes are primarily carried out by proteins, it is the expression patterns of proteins that reflect the state and functioning of the cell. Protein phenotyping has the potential to greatly contribute to our understanding of disease initiation and progression, monitoring response to therapies, improving diagnosis, and other useful applications.

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Clearly one area in which proteomics can offer valuable insight is in the development of cancer. Numerous carcinogenesis studies have been carried out over the last 50 years or more using mouse skin as a model system. Such studies have been extremely valuable in identifying agents, including environmental chemicals and physical agents, that are carcinogens or tumor promoters. In more recent years the emphasis has been on elucidating the molecular mechanisms by which these agents cause or promote the growth of cutaneous malignancies. The identification of specific mutations in oncogenes, e.g., in the 61st codon of H-ras following polycyclic hydrocarbon exposure (1), has been instrumental in understanding the nature of tumor-initiating events. The development of genomic profiling has provided additional information on how these events affect the expression of genes involved in such processes as cell cycle, differentiation, apoptosis. To begin to understand the role of specific genes in skin cancer development, as well as in normal physiological processes, many investigators have generated transgenic or knockout mice of their gene of interest delivered in a keratinocyte-specific manner. This frequently results in macroscopic phenotypes such as alopecia, thickened epidermis, or enhanced responsiveness to ultraviolet light (2). But this alone provides little information on the cascade of molecular changes elicited by the transgene (or knockout) that causes the observed phenotype. An analysis of the types of proteins whose expression are altered can shed light on the molecular processes that have been perturbed in these mouse models.

We have developed a proteomics approach to delineate protein expression profiles on several different transgenic mouse models, as well as on various strains of wild-type mice with different genetic backgrounds that are differentially sensitive to classical initiation-promotion skin carcinogenesis protocols ((3, 4 and unpublished results of Jianjun Shen, Susan Fischer, and John DiGiovanni).

2. Materials

2.1. Preparation of Mouse Skin

1. Mice between 6 and 9 weeks of age.
2. RIPA buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X100, 1 mM EDTA, 1 mM PMSF, 0.66 mg/mL aprotinin, 0.5 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mM Na_3O_4, 1 mM NaF or pre-mixed cocktail tablets (Roche Applied Science, Indianapolis, IN). This buffer can be prepared without protease inhibitors beforehand and stored in aliquot at –20°C. Thaw enough aliquots and add proper amounts of protease inhibitors prior to use.

3. BCA Protein Concentration Measurement kit (Pierce, Rockford, IL).

2.2. Handling of Protein Lysates

Perfect-FOCUS kit from Geno Technology, Inc. (St. Louis, MO) is used to precipitate proteins. The following listed reagents are from that kit.

1. Universal Protein Precipitation Agent-I (UPPA-I).
2. Universal Protein Precipitation Agent-II (UPPA-II).
3. OrgoSol Buffer.
4. Seed.
5. Perfect-FOCUS Buffer-I.
6. Perfect-FOCUS Buffer-II.

2.3. Gel Separation of Proteins

2.3.1. Traditional 2D Gel Electrophoresis

1. Immobilized pH gradient (IPG) dry strips, pH ranges of 3–10, 4–7, 5–8, and 7–10, and strip lengths of 7 cm, 11 cm, or 17 cm. Store at –20°C.
2. Rehydration buffer (with protein sample): 8.0 M urea, 2% CHAPS, 15 mM dithiothreitol (DTT), and 0.5% (w/v) Bio-Lytes (Bio-Rad, Hercules, CA). This buffer can be pre-made without DTT and Bio-Lytes and stored in aliquot at –20°C. Thaw one aliquot and add proper amounts of DTT and Bio-Lytes prior to use (*see* **Note 1**).
3. Rehydration buffer (without protein sample): 8.0 M urea, 0.5% CHAPS, 10 mM DTT, and 0.2% (w/v) Bio-Lytes (Bio-Rad) (*see* **Note 1**).
4. Equilibration buffer I: 6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl, pH 8.8, 20% glycerol, and 130 mM DTT. Prepare immediately prior to use.
5. Equilibration buffer II. 6 M urea, 2% SDS, 0.375 M Tris-HCl. pH 8.8, 20% glycerol, and 135 mM iodoacetamide. Prepare immediately prior to use.
6. Mineral oil.
7. Criterion precast SDS-PAGE gel with an IPG+1 comb (Bio-Rad), 8–16% gradient gel. Other percentage or gradient gels should be used based on the range of protein molecular weights of interest.
8. SDS-PAGE gel running buffer (5 ×): 125 mM Tris-HCl, 960 mM glycine, 0.5% (w/v) SDS. Store at ambient temperature.
9. Protein molecular weight markers: Precision plus protein unstained standards and Precision plus protein dual color standards (both from Bio-Rad).
10. Agarose gel: 0.5% agarose in 1 × SDS-PAGE gel running buffer (*see* **Note 2**).

11. SDS-PAGE gel fixing solution: 30% (v/v) methanol, 7.5% (v/v) acetic acid.
12. SYPRO Ruby protein gel stain (Bio-Rad). Store at ambient temperature and protect from light.
13. Destain solution: 10% (v/v) methanol, 6% (v/v) acetic acid.

2.3.2. Difference Gel Electrophoresis (DIGE)

1. CyDye labeling buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5 (*see* **Note 3**). This buffer can be premade and stored in 1 mL aliquot at –20°C. Prior to use, thaw three aliquots: one for CyDye labeling, and for the other two, add proper amounts of DTT and Bio-Lytes to make 2 × and 1 × sample buffer, respectively.
2. CyDyes: Cy2, Cy3, and Cy5 (GE Healthcare, Piscataway, NJ). They are supplied as powder and needed to be protected from light. Store at –20°C.
3. Dimethylformamide (DMF) (Sigma, St. Louis, MO).
4. Lysine buffer, 10 mM.
5. Sample buffer (2 ×): 1 mL of CyDye labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5), 30 mg DTT, and 10 μL Bio-Lytes (Bio-Rad).
6. Sample buffer (1 ×): 1 mL of CyDye labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5), 15 mg DTT, and 5 μL Bio-Lytes (Bio-Rad).

   All other reagents and/or solutions used for DIGE 2D gels are the same as those used in the traditional 2D gel protocol as described above.

2.4. Spot Picking and Trypsin Digestion

1. Destain solution: 50% methanol and 5% acetic acid in HPLC-grade water.
2. Acetonitrile, HPLC grade (Fisher Scientific, Pittsburgh, PA).
3. Trypsin, sequencing grade (Promega Corporation, Madison, WI): Add 2 mL of 50 mM ammonium bicarbonate to each vial of 20 μg trypsin to make 10 μg/μL trypsin solution (*see* **Note 4**).
4. DTT: Prepare 10 mM DTT solution by adding 3 mg DTT in 2 mL of 100 mM ammonium bicarbonate.
5. Iodoacetamide: Make 50 mM iodoacetamide solution by adding 20 mg iodoacetamide in 2 mL of 100 mM ammonium bicarbonate.
6. Ammonium bicarbonate (100 mM): 0.79 g ammonium bicarbonate in 100 mL HPLC-grade water.
7. Ammonium bicarbonate (50 mM): 0.395 g ammonium bicarbonate in 100 mL HPLC-grade water.
8. Extraction buffer: 5% (v/v) formic acid in HPLC water.

2.5. Sample Preparation for Mass Spectrometry

1. ZipTip μ–C18 Pipette Tip (Millipore, Billerica, MA).
2. Sample preparation solution (5 ×): 0.5% trifluoroacetic acid (TFA) in HPLC-grade water.
3. Acetonitrile: HPLC grade (Fisher Scientific, Pittsburgh, PA).
4. Equilibration solution: 0.1% TFA in HPLC-grade water.
5. Wash solution: 5% methanol and 0.1% TFA in HPLC-grade water.
6. Elution solution: A saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA.

2.6. Mass Spectrometry Analysis of the Proteins and Database Search

1. Calibration Mixture 1 (Applied Biosystems, Foster City, CA).
2. MALDI-TOF target plate.
3. 4700 mix (Applied Biosystem) for external calibration.
4. MALDI-TOF/TOF target plate.

2.7. Validation and Interpretation

2.7.1. Western Blot Analysis

1. SDS-PAGE gel running buffer (5 ×): 125 mM Tris-HCl, 960 mM glycine, 0.5% (w/v) SDS. Store at ambient temperature.
2. Pre-stained protein molecular weight markers: Precision plus protein dual color standards (Bio-Rad).
3. SDS protein loading buffer (5 ×): 300 mM Tris, pH 6.8, 50% glycerol, 10% SDS, a trace amount of bromophenol blue. Aliquot 200 μL into microcentrifuge tubes and store at –20°C. Thaw immediately prior to use as needed. Add 500 mM DTT (6 mg) per aliquot.
4. SDS-PAGE gel running buffer (5 ×): 125 mM Tris-HCl, 960 mM glycine, 0.5% (w/v) SDS. Store at ambient temperature.
5. Criterion precast SDS-PAGE gel (Bio-Rad), 8–16% gradient gel. Store at –20°C. Warm to ambient temperature immediately prior to use.
6. Immun-Blot PVDF membrane (Bio-Rad).
7. 3MM Whatman filter paper (Whatman International Ltd., Maidstone, UK).
8. Methanol.
9. Transfer buffer: 25 mM Tris-HCl, 192 mM glycine, and 3.5 mM SDS. Store at ambient temperature. Chilled in a 4°C refrigerator prior to use.
10. Wash buffer (10 ×): Phosphate buffered saline-Tween (PBS-T), 14.7 mM $NaH_2PO_4(H_2O)$, 80.9 mM Na_2HPO4, 1.37 M NaCl, and 1% Tween.

11. Blocking buffer: 5% (w/v) non-fat dry milk in PBS-T. Prepare it immediately prior to use to prevent bacterial growth.
12. Primary antibody: A primary antibody is selected based on the protein of interest for the validation and its commercial availability. Its dilution factor is initially based on its manufacturer's recommendation and then optimized experimentally.
13. Primary and secondary antibody dilution buffer: 1% (w/v) bovine serum albumin (BSA) in PBS-T.
14. Secondary antibody: It is the primary antibody-specific horse radish peroxidase (HRP) labeled. Its dilution factor is initially based on its manufacturer's recommendation and then optimized experimentally.
15. Enhanced Chemiluminescent Plus (ECL Plus) Western blot detection reagents: ECL Plus is a product of GE Healthcare (Piscataway, NJ).
16. X-ray film: Amersham Hyperfilm (GE Healthcare Limited, Buckinghamshire, UK).

2.7.2. Immunohistochemistry

1. Fixing solution: 10% (v/v) formalin in water.
2. Xylene or xylene substitute.
3. Ethanol, 100% or 95%.
4. H_2O_2: 3% (v/v) in double distilled water.
5. Citrate buffer: 10 mM, pH 6.0.
6. Biocare Blocking Reagent: #BS966M, with casein in buffer.
7. Primary antibody: sometimes a good primary antibody for Western blot analysis can also be used for immunohistochemical analysis. The dilution factor should be determined experimentally.
8. Secondary antibody: biotinylated.
9. SA-HRP (BioGenex).
10. BioGenex DAB.

3. Methods

3.1. Preparation of Mouse Skin

There are several aspects of mouse skin physiology that require consideration in experimental design. The first is concerned with the stage of the hair cycle. Ideally the hair should be in the resting, non-growing phase (telogen). This makes it easier to remove the epidermis and avoids any contribution from proliferating or dying hair follicle cells. Additionally, the area of the skin to be sampled needs to be standardized, e.g., a

rectangular piece of dorsal skin that is cut from the base of the neck to the base of the tail and is centered on the midline of the animal. This is particularly important in situations where the dorsal surface has been topically treated with some agent. Another consideration is the inclusion of appropriate controls, particularly solvent-only controls. To avoid having aqueous solutions run off the back, most solutions are made in quick-drying solvents such as acetone or ethanol or agents are applied in a cream-based product. Either approach has the potential to alter protein expression.

1. Shave the dorsal surface of a mouse that is between 6 and 9 weeks of age (telogen phase of the hair cycle); observe for possible hair re-growth over next 24–48 hours. Discard mice showing hair regrowth.
2. Treat skin topically, if required, with 200 μL of vehicle or test agent, using a pipettor.
3. After the appropriate time, sacrifice mice by IACUC-approved procedures, generally CO_2 asphyxiation.
4. Using sharp scissors and forceps, remove a rectangle of skin from the dorsum, approximately 1.5 cm wide and 3.5 cm long; place on cold glass surface on ice with epidermis side down, subcutaneous side up. Meanwhile, cut a small piece of this skin from each mouse, place in plastic cassette, and fix it in 10% formalin. Use this fixed skin sample for validation by using immunohistochemistry.
5. Using a glass microscope slide to hold one end of the skin in place, quickly scrape off adipose and subcutaneous material with a sharp scalpel blade or razor; this prevents contamination of the epidermal samples with fat or blood cells.
6. Flip skin over so the epidermis is facing up; using the glass slide to hold one end of the skin in place, scrape epidermis off with a sharp scalpel blade; removal of the epidermis will leave a shiny dermis.
7. Immediately transfer scraped material to an Eppendorf tube containing a minimum of 300 μL cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X100, 1 mM EDTA) to which protease and phosphatase inhibitors (1 mM PMSF, 0.66 mg/mL aprotinin, 0.5 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mM Na_3O_4, 1 mM NaF or premixed cocktail tablets from Roche Applied Science, Indianapolis, IN) have been added.
8. Scraped material is homogenized on ice, using a tissue homogenizer at 50% power for several minutes, and then centrifuged at 14,000 × g for 15 min at 4°C.

9. Determine protein concentration using the BCA kit (Pierce, Rockford, IL). Adjust sample volumes with RIPA buffer so that all samples are of the same concentration.
10. Freeze aliquots in the amount of 150 μg to prevent repeated freezing and thawing.

3.2. Handling of Protein Lysates

In order to obtain quality 2D gels, epidermal protein lysates should be treated to remove contaminants such as salts, ionic detergents, nuclei acids (DNA and RNA), polysaccharides, and lipids. The presence of these materials interferes with the isoelectric focusing step of 2D gel electrophoresis. Precipitation of proteins is the method of choice using ice-cold acetone or a commercially available Perfect-FOCUS kit (Geno Technology, Inc., St. Louis, MO) to get rid of these contaminants. In our laboratory, 150 μg of protein is precipitated from RIPA buffer using the Perfect-FOCUS kit (Geno Technology, Inc). The following protocol describes the use of this commercial kit.

1. Add 150 μg of each protein sample into 300 μL of Universal Protein Precipitation Agent I (UPPA-I) in an Eppendorf tube and mix well. Keep the sample on ice for 15 minutes.
2. Add 300 μL of Universal Protein Precipitation Agent II (UPPA-II) into the above mixture of protein and UPPA-I. Vortex and centrifuge at 15,000 × g for 5 minutes.
3. Immediately after centrifugation, remove the entire supernatant carefully with a pipette and avoid disturbing the pellet.
4. Add 25 μL of deionized water and vortex to re-suspend the pellet.
5. Add 1 mL of pre-chilled (at –20°C) OrgoSol buffer and 5 μL of SEED. Vortex to fully suspend the protein precipitate and incubate at –20°C for 30 minutes.
6. During this incubation period, vortex each sample several times, in 20–30 second bursts. Centrifuge the tube at 15,000 × g for 5 minutes to form a tight pellet.
7. Immediately after centrifugation, remove the entire supernatant carefully with a pipette and avoid disturbing the pellet.

3.3. Gel Separation of Proteins

2D gel electrophoresis was discovered by O'Farrell, P.H. over 30 years ago (5). Several recent advancements, such as the completion of the human genome project and numerous other organisms, advanced mass spectroscopic techniques, more powerful and less expensive computers and related software programs, commercially available immobilized pH gradient (IPG) strips, and web-based genomic and proteomics database for database search, have made this technique a most utilized proteomics approach. There are now two widely utilized 2D-PAGE approaches. The first is the more traditional approach, running each individual protein sample on a

separate 2D gel. When comparing two protein samples, they are run on two separate 2D gels, and then protein spots from these two gels are matched extensively to identify differentially expressed proteins. This approach has been used by many laboratories and has some inherent problems, such as gel to gel variation when individual samples are run separately. However, this method can be used to perform 2D Western blot analysis and to elucidate post-translational modification, among many other applications. The second approach is to label two individual protein samples with two different fluorescent-colored dyes (Cy3 and Cy5), respectively. The samples are then combined and run on a single 2D-PAGE gel (the so-called difference gel electrophoresis). Together with an internal control of combining equal amounts of the two individual samples and labeling it with a third fluorescent-colored dye Cy2, this method proves to be superior by eliminating gel to gel variation and the inclusion of the internal control when performing differential protein expression analysis. The following are the descriptions of both methods.

3.3.1. Traditional 2D Gel Electrophoresis

3.3.1.1. First Dimensional Protein Separation

1. Solubilize each protein pellet into 185 μL of immobilized pH gradient (IPG) strip rehydration buffer [8.0 M urea, 2% CHAPS, 15 mM DTT, 0.5% (w/v) Bio-Lytes (Bio-Rad)]. Incubate the sample at ambient temperature for 60 minutes. During this incubation period, vortex the sample about once every 10 minutes, each with 20–30 second bursts, or until protein pellet is completely dissolved in rehydration buffer (*see* **Note 5**).
2. Centrifuge the tube at 15,000 × g for 30 minutes to remove any remaining insoluble debris.
3. During this time period, take out an IPG strip with an appropriate pH range (3–10, 4–7, 5–8, and 7–10) and length (7 cm, 11 cm, or 17 cm), depending on experimental design, from a –20°C freezer. Let it warm up to ambient temperature for several minutes.
4. Transfer each supernatant from step 2 to one channel of the IEF focusing tray. Carefully spread the sample as evenly across the entire length of the channel as possible, but not beyond the +/- electrode wires.
5. Identify the positive (+) end of each IPG strip. Peel the plastic cover off the IPG strip. Lay the strip, gel side down, into the channel of the focusing tray with the protein solution. Make sure that the positive and negative ends just overhang slightly across the top of the (+) and (–) electrodes, respectively, sitting on water-wet wicks. The dried gel from the IPG strip should be in direct contact with the protein solution. Be careful not to trap any air bubbles beneath the IPG strip.
6. Repeat steps 1–5 for all other samples.

7. Overlay each channel of the IEF focusing tray with or without protein sample and IPG strip with mineral oil. This prevents protein solution evaporation during the high-voltage isoelectric focusing step.
8. Carefully place the lid on the IEF focusing tray and put the IEF focusing tray into the IEF Cell without tilting the tray (to avoid protein solution or mineral oil spilling or mixing). Make sure that the (+) and (−) electrodes are aligned properly in the IEF Cell.
9. Program the Bio-Rad IEF Cell with the following active rehydration protocol, followed automatically with an isoelectric focusing program:
   a) Rehydration Step: Rehydrate the IPG strips in the focusing tray at 50 V at 20°C for 12 hours or overnight for active rehydration.
   b) Conditioning Step: A low voltage of 250 V for 15 minutes is applied to remove salt ions and charged contaminants.
   c) Voltage Ramping Step: One of three voltage ramping modes, slow, linear, and rapid, can be used to increase the voltage to the maximum desired level for specific length of IPG strips at 7,000 V for 7 cm strips, 8,000 V for 11 cm strips, and 10,000 V for 17 cm strips. In any case, the current should not exceed the limit of 50 μA/strip.
   d) Final Focusing Step: Select the time to have a total of at least 20,000 vhours for 7 cm strips, 35,000 vhours for 11 cm strips, and 60,000 vhours for 17 cm strips; or select volt-hours option of at least 20,000 vhours for 7 cm strips, 35,000 vhours for 11 cm strips, and 60,000 vhours for 17 cm strips.
   e) Hold Step: If a run will not be stopped immediately after the Final Focusing Step, a Hold Step at 500 V is programmed to avoid focused proteins diffusing until the run is stopped manually.
10. Enter the number of IPG strips in the IEF focusing tray on the IEF Cell display screen. Press "Run" to start the dehydration and isoelectric focusing.
11. Once completed, stop the IEF Cell. Remove IPG strips from the IEF focusing tray. Wrap them individually in Saran wrap and store at –20°C until the second dimensional SDS-PAGE is ready to run.

3.3.1.2. IPG Strip Equilibration

12. Prior to running the second dimensional SDS-PAGE gel(s), focused IPG strips need to be equilibrated in order to exchange detergent with SDS and reducing agent to minimize vertical streaking.

13. Immediately prior to use, freshly prepare DTT containing equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol and 130 mM DTT) and iodoacetamide-containing equilibration buffer II (6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol, and 135 mM iodoacetamide).
14. Fill one channel of a rehydration/equilibration tray for each IPG strip with DTT containing equilibration buffer I and place the IPG strip in the channel with gel side up. Gently shake for 10 minutes on a shaker.
15. Take the IPG strip out and discard the DTT-containing solution. Add the iodoacetamide containing equilibration buffer II into the same channel and place the IPG strip in the channel with gel side up. Gently shake for another 10 minutes on the shaker. The IPG strip is now ready for the second dimensional gel separation.

3.3.1.3. Second Dimensional SDS-PAGE Protein Separation

16. The following instructions assume the use of a Bio-Rad Criterion precast gel system, which includes a flexible 2-gel Criterion cell for 1–2 gels or a 12-gel Dodeca cell for 3–12 gels. A variety of precast gels with an IPG+1 comb and Tris-HCl buffer system for 2D electrophoresis are also available from Bio-Rad. However, other formats can be easily adapted to fit with different lengths of IPG strips with different gel sizes. In the latter case the gel running conditions for a larger gel format and 17 cm IPG strip(s) need to be adjusted accordingly.
17. Prepare the SDS-PAGE gel running buffer by diluting 100 mL of 5 × running buffer with 400 mL of double-distilled deionized water in a graduated cylinder and mix well by inverting the cylinder after covering with parafilm.
18. Take out one precast Criterion 8–16% gradient gel from a 4°C refrigerator for each focused IPG strip. Remove the seals from the top and bottom of the gel cast, then carefully remove the IPG+1 comb. Wash the top of the gel(s) with 1 × SDS running buffer.
19. Assemble the Criterion gel apparatus according to the manufacturer's instructions using either the 2-gel Criterion cell or the 12-gel Dodeca cell depending on the number of SDS-PAGE gels. If the latter 12-gel Dodeca cell is used, cooling water should be connected to the Dodeca cell from a water bath with cooling capacity (VWR Scientific Products, West Chester, PA). Insert the precast Criterion gel(s) into the Criterion cell or Dodeca cell. Add the running buffer to the upper and lower chambers of the gel apparatus.

20. Carefully insert the focused IPG strip on to the top of the SDS-PAGE gel. Ensure the IPG strip is close to the gel with no gap in between. If necessary, prepare 0.5% melted agarose in running buffer, cool down so that agarose would not melt gel on the IPG strip, and fix the IPG strip in place at both ends of the strip.
21. Load the protein molecular weight standard into the well of the IPG+1 comb. The protein standard used is unstained for SYPRO Ruby stain.
22. Complete the assembly of the gel apparatus and connect to a power supply. Start to run the gel at 50 V for 5 minutes to let the focused proteins enter the stacking gel on the top of the resolving gel from the IPG strip. Switch the voltage to 200 V to run the gel for about 1 hour. Stop running the gel before the loading dye reaches the very bottom of the gel.
23. For each gel, prepare 100 mL of a SDS-PAGE gel fixing solution containing 40% methanol and 10% acetic acid in double distilled water.
24. Disconnect the gel apparatus from the power supply. Take out the Criterion gel cassette and carefully break open to remove the SDS-PAGE. Gently place the gel in a clean container with 100 mL of the fixing solution and shake for 1 hour.
25. Carefully remove the fixing solution. Add 75 mL of fresh SYPRO Ruby stain solution for each gel. Gently shake for at least 4 hours or overnight in the dark by wrapping the container first with saran wrap and then with aluminum foil.
26. Remove the SYPRO Ruby stain solution and wash the gel three times with 75 mL of double distilled water each time. Destain the gel in 100 mL of the destain solution (40% methanol and 5% acetic acid in water) for 2–4 hours in the dark (with aluminum foil wrapped around the container). Wash the gel three times with 75 mL of double distilled water each time.
27. The gel image is captured by using mutually exclusive excitation/emission wavelengths of 532/610 on a Typhoon 9410 Variable Mode Imager (GE Healthcare, Piscataway, NJ). Laser voltage should be adjusted to capture the most intense 2D gel image but avoid oversaturation of protein spots.
28. Integrated signal intensities of protein spot features on 2D gel images are analyzed quantitatively using Bio-Rad's PDQuest 2D gel image analysis software. Briefly, a master gel is constructed with all protein spot features from one 2D gel of a control sample and another 2D gel of an experimental sample or many sets of 2D gels in an experiment. This master gel is used as a reference gel to compare all protein spot features on

all 2D gel images in the experiment. A list of protein spot features with differences of at least 2-fold in terms of upregulation or downregulation between two compared 2D gel images is generated after comprehensive analysis of the 2D gel images by using PDQuest. Two independent observers then visually confirm differential expression of identified protein spot features. Disregard those identified protein spot features that appear at the edges of a 2D gel or do not look like true protein spots. An example of the traditional 2D gel images is shown in **Fig. 16.1**.

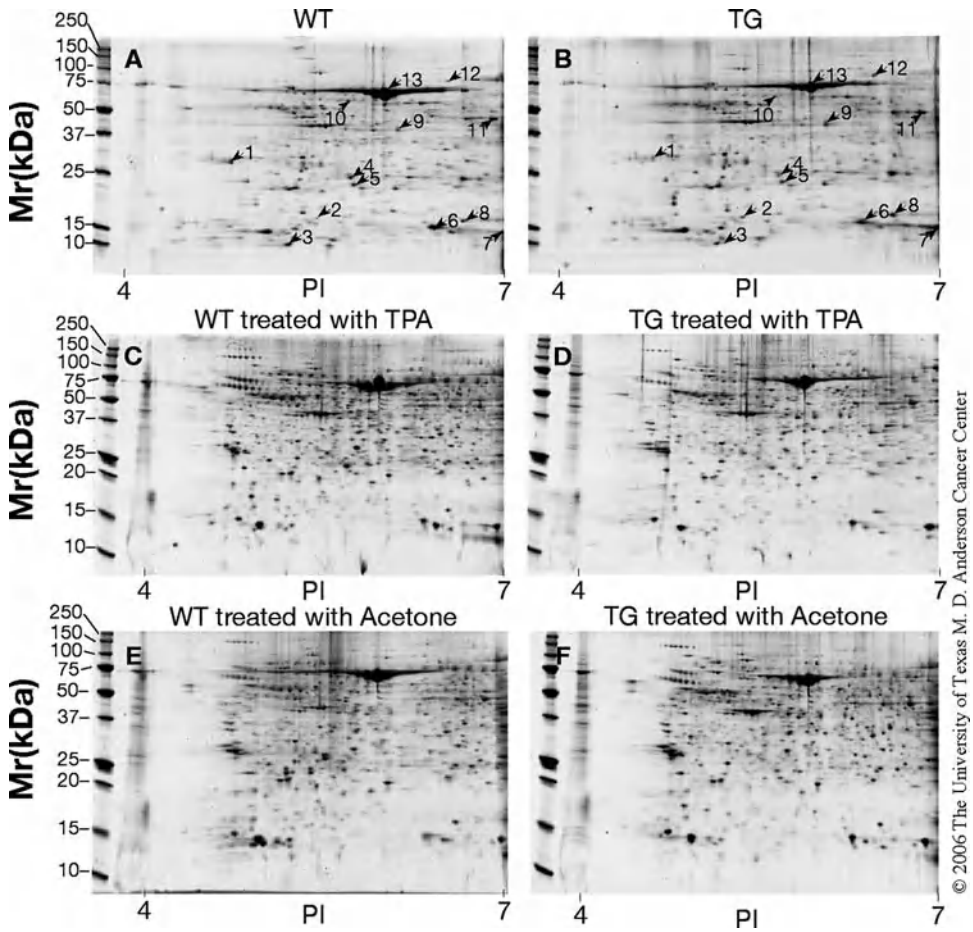

Fig. 16.1. Representative 2D gel images of the epidermal lysates of K14.COX-2 TG and wild-type mice. The pH range of 4–7 for all three treatment groups is shown here. **(A and B)** Mice that did not undergo treatment with differentially expressed proteins marked. The protein identifications for the numbered spots are listed in (*3*, Table 1) (data not shown). **(C and D)** Wild-type and K14.COX-2 TG mice were both treated with TPA. **(E and F)** Wild-type and K14.COX-2 TG mice were both treated with acetone. WT, wild type; and TG, transgenic. This figure demonstrates the more traditional 2D gel approach that runs each individual protein sample on a separate gel. (Reproduced from (3) with permission from the American Chemical Society.)

3.3.2. Difference Gel Electrophoresis (DIGE)

3.3.2.1. Preparation of Mouse Skin

1–6. Steps 1–6 are the same as the steps 1–6 of "Preparation of Mouse Skin" for the traditional 2D gel protein separation (3).

7. Immediately transfer scraped material to an Eppendorf tube containing 500 μL CyDye labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris-HCl, pH 8.5) at ambient temperature.
8. Homogenize scraped material using a tissue homogenizer at 50% power for several minutes and then centrifuge at 14,000 × g for 15 minutes at ambient temperature.
9. Determine protein concentration using the BCA kit (Pierce, Rockford, IL). Adjust sample volumes with CyDye labeling buffer so all samples are at the same concentration. Minimum concentration and maximum volume are determined by the length of the IPG strip used. For an 11 cm IPG strip, the total volume of all labeled products (internal control, experimental control, and sample) plus 2 × buffer cannot exceed 190 μL. Minimum concentration of protein for efficient labeling is 5 μg/μL.

3.3.2.2. Labeling of Protein Sample with CyDyes and Protein Separation

1. Three CyDyes, Cy2, Cy3, and Cy5 are supplied as powder (GE HealthCare) in the amount of 10 nM per vial. Reconstitute them by adding 10 μL of dimethylformamide (DMF) to each vial to make a stock solution at a concentration of 1 nM/ μL. Vortex to dissolve all the powder completely. Spin down and store the stocks at –20°C.
2. Make enough CyDye working solution by combining two portions of each CyDye stock solution with three portions of DMF solvent. Keep working solutions on ice and protect from light by wrapping with aluminum foil.
3. Transfer 50 μg of an experimental protein extract (from the epidermis of either a transgenic or a knockout mouse) to a labeled Eppendorf tube, 50 μg of a control protein extract (from the epidermis of either a wild-type or acetone-treated mouse) to a second labeled Eppendorf tube, and an internal control of 25 μg of the experimental protein extract and 25 μg of the control protein extract to a third labeled Eppendorf tube.
4. Add 1 μL of Cy5 working solution to the first labeled tube, 1 μL of Cy3 working solution to the second labeled tube, and 1 μL of Cy2 working solution to the third labeled tube. Mix each labeling reaction by vortexing and then spin down. Incubate the labeling reaction for 30 minutes on ice.
5. Stop the labeling reaction by adding 1 μL of 10 mM lysine buffer to each tube. Vortex, spin down, and set the reaction on ice. The protein samples labeled with CyDyes can be stored at –80°C for several weeks or be used immediately in the next step.

6. Prepare two types of sample buffer: (1) 2 × sample buffer is prepared by adding 30 mg DTT and 10 μL of Biolytes to 1 mL of CyDye labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris-HCl, pH 8.5) and (2) 1 × sample buffer is prepared by adding 15 mg DTT and 5 μL of Biolytes to 1 mL of CyDye labeling buffer.
7. Pool the three individually labeled protein samples of the internal control, experimental control, and sample from step 5 together in an Eppendorf tube. Measure the total volume of the combined sample. Add the same volume of 2 × sample buffer. For an 11 cm IPG strip, add 1 × sample buffer to a final volume of 190 μL.
8. Protect the sample from light by wrapping with aluminum foil. Gently shake for 1–2 hours in a 27°C incubator.

9–27. Perform steps 2–20 of the traditional 2D gel protein separation as described. One exception is to keep the CyDye labeled protein samples in the dark by wrapping the Bio-Rad IEF Cell with aluminum foil and turning off the lights.

28. After loading the IPG strip on to the top of the SDS-PAGE gel, load the mixture of two protein standards to the one well on the gel. One protein standard is dual-color standard for Typhoon imaging scanning. Another is an unstained standard for eventual SYPRO Ruby stain.
29. Complete the assembly of the gel apparatus and connect to a power supply. Start to run the gel at 50 V for 5 minutes to let the focused proteins enter the stacking gel on the top of the SDS-PAGE gel from the IPG strip. Switch the voltage to 200 V and run the gel for about 1 hour. Stop running the gel before the loading dye reaches the very bottom of the gel.
30. Disconnect the gel apparatus from the power supply. Take out the Criterion gel cassette and carefully break open to remove the SDS-PAGE gel. Carefully place the gel on a flat plate. Wash the gel three times with double distilled water. The gel is now ready to be scanned. The CyDye 2D gel images need to be scanned immediately to avoid fading of the fluorescent dyes.
31. The gel is scanned with three channels of mutually exclusive excitation/emission wavelengths of 488/520 for Cy2, 532/580 for Cy3, and 633/670 for Cy5 on the Typhoon 9410 Variable Mode Imager (GE Healthcare). Two sets of 2D DIGE gel images are scanned with a low voltage of 550 V for Cy2, 530 V for Cy3, and 550 V for Cy5, and a high voltage of 600 V for Cy2, 580 V for Cy3, and 550 V for Cy5 to capture both low- and high-intensity protein spots without oversaturation. Scan the gel using low voltage first, and then scan it with high voltage to avoid photo bleaching (*see* **Note 6**). Select a pixel size of 100 μm.

32. For the purpose of cutting protein spots from 2D gel(s), gently place the gel in a clean container with 100 mL of the fixing solution and shake for 1 hour.
33. Carefully remove the fixing solution. Add 75 mL of fresh SYPRO Ruby stain solution for each gel. Gently shake for at least 4 hours or overnight in the dark by wrapping the container first with saran wrap and then with aluminum foil.
34. Remove the SYPRO Ruby stain solution and wash the gel three times with 75 mL of double distilled water each time. Destain the gel in 100 mL of the destain solution (40% methanol and 5% acetic acid in water) for 2–4 hours in the dark (with aluminum foil wrapped around the container). Wash the gel three times with 75 mL of double distilled water each time.
35. Perform a pair-wise comparison of the experimental (transgenic or knockout) and control protein abundance using the GE Healthcare DeCyder Differential In-gel Analysis module against a mixed experimental/control internal standard. Volume ratios are generated by comparing Cy5-labeled experimental to Cy3-labeled wild-type control spot volumes after normalization against Cy2-labeled internal standard spot volumes. A 2-fold threshold with 2 standard deviation of 1.8 is used as a selection criterion.
36. As stated earlier visually verify differential expression of protein spots identified by the DeCyder Differential In-gel Analysis module to eliminate artifacts erroneously identified by this software program.
37. Manually excise spots that differ consistently in both software analysis and by visual inspection and perform in-gel tryptic digestion as described below. An example of schematic illustration of 2D DIGE gel experiment is shown in **Fig. 16.2**.

3.4. Spot Picking and Trypsin Digestion (6)

1. Roughly estimate the sizes of the protein spots to be cut and prepare a set of an equal number of 1 mL size pipette tips (with no filter) with tip heads cut to fit the sizes of those protein spots (*see* **Note 7**).
2. Carefully place the 2D gel on the clean surface of a Dark Reader (Clara Chemical Research, Inc., Dolores, CO) and turn on the power switch. Initially try to locate all the protein spots that need to be cut. Use a 1-mL pipette tip with proper size opening at its tip (its original tip is cut off as prepared from step 1).
3. Add 400 μL of destain solution (50% methanol and 5% acetic acid in HPLC-grade water) to a labeled siliconized 1.5 mL Eppendorf tube (*see* **Note 8**) and use the pipette tip to cut the desired protein spot from the 2D gel. Dispense the cut gel piece into the destain solution. Make sure that the gel piece is actually transferred from

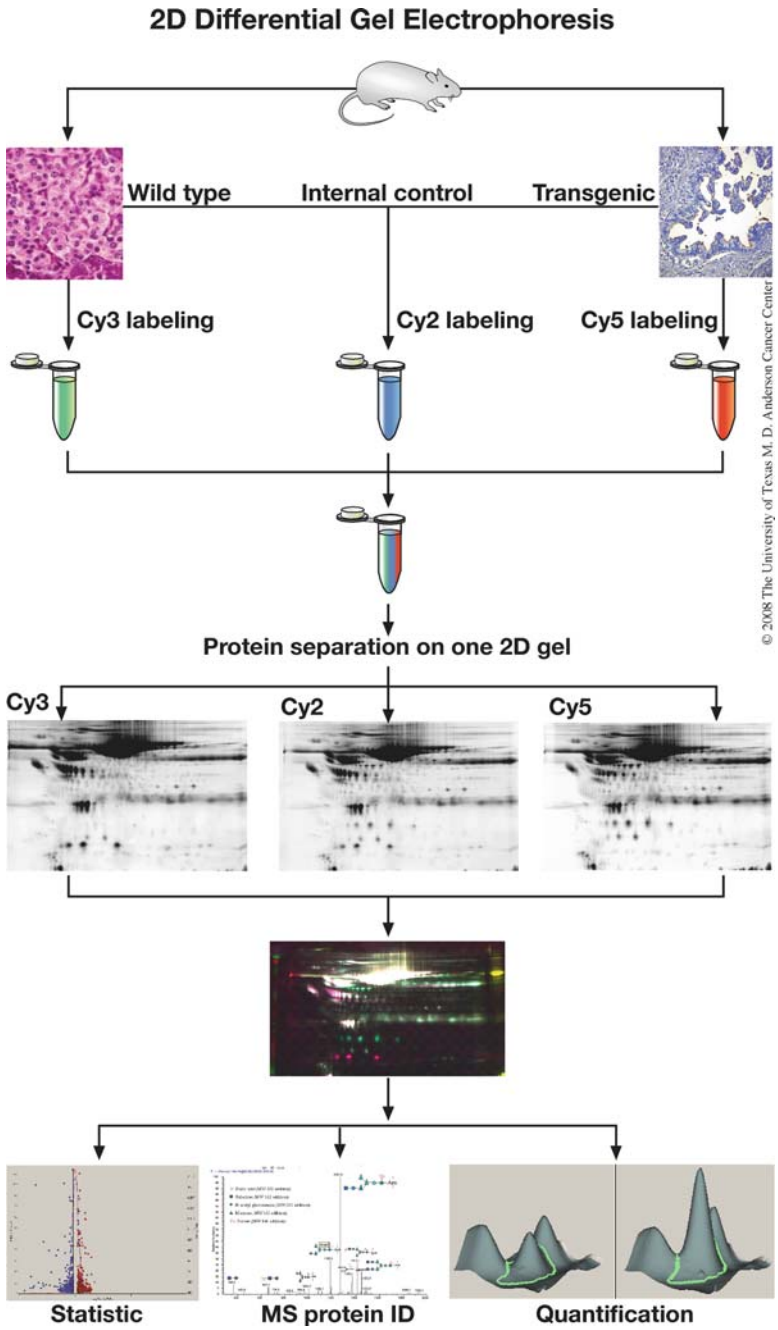

Fig. 16.2. Schematic illustration of 2D DIGE. Epidermal samples are acquired from transgenic (TG) or knockout mice as an experimental sample and from wild-type (WT) mice as an experimental control, respectively. Fifty micrograms of epidermal protein extract from WT and TG are labeled with Cy3 and Cy5, respectively. A combination of 25 μg of the experimental TG protein extract and 25 μg of the WT control protein extract as an internal control is labeled with a third dye Cy2. These three labeled protein samples are pooled together and then separated in the same 2D gel. The gel is scanned with three channels of mutually exclusive excitation/emission wavelengths of 488/520 for Cy2, 532/580 for Cy3, and 633/670 for Cy5. Because all pooled protein samples are electrophoretically separated in the same gel, this eliminates gel to gel variation when two protein samples are compared with one another. The inclusion of the internal control further assists the quantitative protein profiles of differentially expressed proteins between the experimental TG mice and WT control mice for a quick and accurate comparison.

the pipette tip to the Eppendorf tube (*see* **Note 9**). While a SYPRO Ruby stained gel piece does not require destaining, the destain solution is used here to help the small gel piece transfer and for storage.

4. Finish cutting out the rest of the differentially expressed protein spots with at least a 2-fold difference in intensity.
5. Remove the destain solution. Dehydrate with 200 μL of HPLC-grade acetonitrile. Gel pieces should turn to opaque-white. If they do not, remove acetonitrile and repeat this dehydration step with 200 μL of fresh acetonitrile.
6. Remove acetonitrile. Air dry dehydrated gel pieces at ambient temperature to get rid of any residual acetonitrile.
7. Reduce protein in the gel pieces in 100 μL of 10 mM DTT solution for 30 minutes at ambient temperature.
8. Remove and discard DTT solution.
9. Alkylate protein in the gel pieces in 100 μL of 50 mM iodoacetamide and incubate at ambient temperature for 30 minutes.
10. Remove and discard iodoacetamide solution.
11. Wash the gel pieces with 200 μL 100 mM ammonium bicarbonate for 10 minutes.
12. Remove and discard the ammonium bicarbonate wash solution.
13. Dehydrate the gel pieces with 200 μL acetonitrile for 5 minutes twice. Remove acetonitrile and the gel pieces should turn opaque-white after a few minutes of air-drying at ambient temperature.
14. Prepare trypsin in an ice water bath by adding 2 mL ice-cold 50 mM ammonium bicarbonate to a vial of 20 μg sequencing-grade trypsin (Promega Corporation, Madison, WI).
15. Add 20 μL freshly prepared trypsin solution to each tube and keep it on ice water while the gel pieces are rehydrated. This allows trypsin to enter the gel but not to digest protein inside the gel (*see* **Note 10**).
16. Remove and discard 15 μL trypsin solution from each Eppendorf tube and add 20 μL 50 mM ammonium bicarbonate to each tube. Incubate at 37°C overnight.
17. Add 10 μL extraction buffer (5% formic acid in HPLC-grade water) to each Eppendorf tube containing a protein spot. Incubate for 10 minutes at ambient temperature to extract the digested peptides. Collect the extract and transfer to a labeled new siliconized 0.5 mL Eppendorf tube.

18. Repeat the extraction by adding another 10 μL of extraction buffer and incubating 10 minutes at ambient temperature. Collect and combine the extracts in the same Eppendorf tube.
19. Dry the sample to less than 5 μL in a Speed-Vac (Thermo-Savant, Holbrook, NY) and avoid drying to complete dryness (*see* **Note 11**). Store it at –20°C until sample is to be analyzed by mass spectrometry.

3.5. Sample Preparation for Mass Spectrometry

In order to obtain better mass spectrometric data quality, a cleanup step using ZipTip μ–C18 Pipette Tip (Millipore, Billerica, MA) is required to remove contaminants such as salt and detergent from the above in-gel digested peptide sample. ZipTip is a 10 μL pipette tip filled with C18 chromatography media at its tip head so that there is no dead volume.

1. Add 1.0 μL 5 × Sample preparation solution [0.5% trifluoroacetic acid (TFA) in HPLC-grade water] to 4.0 μL in-gel digested peptides solution. The presence of 0.1% TFA will help in maximizing the binding of peptides to the ZipTip.
2. Set a P-10 pipettor at its maximum volume of 10 μL and prewet the ZipTip by aspirating wetting solution (100% acetonitrile) into the tip. Dispense to waste. Repeat.
3. Equilibrate the ZipTip pipette tip by aspirating equilibration solution (0.1% TFA in HPLC-grade water) into the tip. Dispense to waste. Repeat.
4. Fully depress the pipette plunger to a dead stop (at its maximum volume of 10 μL). Aspirate and dispense the in-gel digested peptide sample 7–10 times for maximum binding of peptides to the C18 chromatography media.
5. Aspirate wash solution (5% methanol in 0.1% TFA/HPLC-grade water) into the tip and dispense to waste. Repeat this wash step several times.
6. Dispense 1.5 μL of elution solution with matrix (a saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA) into a clean vial. Carefully aspirate this elution solution with matrix and dispense through a ZipTip pipette tip at least three times.

3.6. Mass Spectrometry Analysis of the Proteins and Database Search (7, 8)

3.6.1. MALDI-TOF Analysis

1. Directly spot eluant onto a MALDI-TOF target plate by dispensing a desired volume of 0.5 μL of desalted sample per spot for three replicates.
2. Spot 0.45 μL of Calibration Mixture 1 (Applied Biosystems, Foster City, CA) for external calibration 1 on alternating rows on the target plate.
3. Allow spots to air dry completely for about 5 minutes at ambient temperature.

4. Load the metal MS sample target plate into a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems).
5. Set the acquisition of MALDI-TOF spectra in reflectron mode over the m/z range of 700–2500.
6. Acquire an initial mass spectrum on an external calibration 1 spot at a laser power much higher than the ionization threshold and then decrease the laser power until a good spectrum is obtained.
7. Acquire one or more spectra of sample adjacent to the external calibration 1 spot. Adjust the laser power to obtain sufficient peak signal intensity without saturation.
8. Perform automated data acquisition by the Proteomics Solutions 1 Utility V1.0.0, with close external calibration performed for each sample spot.
9. Process spectra using Data Explorer 3.5.0.0 for baseline correction and de-isotoping of the mass spectra.
10. Filter the peak list to remove porcine trypsin autolysis, matrix and human keratin masses seen in blank gel digests, or contaminated samples.
11. Enter the remaining 15 or 20 most intense peaks in the 700–2500 m/z range to Auto-MS-Fit (v. 3.2.1) for peptide mass fingerprinting (PMF) matching between the experimentally measured digest peptide masses and those generated by theoretical digest of a mammalian subset of the Swiss-Prot Database (v. May 9, 2004).
12. Carry out the PMF search assuming peptides are the result of tryptic digestion, with no more than two missed cleavages, and carbamidomethylation of the cysteine residues.
13. Perform the search for mammals using an 80 ppm peptide mass tolerance and a minimum of six peptides matched required for a hit.
14. Following Intellical recalibration, perform a second search with 30-ppm peptide mass tolerance and a minimum of five peptides matched. The proteins represented by the greatest number of peptide masses matched and matching the expected molecular weight are reported.
15. For samples with fewer than five peptides matched to one protein, subject at least one ion (one peak) from the mass spectrum to manual postsource decay analysis for protein identification by peptide fragmentation. The protein identified in each case will be the highest scoring match, with the greatest number of ions matching the peptide identified by the database search.

3.6.2. MALDI-TOF/TOF Analysis (3, 9)

1. Directly spot eluant onto a MALDI-TOF/TOF MS target plate by dispensing a desired volume of 0.5 μL of desalted sample per spot for three replicates.

2. Spot 0.45 μL of 4700 mix (Applied Biosystems) for external calibration 1 on alternating rows on the target plate.
3. Allow spots to air dry completely for about 5 minutes at ambient temperature.
4. Load the metal MS sample target plate into a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems).
5. Automatically acquire both MS and MS/MS spectra using 4000 Series Explorer V 3.0 RC1. MS spectra will be acquired with 2000 laser shots over the mass range of 700–4000 m/z and calibrated internally on trypsin autolysis peaks.
6. Select the top 20 MS signals with a minimum signal/noise (S/N) ratio of 20 for MS/MS fragmentation after the exclusion of matrix, trypsin, and keratin peaks.
7. Acquire up to 3750 shots for MS/MS spectra.
8. Perform additional peak processing and a database search using GPS Explorer v3.5. Use MASCOT V2.0 at http://www.matrixscience.com for the database search with the same parameters as before with the following exceptions: 30 ppm MS mass tolerance over the mass range of 700–4000, 0.2 Da MS/MS fragment mass tolerance, up to 100 peaks with a minimum S/N ratio of 15 selected for MS, and up to 65 fragment ions with a minimum S/N ratio of 3. Search both the Swiss-Prot (Dec. 30, 2005) and the mammalian subset of the Trembl (Dec. 6, 2005) databases.
9. Combine both scores from the MS search and the MS/MS search using a probabilistic MOWSE algorithm. The MASCOT score is defined as -10*logP, where P is the probability that the observed match is a random event. Choose a score of 66 corresponding to $P < 0.05$ as the cutoff for a significant hit. The top-ranking mouse hit will be reported for the search.

3.7. Validation and Interpretation

3.7.1. Western Blot Analysis

1. Prepare the SDS-PAGE gel running buffer by diluting 100 mL of 5 × running buffer with 400 mL of double-distilled deionized water in a graduated cylinder and mix well by inverting the cylinder after covering with parafilm (*see* **Note 12**).
2. Add 5 × SDS protein loading buffer to each protein sample in an Eppendorf tube to get a final SDS concentration of 1 ×. Keep the total volume within 25 μL. Heat the sample >95°C for 5 minutes on a heating block and cool down at ambient temperature.
3. Take out a regular precast Criterion 8–16% gradient gel from the 4°C refrigerator. Remove the seals from the top and bottom of the gel cast first and then carefully remove the comb. Wash the top of the gel(s) with 1 × SDS running buffer.

4. Assemble the Criterion gel apparatus according to the manufacturer's instructions using the 2-gel Criterion cell. Add running buffer to the upper and lower chambers of the gel apparatus.
5. Load the protein molecular weight standard into the very first well of the gel. The protein standard used is rainbow protein markers to indicate protein transfer efficiency. Load the protein samples and record the order of sample loading. Remember to load 1 × SDS protein loading buffer in all empty lanes to ensure protein separation in a uniform and straight manner.
6. Complete the assembly of the gel apparatus and connect to a power supply. Start to run the gel at 50 V for 5 minutes to let the proteins enter the stacking gel and get focused on the top of the resolving gel from the wells. Switch the voltage to 200 V and run the gel for about 1 hour. Stop running the gel before the loading dye reaches the very bottom of the gel.
7. Disconnect the gel apparatus from the power supply. Take out the Criterion gel cassette and carefully break open to retrieve the SDS-PAGE gel. Carefully remove and discard the stacking gel portion, leaving the resolving gel portion attached to one side of the Criterion gel cassette.
8. Assemble a protein transfer sandwich by using two transfer fiber pads, six sheets of 3MM filter paper, and one sheet of Immun-Blot PVDF membrane (0.2 μm, Bio-Rad). Briefly, cut six sheets of 3 MM filter paper and one sheet of PVDF membrane with a size slightly larger than that of the resolving gel. Pre-wet the PVDF membrane in 100% methanol, wash it in double-distilled water for 5 minutes, and equilibrate it in transfer buffer for 10 minutes. Submerge two transfer fiber pads and six sheets of 3MM filter paper in the transfer buffer. Put three sheets of 3MM filter paper on top of one transfer foam pad, lay the PVDF membrane on the top, and lay the resolving gel on top of the PVDF membrane. Lay the remaining three sheets of 3MM filter paper on top of the gel, followed by the second transfer fiber pad. Make sure that no air bubbles are trapped in the sandwich, especially between the resolving gel and the PVDF membrane. Place the assembled sandwich in the transfer cassette and close it.
9. Fill the transfer tank with the pre-chilled (4°C) transfer buffer and insert the transfer cassette into the tank. Make sure that the PVDF membrane is between the gel and the anode, so that the proteins will be transferred from the gel to the PVDF membrane.
10. Connect to the cooling water bath (VWR Scientific Products, West Chester, PA) with a super-cooling coil submerged in the pre-chilled transfer buffer for heat dissipation to ensure

uniform protein transfer. Place a stirring bar inside the transfer tank and the transfer tank on a Corning stirrer (Corning Incorporated, Corning, NY) to circulate the transfer buffer. Put the lid on the transfer tank and connect it to the power supply.

11. Transfer the proteins at 100 V for 1 hour. Disconnect the transfer tank from the power supply and remove the lid from the transfer tank. Take the transfer cassette out of the tank, carefully disassemble the sandwich, remove the membrane, and inspect the transfer efficiency by making sure that the colored rainbow protein molecular weight standards are clearly visible on the membrane. Allow the membrane to air dry at ambient temperature. Dehydrated proteins bind more strongly to the membrane.
12. Re-wet the PVDF membrane in 100% methanol, wash it in double-distilled water for 5 minutes, and equilibrate it in 1 × phosphate buffered saline-Tween (PBS-T) for 10 minutes. Block non-specific protein binding sites by incubating the membrane with 100 mL of 5% non-fat dry milk in PBS-T for 1 hour at ambient temperature on an orbital shaker (Ocelot, Fisher Scientific, Pittsburgh, PA).
13. Rinse the membrane twice in PBS-T and wash it in PBS-T twice for 5 minutes each time.
14. Dilute the primary antibody specific to a protein of interest for validation in 1% BSA/PBS-T. The dilution factor should be optimized and determined experimentally. Incubate the membrane with the antibody on the orbital shaker either for one hour at ambient temperature or overnight in a cold room (at 4°C).
15. Discard the primary antibody solution. Rinse the membrane with two changes of PBS-T wash buffer, wash it once for 15 minutes and four times for 5 minutes with fresh changes of wash buffer, each time at ambient temperature.
16. Dilute the primary antibody-specific HRP-labeled secondary antibody with 1% BSA/PBS-T and incubate with the membrane on the orbital shaker for 1 hour at ambient temperature.
17. Discard the secondary antibody solution. Rinse the membrane with two changes of wash buffer PBS-T, wash it once for 15 minutes and four times for 5 minutes with fresh changes of wash buffer, each time at ambient temperature.
18. Warm the ECL Plus Western Blotting Detection Reagents (GE Healthcare) to ambient temperature. Prepare 0.1 mL detection solution per cm^2 of the membrane, enough to cover the entire membrane by mixing Reagent A and Reagent B at a ratio of 40:1.

19. Lay the membrane on a sheet of SaranWrap with protein side up. Absorb excess wash solution with a piece of Kimwipe. Add the mixed ECL-Plus detection solution on to the membrane. Incubate for 5 minutes in the dark (by turning off the lights) at ambient temperature.
20. Drain off the detection solution from the membrane. Carefully wrap the membrane with a new piece of SaranWrap. Gently roll out any air bubbles with a pipette. Place the sealed membrane with protein side up in an X-ray film cassette. In a dark room, place a piece of film on top of the membrane and close the cassette.
21. Expose the film to the chemiluminescent reaction on the membrane for 30 seconds. Remove the film from the cassette and place a second film in the cassette. Immediately develop the first film in an Automated Film Developer, Mini-Medical Series, from AFP Imaging Corp (Elmsford, NY). From the appearance of the first film, decide the exposure time for the second film from 1 minute to 1 hour (*see* **Note 13**). Label each film with protein sample loading order and exposure time. Example of the Western blot analysis for the validation of alpha enolase is shown in **Fig. 16.3**.

3.7.2. Immunohistochemistry

1. Fix skin sample in 10% formalin solution as stated in step 4 of "Preparation of Mouse Skin," and then embed the formalin-fixed skin tissue in paraffin.
2. Cut 5 μm sections for staining.
3. Deparaffinize and hydrate the formalin-fixed paraffin-embedded tissue sections in xylene or xylene substitute followed by graded alcohols (100%, 95% ethanol) to water.
4. Block endogenous peroxidase activity with 3% H_2O_2 in water for 10 minutes and wash.
5. Retrieve antigen with 10 mM citrate buffer (pH 6.0) for 10 minutes in a microwave oven, followed by a 20 minute cooling down. Wash.
6. Block non-specific antibody binding by incubating slides with Biocare Blocking Reagent (#BS966M, with casein in buffer) for 10 minutes. Do not wash this step.
7. Drain slides and incubate with the primary antibody specific to a protein of interest for validation at a dilution factor pre-determined experimentally for 30 minutes at ambient temperature. Wash with buffer five times and change once.
8. Incubate slides with biotinylated secondary antibody at a dilution factor pre-determined experimentally for 20 minutes at ambient temperature. Wash with buffer five times and change once.

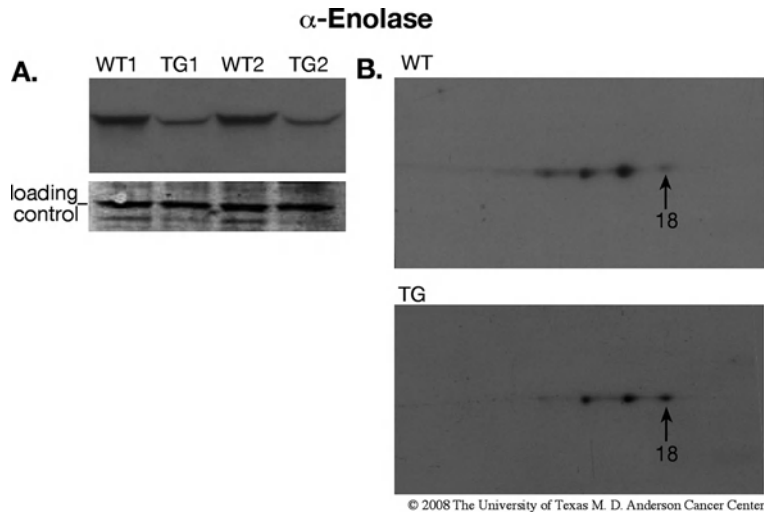

Fig. 16.3. Western blot analysis of alpha enolase. Epidermal protein extracts were prepared from BK5.IGF-1 transgenic mice (TG) and nontransgenic littermates (WT). In (4), alpha enolase had a higher protein expression in TG than in WT at protein spot #18. **(a)** 1D Western blot analysis of alpha enolase. This 1D Western blot experiment showed that the total amount of alpha enolase was less in either TG1 or TG2 than in either WT1 or WT2; **(b)** 2D Western blot analysis with protein spot #18 indicated. At this protein spot location, alpha enolase had a higher protein level in TG than that of WT, which is consistent with the result of 2D gel experiment. This figure illustrates the importance of performing 2D Western blot analysis to validate the result of differentially expressed proteins identified from 2D gel experiment. 1D SDS-PAGE Western blot analysis compares the total amount of a protein of interest, while 2D gel experiment compares a protein of interest between two samples at a particular protein spot position (i.e., possible post-translational modification). (Reproduced from (4) in which the copyright of the figures or artwork belongs to The University of Texas M.D. Anderson Cancer Center.)

9. Incubate slides with SA-HRP (BioGenex) for 30 minutes at ambient temperature. Wash with buffer five times and change the buffer once.
10. Incubate with BioGenex DAB, monitoring staining development.
11. Wash, counterstain, dehydrate, clear and coverslip slides for viewing.
12. Capture images. Example of the immunohistochemical analysis for the validation of S100A8 calcium-binding protein is shown in **Fig. 16.4**.

3.8. Future Direction

In proteomics-related research, 2D gel-based protein separation remains as one of the most utilized protein separation approaches. Like many other protein separation methods, high-abundance protein will interfere with the analysis of low-abundance proteins. To alleviate this problem, the depletion of abundant proteins and/or protein pre-fractionation (such as affinity purification, subcellular

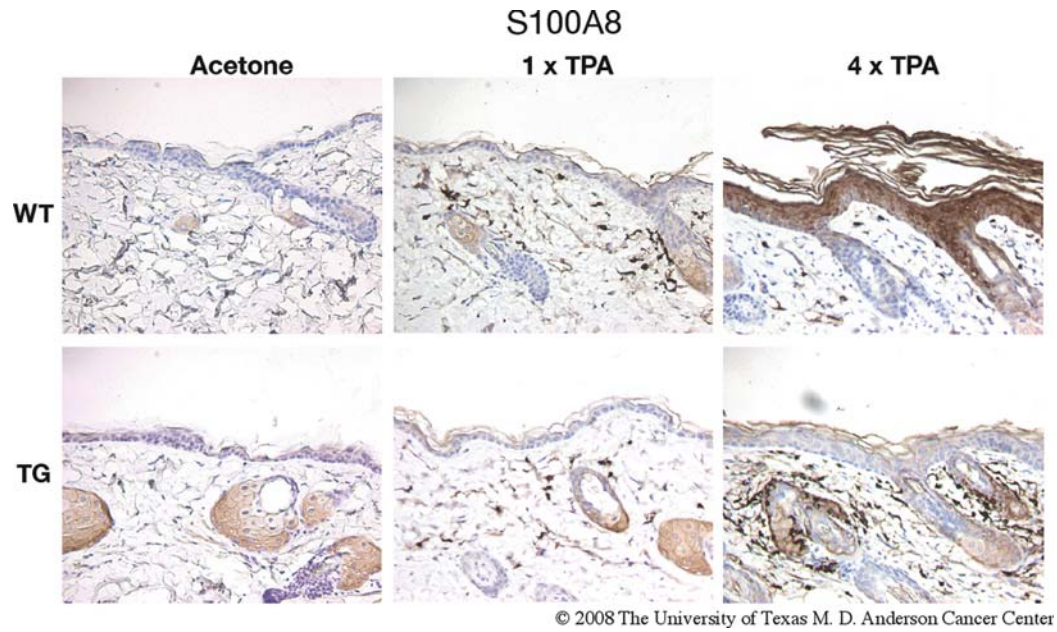

Fig. 16.4. Immunohistochemical analysis of S100A8 calcium binding protein. S100A8 of acetone-, 1 × TPA-, and 4 × TPA-treated K14.COX-2 TG and wild-type mice as indicated. In (3), 2D gel experiment showed that there was no detectable S100A8 protein expression in acetone-treated TG and WT mice. S100A8 had higher protein expression in epidermis of WT mice than TG mice from 1 × TPA treatment (data not shown). Immunohistochemical analysis of S100A8 calcium binding protein was consistent with the results of these 2D gel experiments. Furthermore, S100A8 had a much higher protein expression in epidermis of WT mice than TG mice with 4 × TPA treatment. (Reproduced from (3) with permission from the American Chemical Society.)

fractionation, and liquid phase isoelectric focusing), followed by 2D gel electrophoresis, will help us to analyze low-abundance proteins. However, this traditional protein separation remains labor intensive and cannot be automated. Antibody arrays and shotgun proteomics using technology such as liquid chromatography-based MS/MS profiling have been proved to be powerful alternatives to 2D gel (10). Nevertheless, 2D gel together with mass spectrometry is still the method of choice to study protein post-translational modification such as phosphorylation. The future direction for the molecular profiling of the epidermis is thus to study low-abundance epidermal proteins and their-related post-translational modification, among many other aspects.

4. Notes

1. Two methods of rehydrating IPG strips exist: (1) the most frequently used approach is to add protein sample to rehydration buffer and rehydrate IPG strips while protein enters the

IPG gel matrix; (2) some proteins do not readily enter rehydrated IPG gel matrix; protein samples can be applied to the IPG strips following rehydration just prior to focusing.

2. It is very easy to make a huge mess in a microwave when you try to melt agarose in SDS-containing gel running buffer. You can avoid this mess by using a large flask and keeping a close eye on the flask.
3. The pH of CyDye labeling buffer must be adjusted to pH 8.5 to ensure high CyDye labeling efficiency.
4. It is recommended that trypsin solutions should be freshly prepared prior to use. However, in our experience, the unused portion of trypsin solution can be stored in aliquot at –20°C and is good for use within 1 month of its preparation.
5. Large proteins and membrane proteins are difficult to resolubilize, especially after they are precipitated. It is essential to do everything possible to get these types of proteins and all other proteins back into solution in order to have a complete representation of proteins in a sample. However, it is not recommended to heat the protein in urea-containing rehydration buffer because urea will modify proteins and then change these proteins' isoelectric points.
6. We found that making two sets of 2D gel images, one at a lower voltage and the other at a higher voltage, can capture both low-intensity spots without losing signals and high-intensity spots without saturation, respectively.
7. Manual protein spot picking and in-gel tryptic digestion should be carried out in a clean hood to avoid cross-contamination. The operator who conducts these two tasks should wear a clean laboratory coat and mask, change gloves frequently, and use a different set of pipettes.
8. The use of siliconized 1.5 mL Eppendorf or 0.5 mL tube will prevent peptides and proteins from sticking onto plastic walls.
9. Gel pieces tend to be very small in size especially after drying. One should take extreme care not to lose them during liquid transfer by pipeting.
10. Autolysis of trypsin results in overwhelming trypsin peaks, which will interfere with the protein identification for small and faint protein spots. Therefore, the amount of trypsin used in each in-gel tryptic digestion reaction should be decided based on the size and intensity of a protein spot. We usually use 20 μL of trypsin solution for the most intense spot(s), half the amount (10 μL) for a spot with the median size and intensity, and one-third of the amount (~7 μL) for a small and faint spot.

11. Drying the peptides to complete dryness will result in difficulty in bringing all the peptides back into solution.
12. For 2D gel Western blot analysis, the traditional 2D gel should be performed up to the second dimensional protein separation, followed by protein transfer as described in **Section** 3.7.1.
13. Alternatively, one can also use Typhoon 9410 (GE Healthcare) to acquire signal from the chemiluminescent reaction for Western blot analysis. In fact, this approach gives rise to a larger linear dynamic range of signal intensity and saves the cost of X-ray films, which require a few exposures to obtain right signal intensity.

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Chapter 17

Detection of Gene Expression in Embryonic Tissues and Stratified Epidermis by In Situ Hybridization

Maria I. Morasso

Abstract

Although recent molecular biology advances provide a very effective way in determining localized gene expression, the visualization of the expression by in situ hybridization of whole-mount embryos or paraffin-embedded tissue sections continues to be an excellent method to determine overall gene expression. In this chapter, I review protocols designed to determine mRNA expression by in situ hybridization techniques, in particular, focusing on the developing stratified epidermis.

Key words: DNA, RNA, Gene expression, Epidermis, *In situ* hybridization.

1. Introduction

The epidermis is a stratified squamous epithelia derived from the ectodermal layer during embryogenesis. Postnatally, it serves as an effective protective barrier to the organism from the environment. The epidermis is a continuously renewing tissue, where the proliferative basal cells follow a complex program of differentiation to form the spinous and granular layers culminating in the formation of the cornified layer. The differentiation process is characterized by a stepwise program of transcriptional regulation and is concurrent with the sequential induction and repression of structural, regulatory, and enzymatic strata-specific differentiation markers. We have successfully used an in situ hybridization protocol on paraffin-embedded embryonic and adult tissues to determine the specific expression of highly conserved homeobox transcription factors and epidermal-specific proteins such as Suprabasin and the Ca^{++}-binding protein Scarf (1–3).

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DOI 10.1007/978-1-60761-380-0_17,

2. Materials

1. Diethylpyrocarbonate (DEPC)-treated water.
2. Phosphate-buffered saline (PBS); if made from a 10 × stock, prepare with DEPC-treated water.
3. Buffer-saturated Phenol/chloroform/isoamyl alcohol (Roche cat. no. 03117979001).
4. [^{33}P]-UTP (Amersham cat. no. BF1002).
5. Ribonucleotide triphosphates rATP, rCTP, rGTP, rUTP at 10 mM each in separate tubes (Promega cat. no. P1221).
6. T3, T7, or Sp6 RNA polymerases as required (Promega cat. no. P2083, P2075, P1085, respectively).
7. RNAsin plus RNase Inhibitor (Promega cat. no. N2611).
8. RQ1 DNase 1 U/μl (Promega cat. no. M610A).
9. Yeast tRNA, resuspend to 10 μg/μl.
10. Acetylation buffer: 0.1 M Triethanolamine, 0.9% NaCl (TEA). Add acetic anhydride to a final concentration of 0.25% just prior to use.
11. Hybridization buffer: 50% Formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 8, 5 mM EDTA, 10 mM Na_2PO_4 phosphate buffer, 10% dextran sulfate, 1 × Denhardt's, 0.5 mg/ml yeast RNA. This solution can be prepared and stored at –20°C until use.
12. 1,4-Dithiothreitol (DTT) 1 M.
13. 4% Paraformaldehyde freshly made in PBS.
14. Methanol.
15. Proteinase K, stock dissolved to 10 μg/ml (Roche cat. no. 03 115 879 001).
16. Plastic coverslips (ONCOR cat. no. S1370-14).
17. 20 × SSC.
18. NTE buffer (RNase treatment): O.5 M NaCl, 10 mM Tris-HCl pH 8, 5 mM EDTA.
19. RNase, DNase-free (Roche cat. no. 1579681).
20. KODAK EMULSION NBT3 (KODAK cat. no. 1654441).
21. Developer DEKTOL (KODAK cat. no. 1464726).
22. Fixer (KODAK cat. no. 1971746).
23. Dessicant.
24. Black slide storage boxes (Becton Dickinson cat. no. 423843).

3. Methods

3.1. Tissue and Section Preparation

1. The embryonic and tissue sections are harvested and fixed in 4% paraformaldehyde (freshly made in RNase-free PBS). The length of fixing depends on the size of the tissue, but normally overnight at 4°C is adequate. The tissues are extensively washed in RNase-free PBS and processed for paraffin embedding. Sections are obtained from paraffin-embedded samples on either polylysine or silanized slides. Usually, 9–10 μm sections provide good signals. The sections may be stored in a dust-free area at room temperature (RT) until use. This procedure may also be used for frozen tissue specimens embedded in OCT compound (Tissue-Tek cat. no. 4583). However, the morphology of the tissues is much better preserved in paraffin sections.

3.2. Preparation of the DNA Template

1. The DNA template (20 μg) is linearized with the appropriate restriction enzyme incubating at 37°C for at least 2 h (*see* **Note 1**). The final volume of the digestion should be 100 μl as follows: DNA, 10 μl of the 10 × restriction enzyme buffer, 5 μl of the appropriate restriction enzyme, and complete the volume with H_2O.

   The DNA is linearized in two separate reactions: one at the 5′ end and one at the 3′ end of the cDNA template to generate the sense and antisense probes. The sense probe will be used as a negative control for the experiment.
2. After the digestion is verified as complete (*see* **Note 1**), proceed to purify the digested template by extraction with TE-saturated phenol/chloroform. The template is subsequently precipitated by addition of 10 μl of 3 M NaOAc and 250 μl of 100% EtOH and placed on dry ice for 1 h or at –20°C overnight.
3. Collect the precipitate by centrifugation at 18,000 g for 10 min at 4°C. Remove the supernatant and wash the pellet with 100 μl of cold 70% EtOH. Centrifuge at 18,000 g for 10 min at 4°C, remove the supernatant, and allow the pellet to air dry (*see* **Note 2**).
4. Resuspend the pellet in 15 μl of DEPC H_2O, determine concentration by measuring the absorbance of 1 μl at OD_{260}, and adjust the final concentration to 1 μg/μl. Store at –20°C until use.

3.3. Synthesis of the Riboprobe

1. The riboprobe in vitro transcription reaction is set as follows: 4 μl of the 5 × RNA polymerase buffer, 2 μl of 0.1 M DTT, 1 μl of each rATP, rGTP, rCTP (10 mM), 5 μl of [^{33}P]-UTP,

1 µl RNasin, 1 µl of linearized DNA template (1 µg), 1 µl of the appropriate RNA polymerase (T3, T7, or Sp6 RNA polymerase), and nuclease-free H_2O to a final volume of 20 µl.

2. Incubate for 2.5 h at 37°C.
3. Add 1 µl of RQ1 DNase and incubate 15 min at 37°C.
4. Add 1 µl of 0.5 M EDTA RNase-free to stop the reaction.
5. Add 77 µl of DEPC H_2O and 1 µl yeast tRNA (stock 10 µg/µl) and extract once with an equal volume (100 µl) of phenol/chloroform.
6. To proceed to precipitate the radioactive-labeled riboprobe, add 50 µl of 7.5 M NH_4OAc and 400 µl of 100% EtOH.
7. Place on dry ice for 30 min or at –20°C overnight.
8. Centrifuge thawed sample at 4°C for 20 min and then remove and dispose radioactive supernatant accordingly.
9. Wash the pellet with cold 70% EtOH at 4°C for 5 min. Remove wash.
10. Resuspend the pellet in 20 µl of 100 mM DTT.
11. Check labeling by liquid scintillation counting of 1 µl of the resuspended riboprobe and add 181 µl of hybridization buffer for a final volume of 200 µl.
12. Store the riboprobe at –80°C until use.

3.4. Pretreatment of Tissue Sections

1. To remove the paraffin and rehydrate tissue sections, place slides on rack and carry out the following sequential steps at RT and with slow continuous shaking:
   a. Xylene for 5 min twice
   b. 100% EtOH for 5 min twice
   c. 95% EtOH for 1 min twice
   d. 70% EtOH for 1 min once
   e. 50% EtOH for 1 min once
   f. 30% EtOH for 1 min once
   g. Wash slides in PBS twice
2. After the removal of the paraffin, proceed to the permeabilization/proteinase K treatment. For this, first place the slides in 2 × SCC for 5 min followed by a PBS wash for 5 min.
3. Fix in 4% PFA (freshly made) in PBS for 20 min (save the solution for second fixation, see below), followed by three washes in PBS 5 min each.
4. After fixation, the slides are incubated for 10 min in a Proteinase K solution 2 µg/ml PK made in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA. The amount of time may vary with age of tissue (*see* **Note 3**).

5. Wash in PBS for 5 min.
6. Perform a second fixation step in 4% paraformaldehyde/PBS for 20 min, followed by three washes in PBS 5 min each.
7. Add the TEA acetylation buffer freshly made for 10 min, followed by a wash in PBS for 5 min.
8. Dehydrate the tissue sections by sequential steps of 30% EtOH, 50% EtOH, 70% EtOH, 80% EtOH, 95% EtOH, 100% EtOH, each for 2 min.

3.5. Prehybridization

Prehybridization treatment of the slides is done in an oven and a humidity chamber (container lined with Whatman® paper soaked with 50% formamide, 5 × SSC) at 60°C. Add 100–150 µl of hybridization solution to each slide (**Section 3.1.4**, step 8) and cover with a plastic strip (*see* **Note 4**). Prehybridize for at least 3 h at 60°C.

3.6. Hybridization

1. For preparation of the probe, dilute the riboprobe in hybridization buffer to have 1×10^6 cpm/100 µl/slide. Heat the diluted probe at 75°C for 5 min and load 100 µl of the probe-containing hybridization solution on each slide.
2. Cover each tissue section with a plastic coverslip and incubate in the same humidity chamber used in the prehybridization step (**Section 3.1.5**). Make sure there are no bubbles under the plastic coverslip. Incubate the slides in a chamber in the hybridization oven at 60°C for at least 12 h.

3.7. Post-hybridization Treatment of Tissue Sections

1. The buffers used during the post-hybridization steps should be made in advance and prewarmed to the appropriate temperature (indicated below in each step).
2. The slides are set again on a rack and washed with 5 × SSC at 50°C for 1 h. The plastic coverslip will slide off and remain in the washing chamber (*see* **Note 5**).
3. Wash with a high stringency wash 2 × SSC, 20 mM DTT, 50% formamide at 65°C for 30 min.
4. Wash in prewarmed NTE buffer at 37°C three times for 10 min each.
5. Set the slides in the container for RNase treatment with 20 µg/ml RNase in NTE buffer 37°C for 30 min.
6. Perform a second NTE wash at 37°C for 15 min.
7. Perform a second high stringency wash 2 × SSC, 20 mM DTT, 50% formamide at 65°C for 30 min.
8. Wash in 2 × SSC at RT for 15 min.
9. Wash in 0.1 × SSC at RT for 15 min.

10. Proceed to dehydrate the tissue sections by sequential immersion at RT for 3 min each as follows: 30% EtOH, 50% EtOH, 70% EtOH, 95% EtOH, and 100% EtOH.
11. Allow the slides to air dry in a dust-free area (*see* **Note 6**).

3.8. Photographic Emulsion Preparation, Coating, and Exposure

1. Before initiating the coating of the slides in emulsion, make sure that the dark room to be used has appropriate safelights and that the light indicators of all equipment in the room have been masked. The emulsion preparation, coating, and exposure steps must be performed in the dark.
2. Set a water bath in the dark room at 43°C. Melt KODAK emulsion by placing the emulsion container in a beaker with water, inside the water bath at 43°C for 60–90 min. Once melted, dilute the emulsion 1:1 with H_2O and gently mix avoiding the formation of bubbles. Aliquot the emulsion dilution in 50 ml Falcon tubes. Leave one tube for immediate coating of slides and save the rest of the aliquots wrapped in aluminum foil and inside a light-safe black box at 4°C for future use. These will have to be thawed at 43°C at time of use.
3. Coat the slides by dipping into the diluted emulsion, making sure to cover the complete area of the tissue section (*see* **Note 7**). Let the slides dry by placing vertically in slots of a test tube rack.
4. Dry slides for at least 2 h at RT. Place the emulsion-coated slides in small black storage boxes (that contain dessicant), wrap them in several layers of aluminum foil, and store in safelight container at 4°C until developing (*see* **Note 8**).

3.9. Development of the Emulsion-Coated Slides

1. Set containers with Developer (DEKTOL diluted 1:1 in water) and Fixer solutions and one with distilled water in the dark room. The developer solution should be pre-chilled, since developing is done at 15°C, without agitation. The DEKTOL solution may be stored at 4°C and should be prepared fresh after 1 month.
2. Transfer slides to metal slide holder (24 slides/holder) in the dark room.
3. Place the metal holder in glass container with the DEKTOL solution 16°C, and develop for 2 min.
4. Stop the developing by placing the metal holder in container with distilled water for 15 sec.
5. Fix for 5 min in fixer solution.
6. Wash with slowly running distilled water for 5 min.
7. Let slides dry in dust-free area (i.e., inside a drawer).

3.10. Counterstain and Coverslipping of Slides

1 Wash in distilled water and then dip in 0.35% methyl green solution for 5 min (*see* **Note 9**).

2. Take out slides, let them dry and then wash twice in H_2O to get rid of excess dye.
3. Let the slides air dry overnight. To dehydrate tissue and prepare for mounting, carry out the following sequential steps at RT with no agitation: 70% EtOH for 1 min, 90% EtOH for 1 min, 100% EtOH twice 5 min each, Xylene twice 5 min each. Overlay the tissue section with adequate amounts of Permount (couple of drops) and place glass coverslip on top with care, avoiding any air bubbles.
4. Place slides facing up on a flat surface overnight to dry.

3.11. Photography of Developed Tissue Section Slides

For microscopic examination of the silver grains in the developed emulsion, the images are captured with a dark field setting, using a variable condenser; part of an Axiophot Zeiss microscope. The light images of the same counter-stained area can be obtained utilizing bright field settings.

4. Notes

1. The DNA template is usually digested for 1 h at 37°C for every 10 μg of DNA in the reaction. Following the digestion, an aliquot of the digestion (5 μl, approximately 1 μg) is run on an agarose gel to verify complete linearization of the template. If the reaction is not complete, add 1 μl more of enzyme and incubate further at 37°C. Run again until complete linearization has been verified.
2. It is important not to overdry the pellet, since it will be very difficult to resuspend. About 5 min to air dry at RT is normally sufficient.
3. The length of time and concentration of the enzyme for the Proteinase K digestion is critical for the permeabilization of the tissue. We have found that at the indicated conditions, the riboprobe has good penetration into the tissue. However, longer digestion periods will lead to breakdown of the tissue samples, especially on skin sections.
4. It is important to put a coverslip on top of the probe containing hybridization solution. Although this step is performed in a humidity chamber, the temperature of the oven is high enough to result in evaporation. The tissue sections on the slides must not dry at any moment, since this is a great cause of

artifact background. Given that the hybridization incubation is done at 60°C, special plastic coverslips are used, do not use Parafilm®.

5. This first wash buffer after the overnight hybridization should be considered radioactive waste and disposed accordingly.
6. It is critical to work in a dust-free area. Any dust or powder present during the emulsion preparation and coating of the slides will affect the results. Make sure that all glassware and plastic containers used are dust free and use powder-free_ gloves.
7. The coating of the slides with the emulsion should be done with one single slow dipping movement to try to obtain similar emulsion thickness at all slides.
8. The length of exposure time needed before development will depend on how abundant is the expression of the gene. A couple of slides can be used with the same probe to expose for different lengths of time and develop independently. The use of ^{33}P instead of ^{35}S and addition of DTT through the process has proven, in our hands, to diminish the background substantially.
9. A weak signal might be quenched and difficult to detect if counterstain is performed with hematoxylin/eosin. We have used 0.35% methyl green as an alternative staining. Although the staining is not as nice as with hematoxylin/eosin (particularly the nuclear staining), it is a good compromise that allows for visualization of the tissues on bright field imaging.

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Chapter 18

Embryonic Mammary Anlagen Analysis Using Immunolabelling of Whole Mounts

Heena Panchal, Olivia Wansbury, and Beatrice A. Howard

Abstract

The mouse mammary gland is a unique organ since although the mammary gland primordium forms during embryogenesis, the majority of development occurs postnatally upon hormonal stimulation at puberty and full functionality (i.e. lactation) is not achieved until after parturition. Since both the epidermis and mammary glands share the same developmental origin, many mouse models with epidermal phenotypes often also exhibit abnormal mammary gland development. However, since the most widely used methods to analyse mammary glands are laborious and time-consuming, many mouse models exist that have not been analysed for mammary phenotypes. We have developed a simplified method that allows rapid analysis of embryonic mammary anlagen using immunolabelling of whole mounts that should facilitate more comprehensive studies of mouse mammary glands.

Key words: Mammary gland, Placode, Bud, Anlagen, Confocal, Immunofluorescence.

1. Introduction

The mammalian epidermis is organised as a sheet with appendages attached to it, which makes this tissue amenable to whole mount analysis. Immunolabelling of whole mounts is now routinely used to analyse the expression of markers for stem cells, proliferation and cell death in the epidermis, hair follicles and sebaceous glands from transgenic mice (1–3). The mammary anlagen are epidermal appendages that are first morphologically distinct during E11 as elliptical-shaped placodes and are obvious as distinct buds by E12.5 (4, 5). We have utilised the epidermal whole mount method described previously by Braun et al.(1) and found it is highly suitable for analysing the surface of the ventral flank, including

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the mammary anlagen, from mid-gestation mouse embryos. This technique greatly reduces the labour associated with this analysis since the tissue does not need to be processed, embedded and sectioned, which is especially laborious for small anatomical structures such as the mammary anlagen. In addition, it provides superior resolution of cellular detail when compared to mammary anlagen that have been analysed comprehensively using paraffin-embedded materials (6, 7).

Confocal microscopy of whole mounts provides an excellent opportunity for observation and assessment of epithelial features of cells within the mammary anlagen as it proceeds from the multilayered placode through to the early and later bud stages (**Fig. 18.1**). The epidermal–mesenchymal boundary is obvious throughout all stages of early mammary development, from the first appearance of stratification or multilayering, which is the earliest morphological

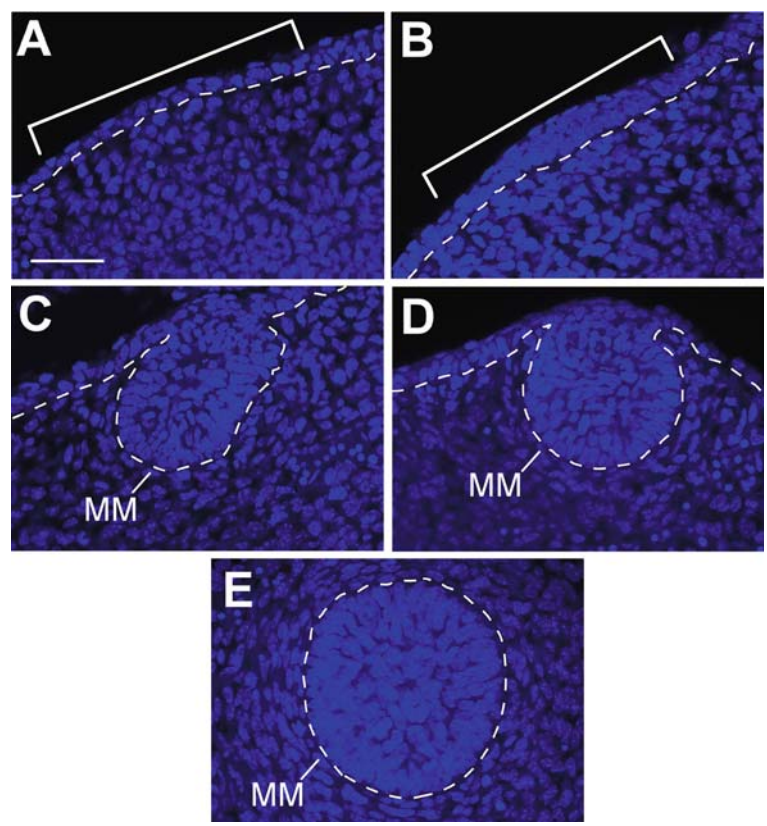

Fig. 18.1. Successive stages of mammary gland development in embryonic mouse epithelium from E11.0 through E14.5. Mammary whole mounts from ventral embryonic skin were stained with DAPI to reveal the nuclei. (**A**) The future site of a mammary placode (bracketed), at E11.0, prior to stratification. (**B**) Mammary placode (bracketed) at E11.5. (**C**) Mammary bud at E12.5. (**D**) Mammary bud at E13.5 (**E**) Mammary bud at E14.5. The epithelial–mesenchymal boundary is denoted by a white dashed line. MM, mammary mesenchyme. Scale bar: 50 μm.

appearance of the mammary anlagen. Mammary buds from E12.5 embryos can repopulate cleared fat pads, indicating that a primitive mammary stem cell population exists within these small clusters of cells. Consistent with previous studies using transmission electron microscopy (TEM) analysis of E15 mammary anlagen (8), we observe a variety of morphological types of cells within the mammary bud from E11.5–E14.5 (**Fig. 18.1**). The majority of cells at the periphery of the mammary bud, including the basal cells, which are in direct contact with the basement membrane, are elongated with a columnar appearance. The cells in the centre of the mammary bud exhibit a wider range of sizes, shapes and fewer cell–cell contacts compared to basal cells, consistent with previous analysis of E13 and E15 mammary buds by TEM (9). Combined with indirect immunofluorescence, confocal microscopy provides an opportunity for both analysis of cell morphology at high resolution and the distribution of cell markers within the mammary bud. This technique should facilitate the analysis of markers of interest within the mammary bud, including prospective stem cell and lineage markers, and may allow identification of unique populations of cells within both the mammary bud epithelium and in the mesenchyme surrounding the mammary bud, a tissue which has been shown to have mammary-inducing capacity when used in tissue recombination studies (10, 11). We found that with minor modifications, this method can also be used for the analysis of postnatal mammary glands.

2. Materials

2.1. Dissection and Fixation of Whole Mounts

1. Phosphate-buffered saline (PBS) pH 7.4 (Roche, Indianapolis, IN, cat. no. 1666789)
2. 10-cm diameter plastic cell culture dishes (Nunc, Rochester, NY, cat. no. 150350)
3. Small scissors
4. Forceps
5. Transfer pipettes (Sigma, St. Louis, MO, cat. no. Z350591)
6. Dissecting iris scissors (FST, Heidelberg, Germany, cat. no. 14060-09)
7. Blunt dissecting forceps 4″
8. Graefe Forceps (FST, cat. no. 11051-10)
9. Dumont #5 Inox forceps (FST, cat. no. 11251-20)
10. Formalin: dissolve 4 g NaH_2 PO_4, 6.5 g Na_2HPO_4, 100 ml 40% formaldehyde, in distilled deionised water and make to final volume of 1 l. Store at 4°C

11. 4% PFA: melt 4 g of paraformaldehyde in 100 ml of PBS at 65°C; filter and store at –20°C
12. Glass microscope slides
13. Cell Safe biopsy capsule (Cell Path Ltd., Newtown, Powys, UK, cat. no. EBE-0301-02A)
14. 50 ml reagent tubes (Sarstedt, Nümbrecht, Germany, cat. no. 62.547.254) or slide rack and container

2.2. Immunofluorescence of Whole Mounts

1. 96-microwell conical bottom plate (Nunc, cat. no. 732-2702)
2. Micro Amp Optical Adhesive Tape (Applied Biosystems, Foster City, CA, cat. no. 4311971)
3. Tris-buffered saline (TBS): 10 × stock is 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl, pH 7.4.
4. Phosphate-buffered saline (PBS), pH 7.4 (Roche, cat. no. 1666789): 10 × stock is 0.01 M KH_2PO_4, 1 M Na_2HPO_4, 1.34 M NaCl, 0.027 M KCl
5. Tween-20 (Sigma, cat. no. P7949)
6. Skim milk powder (Marvel, Premier Foods, St Albans, Hertfordshire, UK)
7. Fish skin gelatin (Sigma, cat. no. G-7765)
8. Triton X-100 (Sigma, cat. no. T9284)
9. PB buffer: 0.5% Skim milk powder, 0.25% Fish skin gelatin (Sigma, cat. no. G-7765), 0.5% Triton X-100 in TBS (*see* **Note 1**).
10. Antibodies α6-integrin (BD Pharmingen, Franklin Lakes, NJ, cat. no. 555734), Collagen IV (Chemicon, Billerica, MA, cat. no. AB756P), Laminin (Sigma, cat. no. L9393)
11. 6.5 mm Transwell plate with 8.0 μm insert (Corning, Corning, NY, cat. no. 3422)
12. Appropriate fluorochrome-conjugated secondary antibodies (Invitrogen, Carlsbad, CA), Alexa 555: goat anti-rat IgG (cat. no. A21434), goat anti-rabbit IgG (cat. no. A21429)
13. DAPI (4,6-diamidino-2-phenylindole) (Invitrogen, cat. no. D21490) 14.3 mM stock
14. Superfrost Plus Slides (VWR International, Lutterworth, Leicestershire, UK, cat. no. 48311-703)
15. Coverslips from VWR International (cat. no. 631-0124 or 631-0137)
16. Vectashield (Vector Labs, Burlingame, CA, cat. no. H-1000)
17. Nail varnish

3. Methods

3.1. Dissections of Tissues and Preparations of Whole Mounts of Embryonic Mammary Anlagen

The age of the embryo and stage of pregnancy are determined by checking vaginal plugs. Noon of the day a plug is observed is counted as day 0.5.

1. Using scissors, remove the uterus with embryos and transfer to a 10 cm cell culture dish filled with ice-cold PBS (*see* **Note 2**).
2. Remove the embryos from the uterus with small scissors and forceps**.**
3. Collect yolk sacs for genotyping if necessary (*see* **Note 3**).
4. Using a dissecting microscope, dissect membranes away from embryos.
5. Cut off the head of the embryo with scissors, forceps or dissecting knives.
6. Using a transfer pipette that has been cut to appropriate size for the embryo stage, place trunks in a Petri dish containing ice-cold PBS. The embryos will lie on their sides.
7. Use dissecting scissors, isolate the right flank of embryo as shown in **Fig. 18.2A**; to keep the embryo in place and provide stability, place forceps held in left hand on the anterior edge, and with dissecting scissors held in right hand, make an incision starting slightly anterior and dorsal to the forelimb bud and cut along the dorsal-lateral ridge until the mid-flank (*see* **Note 4).**
8. Make a second cut from this point to ventral region posterior to the hindlimb bud. Turn flank of embryo over and shell out the internal organs.
9. Turn flank of embryo over and remove limb buds (*see* **Note 5**). The dissected flank with the location of the mammary buds is shown in **Fig. 18.2B**.
10. Turn flank of embryo over. Use dissecting forceps to grip the edge of the flank and peel internal tissues away from the ventral skin (**Fig. 18.2C**, **D**).
11. **Figure 18.2E** shows the flank with the skin-side facing up which still has a thick layer of mesenchymal tissue attached. Remove as much of this as possible with dissecting forceps until the mammary buds are clearly visible (**Fig. 18.2F**) (*see* **Note 6**). **Figure 18.2G** shows ventral skin of suitable thickness for whole mount IF spread on glass slide (*see* **Note 7**).
12. Repeat on other side of embryo.

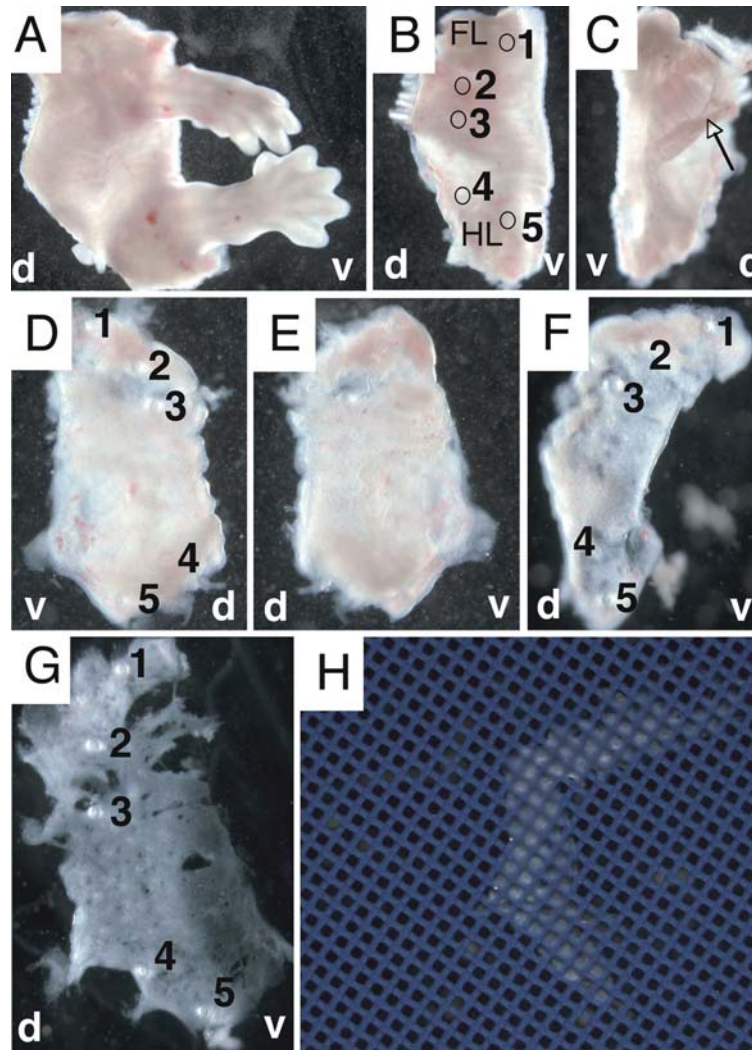

Fig. 18.2. Dissection of E14.5 ventral flank with mammary buds. (**A**) Right ventral flank of female embryo. (**B**) Ventral flank with limb buds removed: approximate location of mammary buds are indicated by black circles. (**C**) Most internal tissues can be removed by peeling them away from the epithelium with forceps: arrow indicates region where tissue is grasped and pulled with forceps to separate. (**D**, **E**) Dense mesenchyme is still visible and must be removed. (**F**) After dense mesenchyme is removed, tissue is suitable for whole mount immunofluorescence protocol. Sample may be spread and fixed on glass microscope slide (**G**) or placed in biopsy cassette in fixative (**H**). d, dorsal and v, ventral are indicated. Numbers (1–5) indicate the locations of the individual mammary buds.

13. Repeat for all embryos in litter.
14. Whole mounts can be fixed individually on glass slides in slide racks or whole mounts from a litter may be put together in a biopsy cassette (**Fig.** 18.2H). After closing biopsy cassette, place in 50 ml falcon tube containing formalin or other suitable fix (*see* **Note 8**).

3.2. Fixation of Whole Mounts of Embryonic Mammary Anlagen

1. Fix tissues for 2 hours at room temperature.
2. Decant used fix into suitable waste receptacle. Rinse twice in PBS and use for whole mount immunofluorescence within a week (or store in PBS and 0.2% sodium azide at 4°C for up to 1 month).

3.3. Preparations of Whole Mounts of Postnatal Mammary Glands

1. After dissecting the mammary gland, spread on a glass microscope slide with blunt forceps.
2. Place slide in a 50 ml corning tube or in slide rack in container filled with fixative and fix glands in formalin or other fixative overnight at room temperature.
3. Rinse twice in PBS and store in PBS at 4°C for immediate use or in PBS with 0.2% azide for storage up to 1 month.
4. Cut into pieces between 3 and 4 mm with dissecting scissors prior to proceeding with whole mount immunofluorescence protocol (*see* **Note 6**).

3.4. Immunofluorescence of Whole Mounts

1. Using forceps, remove samples from slide or biopsy cassette and place in well of 96-well plate containing 150 µl PB buffer (*see* **Note 9**).
2. Block and permeabilise tissues in PB buffer for 30–60 minutes at room temperature on shaker with gentle agitation (*see* **Note 10**).
3. Use micropipettor to remove PB from wells.
4. Dilute primary antibodies in PB buffer and add 150 µl per well, seal plate with adhesive film, and incubate overnight at room temperature with gentle agitation (*see* **Note 11**).
5. Using dissecting forceps, transfer whole mounts to transwell plate with membrane insert filled with PBS + 0.2% Tween-20.
6. Wash in PBS + 0.2% Tween-20; move transwell inserts into wells filled with fresh wash so that wash solution is changed approximately four times over 4 hours.
7. Add secondary antibody diluted 1:1000 and DAPI diluted 1:10,000 in PB buffer (1 ml per well), cover with aluminium foil and incubate overnight at room temperature with gentle agitation.
8. Wash in PBS + 0.2% Tween-20, changing solution three to four times over 1 hour.
9. Fix in 4% PFA at room temperature for 1 hour, then wash in PBS three to four times over 1 hour, with gentle agitation.
10. The samples are ready to be mounted. The whole mount is spread out on a microscope slide and the tissue is oriented so that surface epithelium, mammary epithelium and mesenchyme can be assessed (*see* **Note 12**) (**Fig. 18.1**).

11. A drop of Vectashield mounting medium is added and then a coverslip is placed on top. Nail varnish is used to seal the edges of the coverslip. The sample can be viewed once the varnish has dried and may be stored in the dark at 4°C for a month or at –20°C for longer-term storage.
12. The stained whole mounts are viewed with a Leica SP2 confocal scanning microscope. The laser outputs are controlled via the Acousto-Optical Tunable Filter. The collection windows were set with the Acousto-Optical Beam Splitter. Images are collected with a $\times 20$ dry lens or with a $\times 40$ oil immersion lens. Leica confocal software can be used to overlay the fluorescence images. Images are exported into Adobe Photoshop CS2 v8. Examples of whole mounts stained for DAPI are shown in **Fig. 18.1**. Examples for whole mount immunofluorescence with Collagen IV, α6-integrin and Laminin are shown in **Fig. 18.3**.

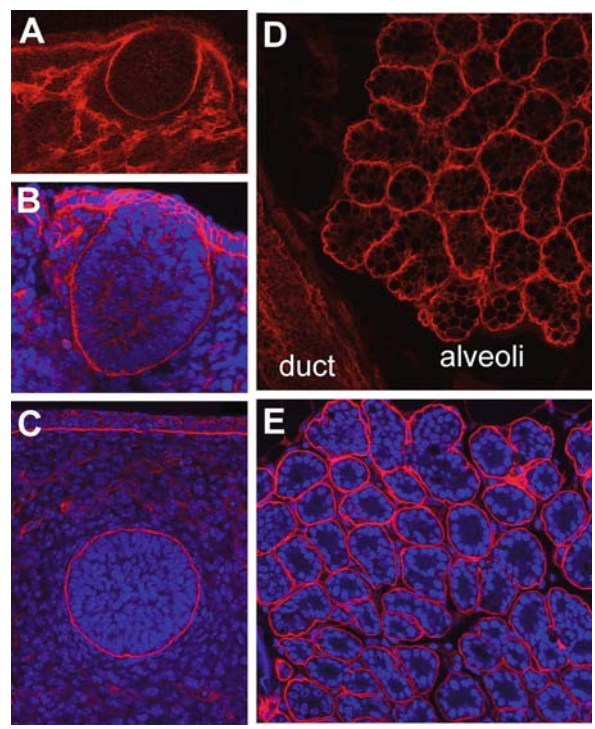

Fig. 18.3. Examples of immunofluorescence of mammary whole mounts. Immunofluorescence for (**A**) Collagen IV; E11.5 mammary placode. (**B**) α6-integrin with DAPI overlay; E12.5 mammary bud. (**C**) Laminin with DAPI overlay; E13.5 mammary bud. (**D**) α6-integrin; duct and alveoli from P17.5 mammary gland. (**E**) Laminin with DAPI overlay, alveoli from P15.5 mammary gland.

4. Notes

1. PB may be stored for up to 2 days at 4°C.
2. E11.5–E14.5 stages have been successfully used.
3. Sexual dimorphism occurs as the mouse mammary bud undergoes androgen-mediated destruction at E14.5. At stages prior to E14.5, there are no obvious morphological differences in the mammary buds between male and female mice. Embryos can easily be sexed by the appearance of the gonads: testes are recognized by the presence of testis cords, ovaries lack any morphological distinction. Alternatively PCR can be used to establish the presence of the Y chromosome using the primers Zfy1: 5′-GACTAGACATGTCTTAACATCTGTCC-3′ and Zfy2: 5′-CCTATTGCATGGACAGCAGCTTATG-3′ with the following cycling conditions:

   92°C for 2 min; 35 cycles: 92°C for 2 min, 58°C for 30 sec, 68°C for 1 min; then 72°C for 7 min and hold at 4°C degrees. Run product on a 2% agarose gel to detect the presence of 200 bp band in male embryos (12). As a positive control, *Hprt* is PCR amplified with the primer set, HprtF: 5′-GACTGAAAGACTTGCTCG-3′ and HprtR: 5′-CTAGGATGAATGAAGCTCGG-3′ which should produce a band in male and female embryos.
4. Alternatively, dissecting forceps can be used to make cuts using a snipping motion.
5. Mammary buds 1 and 5 are not visible until the limb buds have been removed.
6. This is a critical step that will impact both the antibody staining and the quality of confocal images. If the whole mount is too thick, permeabilisation is inefficient and both antibody and DAPI stain will be sub-optimal.
7. Alternatively, the sites of the five pairs of mammary buds can be ascertained so that the individual mammary buds can be dissected separately if required.
8. 4% PFA, formalin, and IHC zinc fixative have been used with this protocol with other antibodies.
9. Up to five whole mounts may be processed together in one well.
10. Whole mounts from postnatal mammary glands and older embryonic stages require longer incubation in PB for optimal results.
11. Recommended dilutions are α6-integrin 1:100; Collagen IV 1:100; Laminin 1:5000 which are used with tissue fixed in formalin.

12. Using a dissecting microscope, whole mounts should be spread out as thinly as possible (*see* **Fig. 18.2G**). Whole mounts from postnatal mammary glands should be cut to pieces between 1 and 2 mm before adding mounting medium and coverslip.

Acknowledgments

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Chapter 19

Whole-Mount Assays for Gene Induction and Barrier Formation in the Developing Epidermis

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Abstract

The skin as a surface organ is uniquely accessible for whole embryo/foetal analyses of developmental changes, such as gene induction, protein expression, formation of epidermal-derived appendages such as hair follicles and formation of the protective barrier. Such analyses have emphasised the heterogenous nature of skin development, perhaps not surprisingly because epidermal development is programmed by heterogenous underlying mesenchyme. It is necessary to account for this heterogeneity by precisely matching body sites when correlating sequential events during development, for example, the activation of gene expression, or comparing wild-type with mutant/knockout animals. In this chapter protocols designed to assay whole-mount in situ hybridisation and whole-mount barrier formation are presented. Formation of the protective barrier is the endpoint of epidermal terminal differentiation and defects in this process are reflected in failure, acceleration, or delay in barrier formation. Hence, these latter assays are of particular value as a rapid initial assay for epidermal developmental defects in genetically modified mice.

Key words: Whole-mount in situ hybridization, Dye penetration, Dye exclusion barrier assays.

1. Introduction

Whole-mount assays for epidermal gene induction, protein expression and barrier function can be applied to epidermis, uniquely, until very late in gestation without penetration problems associated with other tissues. Whole-mount approaches are crucial when studying skin because of the heterogeneity of developing skin and hair follicles. This heterogeneity must be taken into account when comparing sequential developmental events or interpreting mutant/transgenic phenotypes. In this chapter, whole-mount in situ hybridisation, modified from standard

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protocols, for use on embryonic/foetal epidermis is described. The robustness and sensitivity of the whole-mount in situ hybridisation, compared with section in situ hybridisation, is probably caused by a lack of tissue manipulation and consequent cellular damage and RNA degradation before hybridisation. Hence, whole-mount RNA analysis linked to highly sensitive cellular-level RNA detection by post-hybridisation sectioning and hapten detection is described. The outcome of epidermal development is the formation of the protective barrier. Therefore, whole-mount assays for barrier function (1), described in this chapter, report defects in epidermal development and are widely used as a rapid screen for epidermal developmental change in genetically modified animals.

2. Materials

1. Glassware and plasticware used to prepare reagents for in situ hybridisation are often contaminated with RNases. To avoid this, several precautions must be taken to destroy RNases:
   a. RNase-free glassware can be prepared by baking at 180°C for 8 hours or more.
   b. Bench tops, Gilson pipettes and gel tanks can be cleaned with RNA Zap (Ambion, Austin, TX, RNase*Zap*® Wipes, cat. no. AM9786; RNA RNase*Zap*® spray, cat. no. AM9780).
   c. Use clean sterile plasticware (preferably RNase free, but this is not essential).
   d. Use sterile filter tips for pipetting.
2. Transcription buffer ($5\times$ or $10\times$) is available with most commercial preparations of RNA-dependent DNA polymerases. A typical $1\times$ transcription buffer may contain 40 mM Tris-HCl, pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM spermidine and 50 μg/ml BSA (2).
3. $10\times$ nucleotide labelling mix for riboprobe production: 10 mM ATP, 10 mM CTP, 10 mM GTP, 3.5 mM UTP and 6.5 mM of a hapten-labelled-UTP (Roche Applied Science, Indianapolis, IN: digoxigenin-11-UTP, cat. no. 1120925 6910; fluorescein-12-UTP, cat. no. 11427857910; biotin-16-UTP, cat. no. 11388908910) in 10 mM Tris-HCl, pH 7.6. Alternatively, purchase pre-mixed nucleotides (Roche Applied Science, Indianapolis, IN: DIG RNA labelling mix, cat. no. 11277073910; fluorescein RNA labelling mix, cat. no. 11685619910; biotin labelling mix, cat. no. 11685597910).

4. Enzymes and reagents for riboprobe production: T7, T3, or SP6 RNA polymerases (from a variety of commercial suppliers, e.g. Promega, Madison, WI, cat. no. P2075, P2083, P1085, respectively). RNasin ribonuclease inhibitor (Promega, Madison, WI, cat. no. N2611), dithiothreitol (often supplied with polymerases or Sigma, St. Louis, MO, cat. no. 43815) and RQ1 RNase-free DNase (Promega, Madison, WI, RQ1 RNase-free DNase, cat. no. M6101; optional, *see* **Note 1**).
5. 4 M LiCl (Sigma, St. Louis, MO, cat. no. L9650) for RNA precipitation: Dissolve 16.96 g LiCl in 100 ml of diethyl pyrocarbonate (DEPC)-treated water (*see* **Note 2**), aliquot and store at –20°C.
6. 10 × PBS buffer (phosphate-buffered saline): Per liter, mix 80 g NaCl, 2 g of KCl, 11.5 g Na_2HPO_4 and 2 g KH_2PO_4, pH to 7.4 with NaOH. Treat with DEPC (*see* **Note 2**). Dilute to 1 × with DEPC-treated water.
7. 1 × PBST (phosphate-buffered saline with Tween-20; Sigma, St. Louis, MO, cat. no. P1379): 1 × PBS with 0.1% Tween-20.
8. Proteinase K solution for permeabilisation of embryos/foetuses: For convenience, pre-made solutions can be purchased (e.g. Roche Applied Science, Indianapolis, IN; cat. no. 03115887001).
9. 2 mg/ml glycine (Sigma, St. Louis, MO, cat. no. G2879) for proteinase K inactivation. Prepare fresh before each use in 1 × PBST.
10. 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for refixation of embryos/foetuses after proteinase K treatment. Prepare by heating PBS to approximately 80–90°C, then add 4 g paraformaldehyde (Sigma, St. Louis, MO, cat. no. P6148) per 100 ml. Paraformaldehyde is dissolved on a heat stirrer. Dissolution can be assisted by adding a few drops of 1 M NaOH. After cooling, 50% glutaraldehyde stock solution (Sigma, St. Louis, MO, cat. no. G7651, stored frozen) is added to a concentration of 0.2% (e.g. 0.4 ml per 100 ml).
11. 0.1% sodium borohydride (Sigma, St. Louis, MO, cat. no. S9125) in 1 × PBST is freshly prepared for blocking free aldehyde groups after fixation. This solution will bubble and evolve hydrogen so it should be prepared in tubes with loose-fitting caps.
12. 10 × PE buffer: 100 mM Pipes, pH 6.8, 10 mM EDTA. Per liter, mix 30 g Pipes (Sigma, St. Louis, MO, cat. no. P6757) and 3.7 g EDTA (disodium salt), pH to 6.8.

13. Hybridisation buffer: 50% formamide (Sigma, St. Louis, MO, formamide BioUltra for molecular biology, cat. no. 47671) 1 × PE buffer, 0.75 M NaCl, 1% sodium dodecyl sulphate (SDS), 0.05% heparin (Sigma, St. Louis, MO, cat. no. H3393), 100 μg/ml transfer RNA (Sigma, St. Louis, MO, cat. no. R8759) and 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO, cat. no. A3294).
14. Hybridisation washes (*3*, *see* **Note 3**).
    Wash1: 1 × PE buffer, 1% SDS, 300 mM NaCl
    Wash2: 1 × PE buffer, 0.1% SDS, 50 mM NaCl
    Wash3: 50% formamide, 1 × PE, 1% SDS, 300 mM NaCl
    Wash4: 50% formamide, 1 × PE, 0.1% Tween-20, 150 mM NaCl
    Wash5: 1 × PE, 0.1% Tween-20, 500 mM NaCl
15. Embryo powder (2): Embryo powder should be prepared from the same species as being hybridised and from a similar or later developmental stage. However, for very late gestation foetuses use mid-gestation embryos (e.g. E14.5 in mouse; *see* **Note 4**) to include tissue differentiation products but avoid inclusion of excessive fat.

    Prepare embryo powder exactly as in (2). *Note*: pre-absorption of the antibody with embryo powder may be optional since many laboratories omit this step unless background is a problem (4).

    a. Homogenise in minimum cold PBS using a Dounce homogeniser or equivalent.
    b. Add four volumes of ice-cold acetone and incubate on ice for 30 min.
    c. Spin on a bench top centrifuge for 5 min to pellet, remove acetone.
    d. Wash pellet by resuspending in ice-cold acetone and re-centrifuging.
    e. Air dry pellet in an open glass or acetone-resistant vessel with continuous grinding (e.g. with a smooth glass rod) to produce a fine powder.
    f. Store in an air-tight container at –20°C.

    Before use, embryo powder should be heat inactivated. For every 5 ml of diluted antibody solution used (*see* **Section 3.1.5**, **step 1**) 1–3 mg of embryo powder is weighed into 1 ml of 1 × TBST, then heated at 70°C for 30 min.
16. Antibodies to hapten-labelled probes: Hybridisation is detected with enzyme-conjugated antibodies to the hapten incorporated into the RNA probe (riboprobe). Enzymes commonly used are alkaline phosphatase or horseradish

peroxidase (POD). Highest sensitivity is attained with alkaline phosphatase-conjugated antibodies, e.g. alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science, Indianapolis, IN; cat. no. 11093274910, anti-digoxigenin-AP, Fab fragments), or an anti-fluorescein antibody (Roche Applied Science, Indianapolis, IN, cat. no. 11426338910, anti-fluorescein-AP, Fab fragments). Alkaline phosphatase catalyzes production of a purple/brown insoluble precipitate from the colourless substrates nitroblue tetrazolium and 5-bromo, 4-chloro, 3-indolyl phosphate (*see* **step 20** below). However, there are a range of alternative substrates for alkaline phosphatase giving different coloured precipitates (e.g. BM Purple, Roche Applied Science, Indianapolis, IN, cat. no. 11442074001; HistoMark® Red, KPL, Gaithersburg, MD, cat no. 55-69-00; Vector Red™, Vector Laboratories, Burlingame, CA, cat. no. SK-5100). Horseradish peroxidase-conjugated antibodies (e.g. Roche Applied Science, Indianapolis, IN, anti-digoxigenin-POD, Fab fragments, cat. no. 11207733910; anti-fluorescein-POD, Fab fragments, cat. no. 11426346910) can be used with diaminobenzidine (DAB) substrates to give a black/brown stain or alternative substrates to give other precipitates (e.g. see Vector Laboratories under "Precipitating Peroxidase Substrates"). These reactions can be used in multiple labelling applications involving simultaneous detection of digoxigenin and fluorescein-labelled riboprobes (5).

17. 10 × TBS (Tris-buffered saline with Tween-20), *see* **Note 5**: Per liter, 30 g Tris base, pH to 7.6 with NaOH.
18. 1 × TBST (Tris-buffered saline with Tween-20): Dilute 10 × TBS and add Tween-20 to a final concentration of 0.1%.
19. 1 × TBST containing 2 mM levamisole (Sigma, St. Louis, MO, cat. no. L9756) is used when inhibition of endogenous alkaline phosphatase is required (e.g. when using an alkaline phosphatase-conjugated antibody for visualisation of the RNA signal and not a peroxidase based assay). Make up just before use.
20. Visualisation of RNA signal: BCIP (5-bromo-4-chloro-3-indolyl-phosphate) is used in conjunction with NBT (nitro blue tetrazolium) for the colorimetric detection of alkaline phosphatase activity (Promega, Madison, WI, cat. no. S3771, separate vials of BCIP and NBT are supplied, each at 50 mg/ml). Alternatively, BCIP (Sigma, St. Louis, MO, cat. no. B6777 powder or B0274 tablets) and NBT (Sigma, St. Louis, MO, cat. no. N6639 powder or N5514 tablets) can be ordered individually and reconstituted in dimethylformamide (Sigma, St. Louis, MO, cat. no. D4551) at a concentration of 50 mg/ml each. Store at –20°C.

21. NTMT solution: 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-
22. This solution is mixed from stock solutions just prior to use to prevent precipitation.
23. For detection of alkaline phosphatase activity on whole embryos mix 70 µl of NBT solution (final concentration; 350 µg/ml) and 35 µl of BCIP (final concentration; 175 µg/ml) to 10 ml of NTMT solution just before use. Stop reaction by rinsing with TBST.
24. 10% (w/v) polyvinyl alcohol (PVA) solution for alkaline phosphatase detection on sections (6): PVA reduces the development time by concentrating the substrates in a smaller area and reducing their diffusion. Per 20 ml, heat 18 ml of 100 mM Tris-HCl, pH 9.5 and 100 mM NaCl to 90°C using a hot plate. Turn off heat and add 2 g of PVA 70–100 kDa (Sigma, St Louis, MO, cat. no. P1763) to the solution while mixing with a stir bar. The PVA should take about 10–15 min to mix. Let cool.
25. For detecting alkaline phosphatase activity on sections using PVA enhancement: Mix 70 µl of NBT solution (final concentration; 350 µg/ml), 35 µl of BCIP solution (final concentration; 175 µg/ml) and 100 µl 1 M $MgCl_2$ to 20 ml of cooled PVA solution just before use (6). Colour reaction should be done in the dark (e.g. in a covered box).
26. DAB (3,3′-diaminobenzidine tetrahydrochloride) staining solution: DAB is a potent carcinogen so it is preferable to buy pre-mixed solutions or pre-weighed tablets (e.g. Vector Laboratories, Burlingame, CA, cat. no. SK-4100, Liquid DAB Substrate Kit; Sigma, St Louis, MO, cat. no. D5905, tablets). Alternatively, 0.5 mg/ml DAB (DAB, Sigma, St Louis, MO, cat. no. D5637) is dissolved in PBT. Just before use, H_2O_2 is added to a final concentration of 0.01%.
27. For detecting horseradish peroxidase activity on whole embryos incubate in DAB staining solution and monitor colour development. Stop reaction by rinsing with PBST or TBST.
28. Serum from the same species as the antibody for hapten detection is used as a blocking agent, e.g. for anti-digoxigenin antibodies raised in sheep, use sheep/lamb serum (Sigma, St Louis, MO, cat. no. S2263). Heat inactivate the serum by diluting one in ten in TBST then heating at 70°C for 20 min (*see***Note 6**). Store aliquots at –20°C.
29. 1% toluidine blue staining solution (barrier assay): Dissolve 1 g toluidine blue O (Sigma, St Louis, MO, cat. no. T3260) in 100 ml of water. It is important to dissolve in water. Commercial buffered toluidine blue solutions or stocks made in salt solutions should not be used.

30. Staining mix for endogenous glycosidases (barrier assay): 100 mM sodium phosphate pH 5.5, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 1 mg/ml X-gal (5-bromo, 4-chloro, 3-indolyl β-D-galactopyranoside; Sigma, St Louis, MO, cat. no. B4252; dilute from a 20 mg/ml stock in N,N-dimethylformamide), 0.1% sodium deoxycholate (Sigma, St Louis, MO, cat. no. D6750) and 0.2% Igepal Ca-630 (Sigma, St Louis, MO, cat. no. I8896). Make up using distilled water. Since X-gal is light sensitive it should be stored in the dark.

3. Methods

3.1. Whole-Mount In Situ *Hybridisation*

This section describes whole-mount in situ hybridisation (*see* **Note 7**) protocols (2, 3, 5) modified from the version of Conlon ((3, 5); Rossant Lab Protocols page, Conlon method) for use on embryonic/foetal epidermis, effective until the protective barrier forms at embryonic E16.5–17.5 in mouse and E17 in chick (*see* **Note 4** for staging). At this point riboprobes and antibodies are excluded from the epidermis by newly formed stratum corneum. For skin analyses, proteinase K levels are much lower than in standard protocols as it is unnecessary to penetrate underlying tissues while fragile surface ectoderm has to be protected from damage. In addition, greater gentleness is required in manipulation steps to protect ectoderm.

3.1.1. Riboprobe Generation

Riboprobes are generated by transcription using bacteriophage T7, T6 or SP6 DNA-dependent RNA polymerases from cDNAs cloned downstream from bacteriophage promoters. A wide range of commercially available cloning vectors contain these promoter sequences flanking the multiple cloning site. cDNAs available from Expressed Sequence Tag (EST) collections (e.g. I.M.AG.E. clones, http://image.llnl.gov/) are usually in this format. Normally, an anti-sense RNA probe that is complementary to the sense strand (coding) of the cDNA of interest is synthesised. As a control, a sense RNA probe is made that is complementary to the anti-sense strand (non-coding) of the cDNA of interest. Plasmids are linearised with a restriction enzyme at the 5′ end of the cDNA insert to prevent vector sequence from being transcribed. Furthermore, the plasmid should be cut with a restriction enzyme that generates a 5′ overhang, because 3′ overhangs often result in transcripts of different sizes (2).

Alternatively, DNA templates for transcription can be generated by PCR from an appropriate source using primers modified to incorporate a T7, T3 or SP6 promoter sequence. DNA should be

purified prior to transcription (e.g. Qiagen MinElute Cleanup Kit, Qiagen, Valencia, CA, cat. no. 28204) and collected in water at approximately 1 μg/μl.

Riboprobe RNA can be synthesised using commercially available kits with detailed instruction manuals (e.g. Promega, Madison, WI, see Riboprobe Systems; Roche Applied Sciences, Indianapolis, cat. no. 11175025910, Dig RNA labelling kit SP6/T7) using hapten-labelled nucleotides, most commonly digoxigenin, fluorescein or biotin-labelled UTP (*see* **Section 2**, **step 3**). Alternatively, the protocol below can be used.

Production of riboprobes by in vitro transcription.

Mix as below:

1. H_2O (not diethyl pyrocarbonate treated) with volume adjusted to 20 μl
2. 4 μl of 5 × transcription buffer
3. 2 μl of 10 × nucleotide labelling mix
4. 2 μl 0.1 M DTT (available with most commercial polymerase preparations)
5. 1 μl of RNasin ribonuclease inhibitor (10–50 U/μl)
6. 1 μl of DNA-dependent RNA polymerase (T7, T3 or SP6 RNA polymerase)
7. 1 μg of linearised plasmid template or 0.1–0.3 μg of amplified DNA

Incubate at 37°C for 2–4 hours (40°C when using SP6). Stop the reaction by adding 2 μl of 0.2 M EDTA pH 8.0. Unincorporated nucleotides can be removed for estimation of yield or riboprobe can be added directly to the hybridisation mixture without further "clean up". For removal of unincorporated nucleotides precipitate RNA by adding 2.5 μl of 4 M LiCl and 75 μl of ethanol. Mix and hold at –20°C for 5 min overnight and collect RNA by centrifugation at top speed in a microfuge for 20 min. Alternatively, use a commercial column (e.g. Qiagen, Valencia, CA, Qiagen RNeasy MinElute Cleanup Kit, cat. no. 74204; Clonetech, Mountain View, CA, Chroma Spin-30+DEPC-H2O Columns, cat. no. 636087) according to the manufacturer's instructions.

Yield can be estimated by running an aliquot (e.g. 1 μl) of the reaction mix before and after the incubation on a 1% agarose gel and visualising the synthesised RNA under ultraviolet light using ethidium bromide. An RNA band which appears after reaction with an intensity ten times that of the plasmid template indicates approximately 10 μg of RNA was synthesised (2). It is usually unnecessary to degrade template with DNases (*see* **Note 1**).

3.1.2. Collection of Embryos

1. Freshly excised embryos are fixed in ice-cold 4% paraformaldehyde in PBS for 4 hours overnight with very gentle rocking on ice (*see* **Note 7**).

2. Rinse in ice-cold PBS 3 × , approximately 5 min each.
3. Embryos/foetuses are dehydrated through a methanol gradient, approximately 5 min per step (use PBST for making methanol dilutions: (1) 25% methanol, (2) 50% methanol and (3) 75% methanol (*see* **Note 7**).
4. Embryos are rinsed in 100% methanol 3 × , 10 min each, and can then be stored at –20°C for several months.

3.1.3. Pretreatment of Embryos/Foetuses

1. Embryos/foetuses are incubated (all incubations at room temperature unless stated) in five parts methanol: 1 part 30% H_2O_2 (Sigma, St. Louis, MO, cat. no. H1009) for 1–2 hours with gentle rocking to inactivate endogenous peroxidases and to bleach the pigments from the skin.
2. Embryos are rehydrated through a methanol series: 75% methanol, 50% methanol and 25% methanol. Incubate for 5 min each. Then wash in PBST 3 × for 5 min each.
3. Embryos are incubated with varying concentrations of proteinase K in PBST for exactly 10 min (*see* **Note 8**). Proteinase K concentrations have to be determined empirically for each batch of proteinase K. A sample protocol for CD1 mouse embryos is shown: E9.5 (2 μg/ml), E11.5 (3 μg/ml), E13.5 (6 μg/ml), E14.5 (12 μg/ml), E15.5 (24 μg/ml), E16.5 (32 μg/ml).
4. Wash with freshly prepared glycine (2 mg/ml) in 2 × PBST for 5 min to inactivate proteinase K.
5. Refix embryos in 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for 20 min, then wash 3 × X with PBST, 5 min per wash.
6. Treat with freshly prepared 0.1% sodium borohydride in PBST for 20 min (this step blocks free aldehyde groups), then wash with PBST 3 × , 5 min per wash (note that tube lids have to be loose during this step to release evolving gas).
7. Wash twice with hybridisation buffer containing formamide. After equilibration in formamide, embryos/foetuses can be stored at –20°C until ready to hybridise.

*3.1.4. Hybridisation (see **Notes 2 and 3** Regarding Post-hybridisation Solutions and Optional Post-hybridisation Ribonuclease Treatment, Respectively)*

1. Prehybridise at 63°C for at least 1 hour (*see* **Note 9**).
2. Replace with fresh hybridisation buffer and add probe to approximately 1–2 μg/ml. Hybridise at 63°C overnight with gentle rotation (*see* **Notes 10** and **11**).
3. Rinse briefly 3 × times in Wash1, then rinse 2 × in Wash1 using gentle rotation at 63°C, for 30 min each.
4. Rinse briefly 2 × in Wash2, then rinse 2 × in Wash2 using

gentle rotation at 50°C for 30 min. If using RNase A to reduce background, *see* **Note 3** (optional). If not, proceed to step 5 below.

5. Rinse briefly 2 × in Wash3, then rinse 1 × in Wash3 using gentle rotation at 50°C for 30 min.
6. Rinse briefly 2 × in Wash4, then rinse 1 × in Wash4 using gentle rotation at 50°C for 30 min.
7. Rinse briefly 2 × in Wash5 (to remove formamide from Wash4 before raising the temperature), then incubate in Wash5 at 70°C for 20 min. This step is to inactivate endogenous phosphatases if using a phosphatase-linked enzyme for riboprobe detection.

3.1.5. Antibody Detection of Hybridised Probe

1. Pre-absorption of antibody: The antibody to hapten-labelled probe is pre-absorbed with embryo powder to remove anti-embryo activity. Antibody is diluted 1/2000–1/5000 in cold TBST (*see* **Note 12** if using sections), containing 2 mM levamisole (*see* **Note 5**), 1% heat-inactivated serum (*see* **Note 6**) plus inactivated embryo powder. Incubate at 4°C for 30 min with gentle rotation, then centrifuge at 4°C for 10 min in a microfuge to pellet and remove embryo powder.
2. Embryos/foetuses are blocked with heat-inactivated 10% serum (*see* **Section 2**, **step 28** and **Note 6**) in TBST containing 2 mM levamisole (*see* **Note 5,** add fresh) for 1 hour with gentle rocking.
3. Embryos/foetuses are incubated with secondary antibody solution overnight at 4°C with gentle rocking.
4. Rinse 3 × with TBST plus 2 mM levamisole at room temperature.
5. Wash with TBST plus 2 mM levamisole, 6 × for 1 hour each. It is often convenient to let the final wash go overnight.

3.1.6. Colour Development

For detection of alkaline-phosphatase-conjugated secondary antibodies, rinse 3 × with freshly prepared NTMT, containing 2 mM levamisole for 10 min with rocking. Incubate in NBT/BCIP in NTMT (*see* **Section 2**, **steps 20–22**). Perform colour development in the dark in glass dishes. Monitor reaction and terminate by washing in TBST, then transferring to 4% paraformaldehyde. Store at 4°C indefinitely.

For detection of peroxidase-conjugated secondary antibodies incubate with DAB until colour develops (*see* **Section 2**, **steps 26–27**). Wash with PBST or TBST, then transfer to 4% paraformaldehyde. Store at 4°C indefinitely.

3.1.7. Photography of Embryos/Foetuses

Secure embryos/foetuses to the base of a Petri dish with 1% agarose made with water. Either position the embryos on a thin layer of molten agarose or pour agarose plates and then use a hot wire/glass rod to melt the surface locally before attaching the embryos. Adhesion to agarose prevents vibration during photography and permits photography at different angles. Cover with TBS or PBS, as appropriate, and photograph through the liquid. It is necessary to completely submerge the embryo/foetuses for photography to prevent reflection from shiny surfaces and dehydration of the skin. Photograph under a dissecting microscope with fibre optic illumination.

3.1.8. Post-hybridisation Sectioning of Skin

Whole-mount in situ hybridisation provides a very robust and sensitive method of detecting RNA compared to section in situ hybridisation, possibly due to reduced RNA degradation as dissection and handling prior to hybridisation is minimised. Post-hybridisation sectioning is used to locate RNA to cells.

Wax sections of hybridised embryos/foetuses can be prepared by standard techniques, with the caveat that some colour precipitates are partially alcohol or xylene soluble, resulting in reduced signal.

Frozen sections provide the quickest and simplest method for locating RNA at the cellular level after whole-mount in situ hybridisation, with some sacrifice of tissue integrity. For greater sensitivity prepare frozen sections after post-hybridisation washes but prior to antibody detection (i.e. complete **Section 3.1.4**, freeze and section embryos, then resume blocking and antibody detection as in **Section 3.1.5**). Antibody detection is carried out as for whole embryos on the frozen sections, giving a stronger signal, probably due to facilitated reagent access.

Alkaline phosphatase detection on sections, rather than whole foetuses, permits inclusion of high molecular weigh polyvinyl alcohol to the BCIP/NBT colour reaction (6), substantially enhancing and concentrating signal and permitting detection of rare mRNAs ((6); *see* **Note 12**).

3.2. Barrier Assays

Whole-mount dye penetration or dye exclusion barrier assays report defects/acceleration in developmental barrier formation (**Fig. 19.1A**) or barrier defects in adult skin (*see* **Note 13**). The first assay modifies the skin by brief treatment with methanol. This procedure probably removes skin surface lipid. Entry of histological dyes is then dependent on barrier integrity. The second assay detects endogenous glycosidases, most probably lysosomal in origin, active at pH 3.0–6.0 (with an optimum of approximately pH 5.5). Entry of the artificial substrate, 5-bromo, 4-chloro, 3-indolyl β-D-galactopyranoside or X-gal, is dependent on a dysfunctional barrier. It is necessary to use controls, e.g. postnatal skin as barrier positive control, pre-E16.5 day murine skin or wounded adult skin as a negative control.

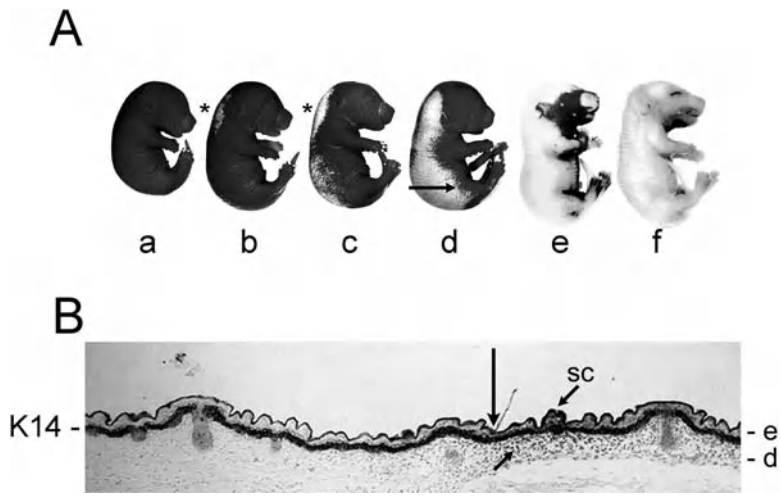

Fig. 19.1. Dye penetration/exclusion barrier assay on mouse embryos during barrier formation. (**A**) Whole-mount barrier assay on embryos from E15.5 to E18.0. Toluidine blue penetrates and stains embryos blue prior to barrier formation (embryo (**a**). Specific initiation sites, in white where the dye is being excluded, can be seen where barrier is forming (denoted by an asterisk; embryos **b** and **c**). Barrier formation crosses foetal skin in a wave-like fashion (embryos **d** and **e**; see arrow) and is completed by E18.0 (embryo **f**). Embryo a (<E16.5), embryos b–e (E16.5–E17.5), and embryo f (>E17.5). Reproduced with permission from (1). (**B**) Barrier assay (haematoxylin staining) in conjunction with K14 immunostaining. Sectioning was carried out after whole-mount barrier assay and then immunostaining was performed. The barrier front is indicated by the long vertical arrow. To the right of the arrow the barrier has not formed, shown by the more intense dye penetration in both the epidermis and the dermis (small arrows). K14 expression is independent of barrier status. Stratum corneum (sc), epidermis (e), and dermis (d).

Formation of the protective barrier in mice is strain dependent and correlates more closely with foetal weight than estimated gestational age. In CD1 strain mice, barrier forms at embryonic day E16.5, corresponding to a foetal weight of 0.6–0.9 g. However, in hybrid offspring of C57Bl6 × CBA mice that are often used to generate transgenic mice, barrier forms at E17.5 over a similar weight range. In Sprague–Dawley rats barrier forms at E18.5 (1) and in chicken barrier forms at day 17 (Byrne and Hardman, unpublished).

Barrier assays can be used with immunohistochemistry to visualise protein distribution at the barrier front (*see* **Note 14, Fig. 19.1B**).

3.2.1. Whole-Mount Barrier Assay, Method 1

This barrier assay modifies skin to permit barrier-dependent penetration by histological dyes. The nature of the modification is unknown but likely involves extraction of surface polar lipids. This assay can be carried out on tissue previously fixed with a variety of standard fixatives (e.g. formaldehyde, glutaraldehyde, and Bouin's fixative).

1. Tissue is transferred up and then down through a methanol gradient, about 30 sec–1 min per step (use water for making methanol dilutions):

(1) 25% methanol, (2) 50% methanol, (3) 75% methanol, (4) 100% methanol, (5) 75% methanol, (6) 50% methanol, (7) 25% methanol, (8) equilibrate in PBS or water.

2. For embryos/foetuses: Add dye mix (1% toluidine blue in water, *see* **Section 2**, **step 29**) to embryos and stain usually for about 0.5–1 min, destain in PBS pH 7.4 until the pattern appears. The high pH of PBS facilitates the rapid destain necessary to see the pattern. Alternative histological dyes can be used (*see* **Note 14, Fig. 19.1B**).
3. For skin pieces: Seal the dermal side by placing the skin piece (embedding slightly so that only the epidermal side is exposed) in a Petri dish containing petroleum jelly. Either smear the jelly onto the dish or add to the plate then melt in a microwave to get a smooth surface upon setting, like a bacterial agar plate. Add dye mix topically (e.g. use freshly prepared 1% toluidine blue in water), then destain in PBS pH 7.4 until the pattern appears.
4. Photograph as in **Section 3.1.7**, as soon as possible to prevent diffusion of dye and blurring of pattern.

3.2.2. Whole-Mount Barrier Assay, Method 2

This barrier assay exploits endogenous glycosidase activity present in murine skin at low pH (pH 3.0–6.0, with an optimum at approx. pH 5.5). This glycosidase activity will cleave the substrate X-gal to produce a blue, insoluble precipitate provided X-gal can penetrate the skin barrier. Hence, the glycosidase activity is only accessible topically if barrier is unformed or dysfunctional. Do not go up and down a methanol series, simply add the solution to the embryos/foetuses or seal the dermal side of explanted skin as above and add the solution topically. Incubate for approximately 30 min to several hours (reaction proceeds faster at 37°C; however, tissue morphology will be poorer than a room temperature incubation). The endogenous glycosidases are sensitive to fixative so this assay should be carried out on unfixed or partly fixed fresh tissue (*see* **Note 15**). Tissue can be fixed post-staining and staining is stable. Photograph as in **Section 3.1.7**.

4. Notes

1. If the RNA signal is low and/or the background is high it may help to treat the in vitro transcription reaction with DNase to prevent non-specific background and/or complementary plasmid cDNA from binding to the RNA of interest. Furthermore, if the DNA and the nucleotides have been removed,

then the RNA concentration can be determined directly using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE).

DNase treatment: After the in vitro transcription reaction, add 2 µl of DNase (2 units) and incubate for 15 min at 37°C. To remove nucleotides and DNase, use a spin column or precipitation (*see* **Section 3.1.1**).

2. Aqueous solutions are pre-treated in 0.1% diethyl pyrocarbonate (DEPC, Sigma, St Louis, MO, cat. no. D5758) for 2 hours overnight to inactivate ribonucleases, followed by autoclaving under standard conditions for 40 min to breakdown diethyl pyrocarbonate. It is important to autoclave for the specified time since intact diethyl pyrocarbonate can degrade RNA. There is no need to treat post-hybridisation solutions with DEPC.
3. Many authors include an RNase A treatment during the post-hybridisation washes, (e.g. between washes 2 and 3) to degrade single-stranded RNA that has not hybridised (2, 3). This step reduces background but it can be omitted for many skin applications. To carry out RNase A treatment: Embryos/foetuses are rinsed briefly a few times in RNase buffer (10 mM Pipes pH 7.2, 0.5 M NaCl, 0.1% Tween-20), then incubated with heat-treated RNase A at 50 µg/ml at 37°C for 30 min (3). To prepare RNase A, ribonuclease A from bovine pancreas (Sigma, St Louis, MO, cat. no. R4875) should be resuspended in TE at 10 mg/ml, heat-treated by boiling for 10 min and then cooled. Make aliquots and store at –20°C.
4. Mouse and rat embryos are staged so that the day the vaginal plug is detected is at day 0.5 (e.g. approximately 12 hours after insemination also known as embryonic day 0.5 (E0.5). Chick embryos/foetuses are staged so that the start of incubation is day 0 (E0).
5. Tris-buffered saline replaces phosphate-buffered saline in later stages of the whole-mount in situ procedure when alkaline phosphatase enzymic reaction is to be used. This replacement is to remove inhibitory phosphate. Levamisol is added (fresh each day as it is unstable in solution) to washes to suppress endogenous phosphatase activity. TBS and levamisol do not have to be included if a peroxidase visualisation technique is being used.
6. Heat inactivation of serum is required to destroy the complement system proteins involved in non-self-recognition. If the serum is not heat inactivated the sheep complement proteins will recognise the mouse antigens on the cell surface as foreign and cause cell lysis. An alternative to the protocol provided is to heat undiluted serum in the original packaging at

56°C for 1 hour. Avoid using higher temperatures with undiluted serum since this will increase the amount of proteins precipitating out of solution. Aliquot and store at –20°C.

7. Embryonic epidermis is particularly fragile. Manipulation steps must be minimised and rocking carried out gently. Smaller embryos can be transferred by suction using the wide end of an inverted Pasteur pipette or plastic pipette tip to avoid damage to the surface ectoderm.
8. Other protocols use fixed concentrations of proteinase K for varying periods of time. The concentration of proteinase K is crucial for success of this method and has to be determined empirically for each batch.
9. Hybridisation and post-hybridisation washes can be carried out in a standard rotating hybridisation oven with a rotor or attachment for 50 ml sterile, disposable plastic tubes. For hybridisation with small volumes embryos/foetuses can be placed in smaller tubes within the 50 ml tube. Post-hybridisation washes can be carried out directly in 50 ml tubes.
10. Some labs denature the probe before addition which may improve hybridisation and may be important for longer probes. Just before addition to hybridisation buffer, the RNA probe is diluted in a microtube with approx. 2 vol of formamide (RNA is stable in formamide), heated to 65°C for 5–10 min, quick chilled on ice, followed by a quick spin in a microfuge to pellet any condensation on the cap.
11. 63°C hybridisation temperature is from (3) and has been found useful in this protocol for probes of approximately 0.5–2 kb. However, if a probe is not successful it may be helpful to lower the hybridisation temperature.
12. When carrying out antibody detection on sections of hybridised embryos the washing times can be reduced to at least half.

    The use of high molecular weight PVA solutions when performing the alkaline phosphatase colour reaction on whole embryos can result in unacceptably high levels of background. Therefore, this type of signal enhancement should only be used on sections.
13. Whole-mount dye penetration or colorimetric barrier assays report major defects or changes in barrier. In reality, the barrier develops and is refined until quite late in development and, possibly, during early postnatal life. Late or subtle defects in barrier formation may not be detected with these assays. In order to detect these changes transepidermal water loss (TEWL), a measure of barrier function, can be assayed quantitatively on newborn or young (hairless) animals using

instruments (e.g. Tewometer, CK Electronics GmbH, Berlin; Evaporimeter, Servo-Med, Cairo, EG) that measure the water vapour pressure gradient at the skin surface.

14. Immunohistochemistry (this volume) can be carried out on barrier-stained tissue if the dye haematoxylin is used instead of toluidine blue (*see* **Section 3.2.1**). Haematoxylin stains nuclei of pre-barrier epidermis and dermis and staining persists through tissue embedding, sectioning and immunohistochemistry steps (**Fig. 19.1B**). After fixation and methanol treatment, haematoxylin (1% in water) is used instead of toluidine blue. Fixation time will have to be determined empirically for the antigen of interest.
15. When detecting endogenous glycosidase activities (and transgene-encoded β-galactosidase activity) cross-linking fixatives such as glutaraldehyde and formaldehyde will reduce enzyme activity as the enzymes also become cross-linked. Omission of fixatives, or fixation delay until colour development is complete, will reduce tissue integrity. However, epidermis is a particularly robust tissue and epidermal keratinocytes and the epidermal strata can still be clearly distinguished after sectioning even when tissue is unfixed or poorly fixed.

Acknowledgments

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Chapter 20

Tetracycline-Regulated Gene Expression in Transgenic Mouse Epidermis

Rose-Anne Romano and Satrajit Sinha

Abstract

The ability to specifically manipulate gene expression in vivo using mouse models has been one of the most important advances in understanding gene function over the last few decades. Methods used to control gene activities in the mouse include gene targeting and transgenic approaches. While gene targeting methods have proven to be powerful genetic tools designed to eliminate gene function by creating a "knockout" or "null mutant," transgenic studies offer gain-of-function capabilities, expression of dominant negative or knock-down of specific target genes and thus have often served a complementary and useful role. This chapter provides an overview for the generation of transgenic mouse models to study important questions in skin biology by taking advantage of the tetracycline-inducible gene expression system.

Key words: Keratinocyte, Transgenic, Tet-Effector, Tet-Responder, Skin, Inducible.

1. Introduction

It is relatively easy (with practice and skill!) to insert a foreign piece of DNA, i.e., a transgene, into the mouse genome to generate a transgenic mouse. For this purpose the foreign DNA is introduced using a fine needle directly into the mouse egg, where the DNA integrates at a random position in the genome – a procedure referred to as "pronuclear injection." This typically results in the incorporation of many tandemly arranged copies of the transgene in the genome and thus generates animals with increased levels of expression of the gene or gene product of interest. In addition, this system can also be used to generate dominant negatives or deliver siRNA to inhibit the expression pattern of a specific endogenous gene by using cell- and tissue-specific promoters. However, one of

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the major shortcomings of this system is that constitutive expression of the transgene can sometimes have toxic and/or deleterious effects on the animal resulting in embryonic or early postnatal lethality. Furthermore, this technique does not allow for the manipulation of transgene expression in a spatially or temporally defined manner or to alter the desired expression levels of the transgene. These drawbacks can make studying in vivo gene function rather challenging.

It was not until the introduction of the tetracycline-inducible system by Gossen and Bujard in 1992 that some of the limitations of controlling expression of the transgenic systems could be addressed (1). Briefly, there are two important components to this system. The first component involves transgenic animals expressing a tetracycline repressor or regulator (TetR), fused to the herpes simplex virus VP16 transcriptional activation domain, which is under the control of a cell or tissue-specific promoter. There are two complementary Tet control systems available. One is the tTA (tetracycline-controlled transactivator) or Tet-Off system where tTA will bind to a Tet-regulated promoter in the presence of the antibiotic tetracycline or a common derivative Doxycycline (Dox), to repress gene expression. Conversely, withdrawal of Dox prevents binding of tTA to a tetracycline-regulated promoter, thus permitting transcription of the transgene. The second system, the Tet-On system, utilizes the rtTA (reverse tetracycline-controlled transactivator) which requires the addition of Dox to activate transcription of the transgene (**Fig. 20.1**) (2).

The second component consists of animals expressing an inducible transgene of interest under the control of a tetracycline-regulated promoter or operator (*tetO*) (also known as a tetracycline-responsive element, or TRE). In this case, binding of the TetR to the TRE regulates gene expression. Thus, the tetracycline-inducible system is a binary system in that it requires the generation of two separate transgenic lines of mice: one carrying the tTA or rtTA transgene (Tet-Effector mice) and another carrying the transgene of interest driven by the TRE (Tet-Responder mice). These transgenic animals can then be cross-bred to produce bi-transgenic animals and transgene expression can be tightly regulated by the administration or withdrawal of Dox (*see* **Fig. 20.1**). For example, in the previously described Tet-On system, bi-transgenic animals can be administered Dox through their diet or in their drinking water, allowing transcriptional activation of the transgene. Upon the withdrawal of Dox, transgene expression is abolished. Conversely, in the Tet-Off system, in the absence of Dox, transgene expression is induced.

One of the major benefits of the Tet-inducible system is that it allows for varied expression levels of the transgene and provides the option to rapidly and reversibly switch the transgene on or off at any time point during the lifetime of the animal. Stringent

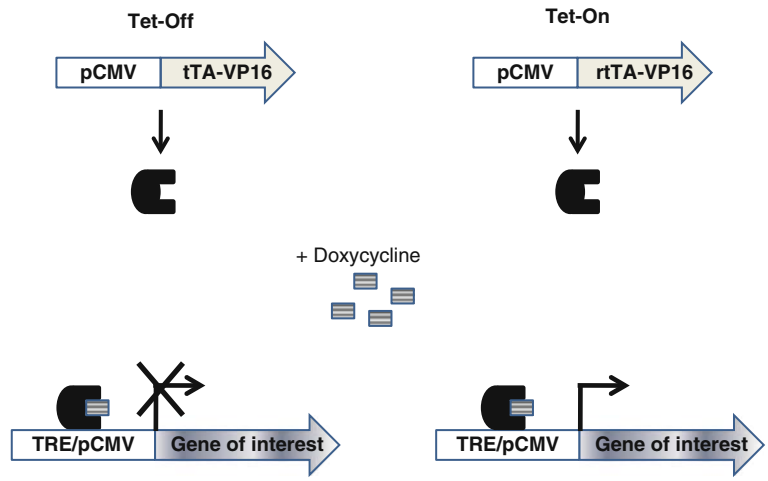

Fig. 20.1. Schematic of the Tet-Off and Tet-On inducible expression system in transgenic mice. This system requires two independent types of transgenic animals: Tet-Effector mice, which express a Tet-Regulator (TetR) under the control of a cell- or tissue-specific promoter, and Tet-Responder mice, in which expression of the gene of interest is under the regulation of a tissue- or cell-specific promoter. Breeding of the two transgenic lines generates bi-transgenic animals where the addition of Dox in the Tet-Off system (in left panel), results in silencing of the transgene of interest. In the absence of Dox, expression is induced. Conversely, in the Tet-On system (right panel), addition of Dox results in transgene induction. Upon Dox withdrawal, rtTA dissociates from the TRE, subsequently terminating expression of the gene of interest.

control over transgene expression can be achieved by changing the concentrations of Dox that are administered to the bi-transgenic animals. Another advantage is the highly effective tissue- and cell-penetrant properties of Dox; it is transferred across the placenta and through the breast milk of lactating mothers. These factors make this system ideal for inducing transgene expression during embryonic development as well as in the offsprings before weaning (3). Finally the expression of the TetR can be driven by a cell- or tissue-specific promoter allowing restricted and targeted expression of the transgene, thereby avoiding systemic effects. The versatility of this system is further enhanced by the fact that a growing number of Tet-Effector transgenic animals are available commercially and from different research laboratories.

2. Materials

2.1. Plasmids for Gene Cloning

2.1.1. TRE Plasmids

There are a large number of commercially available plasmids that contain the TRE upstream of a ubiquitously expressed promoter such as the cytomegalovirus (CMV) minimal promoter. Often these plasmids also contain sequences that allow

addition of an epitope tag for expressing tagged fusion proteins, and a Multiple Cloning Site (MCS) for cloning the cDNA of interest. Some commonly used epitope tags include Hemagglutinin (HA) and *c-myc*. Expressing the transgene as a fusion protein provides the advantage of monitoring expression and subcellular localization of the transgene *in vivo* in the event there are no or poor antibodies available against the protein being studied, as well as to discriminate the transgene from the endogenous gene. In addition, incorporating a fluorescent epitope-tagged fusion protein such as Green Fluorescent Protein (GFP) allows cells expressing the transgene to be isolated or sorted using FACS (fluorescent-activated cell sorting) for additional analysis (4). A partial list of commercially available plasmids includes

1. pTRE-HA,-Myc plasmids contain a TRE, a CMV minimal promoter, and an N-terminal epitope tag as indicated, followed by a MCS (Clontech Cat. no. 631012 and 631010, respectively).
2. pTRE-Tight vectors contain a modified TRE and minimal CMV promoter that provide better control of gene expression by eliminating leaky transgene expression in the absence of inducer. However, this plasmid lacks both an epitope tag and an initiating ATG, both of which can be easily incorporated by routine molecular biology techniques (Clontech Cat. no. 63159).

2.2. Keratinocyte-Specific Tet-Effector Transgenic Animals

The following is a list of Tet-Effector transgenic animals expressing various TetR under the control of different keratinocyte-specific promoters.

1. bK5 (bovine keratin5) – tTA and rtTA (5).
2. bK5NLS (bovine keratin5-NLS) – rtTA (6).
3. bK6 (bovine keratin6) – tTA (7).
4. hK14 (human keratin14) – tTA and rtTA (8–10).
5. hK18 (human keratin 18) – rtTA (11).
6. hInv (human involucrin) – tTA and rtTA (12).

A more comprehensive database of Tet-Effector and Tet-Responder transgenic animals is available at the following site http://www.zmg.uni-mainz.de/tetmouse.

2.3. Other Key Reagents

1. Doxycycline (Sigma-Aldrich, St,Louis, MO; Cat. no. D9891) is a tetracycline derivative commonly used by most laboratories. For long-term storage, prepare a sterile 1 mg/ml stock in sterile water, filter sterilize, aliquot, and store at –20°C. The final concentration used in a typical cell culture experiment is typically 0.2–1 μg/ml of culture medium.

2. OmniPrep Kit for Extraction of High-Quality Genomic DNA (GBiosciences Cat. no. 786-136). This kit can be used for extracting genomic DNA from animal samples quickly and with relative ease.
3. Histo-ClearII (National Diagnostics Cat. no. HsS-202). This is used for de-paraffinization of slides which have paraffin-embedded tissue samples on them.
4. QIAfilter Plasmid Midi Kit (Qiagen, Cat. no. 12243).
5. QIAquick Gel Extraction Kit (Qiagen, Cat. no. 28704).
6. Tet system approved Fetal Bovine Serum (Clontech, Cat. no. 631106).

2.4. Genomic DNA Extraction Buffer

1. Proteinase K stock solution at 20 mg/ml (Novagen Cat. no. 70663-4). Dissolve in 10 mM Tris HCL, pH 7.5, 20 mM calcium chloride, and 50% glycerol. Store in 0.5 ml aliquots at –20°C.
2. DNA Extraction Buffer:50 mM Tris (pH 8.0), 10 mM EDTA, 200 mM NaCl, 0.5% SDS.

2.5. Mouse Dissections and Skin Tissue Specimen Preparation

1. Following the animals care guidelines set out by the Institutional Animal Care and Use Committee (IACUC), both a wild-type and bi-transgenic mouse must be sacrificed.
2. Using an electric shaver, gently shave the area of the skin that needs to be harvested (typically the dorsal region of the mouse). Working quickly to minimize tissue damage and necrosis, harvest the skin. Spread the skin out flat with the dermis side down on a paper towel. Trim the excess paper towel and cut into a small-sized piece and proceed directly to fixation.
3. Tissues can be fixed in 10% Neutral Buffered Formalin (NBF) overnight at room temperature (RT). The tissues are then processed and embedded in paraffin and sectioned to 4–10 μm thickness. Tissues can alternatively be fixed in 4% paraformaldehyde overnight at RT.

2.6. Blocking Solutions

20% Normal Goat Serum (NGS) in 0.1% TX-100 in PBS (blocking solution):

For 50 ml: NGS (10 ml), 10% TX-100 (0.5 ml),10 × PBS (5 ml), MilliQ water (34 ml), 2% NaN3 (0.5 ml), make the solution first, then heat inactivate at 56°C for 30 min. Store at 4°C.

2% NGS in 0.1% TX-100 in PBS

For 10 ml: Blocking solution (see above) (1 ml), 10% TX-100 (90 μl), 10 × PBS (900 μl),

MilliQ water (7.92 ml), 2% NaN3 (90 μl). Store at 4°C.

3. Methods

3.1. Cloning the Transgene and Preparing the Plasmid for Microinjection

3.1.1. Plasmid Design

The transgene requires a number of essential elements for successful gene expression. The transgene should include a promoter, intron sequences, the coding sequence of the gene of interest, and a termination/polyadenylation signal. Inclusion of a heterologous intron between the promoter and the gene is thought to improve the levels of transgene expression. Similarly, termination/polyadenylation sequences are essential for ensuring transgene expression and must be included. Most mammalian expression plasmids contain these elements, which are typically heterologous in nature (such as the SV40 intron and polyA sequences). A schematic depicting an example of a transgene used to generate transgenic animals is shown in **Fig. 20.2**.

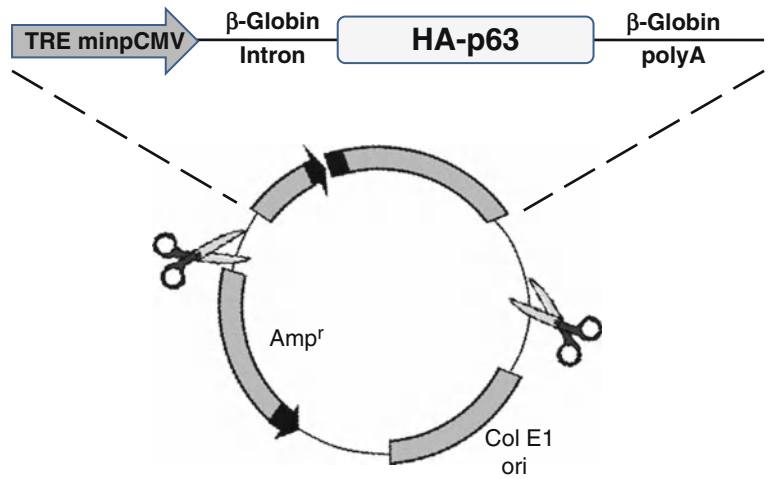

Fig. 20.2. Components required for designing a construct for expression in transgenic mice. Upper panel shows an example of a construct used for the generation of transgenic mice. Transgene expression includes the Tet-Response Element (TRE) followed by the pCMV minimal promoter (minpCMV). The TRE is made of several Tet-operator (Tet-o) repeats to which the TetR will bind. Downstream of the TRE is the β-globin intron followed by the coding sequence of the gene of interest. The transgene should also contain a polyadenylation sequence downstream of the gene of interest. In the lower panel is a cartoon of the final cloned plasmid. The scissors represent regions, which should be targeted for restriction enzyme digestion to excise the transgene from the plasmid backbone.

1. When cloning the transgene into a TRE plasmid containing an epitope tag, the coding sequence of the gene must be placed in frame with the epitope tag. This can be done using simple PCR strategies. It is important to then sequence the cloned transgene for errors, which may have occurred during the PCR, and to ensure the transgene is in frame with the epitope tag.

2. Once the transgene sequence has been verified, Tet-regulated expression of the designed transgene can be tested in cell culture by Western Blot analysis. Tet-On and Tet-Off plasmids are available from Clontech and can be used in co-transfection experiments with the TRE plasmid containing your transgene. This is a critical preliminary step whose importance cannot be stressed enough. This ensures that the transgene is expressed as expected and thus can save valuable time and resources associated with the generation of transgenic mice.

3.1.2. Preparing the Plasmid for Microinjection

Once Tet-regulated expression of the transgene has been confirmed, the plasmid can be prepared for microinjection by excising the transgene from the plasmid backbone using appropriate restriction enzymes and gel electrophoresis. Linearization of the transgene has been shown to improve integration efficiency and removal of the extraneous plasmid sequences can improve the frequency of expression (13). When considering removal of the plasmid backbone, be sure not to remove the polyadenylation signal within the backbone, as this will prevent transgene expression in vivo (*see* **Fig. 20.2**). Techniques and protocols for the purification of the plasmid for microinjection should follow the guidelines set out by the transgenic facility performing the microinjection. There are several factors that may influence the success rate of generating transgenic animals with the most important being the purity and the quality of the DNA used for microinjection (*see* **Note 1**). DNA concentration is another factor influencing the success rate of generating transgenic animals.

Injection of the DNA-containing solution into the pronuclei of fertilized eggs is the most common and efficient method used for generating transgenic animals. With this approach, the transgene undergoes random integration into the mouse genome, often allowing multiple transgene copies to be inserted at a single locus in a head-to-tail fashion. There are a number of shortcomings using this technique in that due to the random nature of insertion of the transgene within the mouse genome, it is possible this method can result in potential integration into sites of permanently silenced chromatin. In this case, the transgene will not express. Another possibility to consider is the potential for mosaicism. Although the transgene is generally transmitted in a Mendelian fashion, in the case of a mosaic mouse, transmission may not follow Mendelian frequencies, and one may have to go though several breeding cycles to generate an offspring carrying the transgene. As a result, it is wise to work with multiple (preferably three to five or more) different founder lines to ensure an expressing animal and to confirm the observed phenotype.

3.2. Screening and Identification of Founder Transgenic Lines

Once the litters of the potential founders are born, the animals must be screened to identify founders. The two most commonly used techniques for founder screening are Southern Blot and PCR analysis. The DNA used for both these methods are derived from small tail clippings. PCR is the quickest and easiest method for identifying transgenic founders.

1. Take a 1–2 mm tail biopsy from a 2-week-old mouse.
2. Extract genomic DNA: Add 250 µl of DNA extraction buffer and 3.5 µl of proteinase K solution (20 mg/ml) to the tail biopsy and incubate at 55°C overnight. Add an equal volume of phenol/chloroform (1:1), mix well and centrifuge at 15,000*g* for 5 min at room temperature (RT). Remove the supernatant and place in new centrifuge tube and add 500 µl of 95% ethanol. Centrifuge for 5 min at 15,000*g* at RT. Wash the DNA pellet with 70% ethanol, decant the pellet, and let air dry. The pellet can be dissolved in 100 µl of ddH_2O and stored at 4°C for several weeks. For PCR reactions, 1 µl of genomic DNA can be used.
3. Conversely, the OmniPrep Kit for Extraction of High Quality Genomic DNA available from GBiosciences can also be used. See materials section above.

3.2.1. Genotyping Potential Founders Using PCR

Once genomic DNA has been isolated, PCR can be performed to identify transgenic founders. Although the quality of genomic DNA is a contributing factor in the identification of false positives or false negatives, an equally important factor is the primer sets used for genotyping. When designing primers for use in PCR genotyping reactions, it is important to use a set of primers that will amplify a region within the transgene. Potential regions of amplification include the polyadenylation signal, or sequences spanning the MCS and the 5′ region of the gene. A reason these areas are ideal for primer design is that they generally lack any homology to the mouse genome and are less likely to give non-specific amplification. Given this, primers should not be designed within the coding region of the transgene as in some cases, this may not distinguish between the endogenous genomic DNA and the transgene.

3.3. Confirming Expression of the Transgene In Vitro

The identification of founder transgenic lines does not guarantee successful transgene expression in vivo. This depends on many factors, some of which have been addressed in the above sections. Although the most straightforward method to confirm expression of the transgene is to cross-breed the Tet-Responder transgenic founders to a Tet-Effector transgenic line, this may not be feasible if many founders have been identified. A simpler and faster approach to screen through a high number of transgenic founder lines takes advantage of the relative ease with which keratinocytes

and fibroblasts can be isolated from mouse skin and maintained in culture. Fibroblast cell lines from transgenic founders can be generated from tails and subsequently tested for transgene induction by transfecting the cells with a TetR plasmid driven by the ubiquitously expressed CMV promoter (4). Transgene expression can be confirmed by performing a Western Blot to detect expression of the epitope tag. If multiple expressing founders have been identified, relative expression levels of the transgene can be extrapolated from Western blots and matings can be set up accordingly. Another advantage of utilizing the cell culture method for confirming transgene expression is that it can be used for monitoring the inherent leakiness of this system. One of the limitations of the Tet-inducible system is the high basal activity of the TRE promoter in uninduced states, resulting in leaky expression of the transgene. These conditions are generally unfavorable, particularly if the transgene is highly toxic. Transgenic founder lines can be screened for levels of leakiness by examining transgene expression levels in the absence of inducer (14).

3.4. Confirming Expression of the Transgene In Vivo

Once expressing founders have been identified, they can then be cross-bred to Tet-Effector transgenic animals expressing a TetR under the control of the keratinocyte-specific promoter of your choice. When choosing Tet-Effector animals to be used for cross-breeding, important factors to consider are some of the inherent limitations of both the rtTA and the tTA systems. While the tTA or Tet-Off system is very convenient in that in the absence of Dox, the transgene is induced, if the transgene requires a period of silencing followed by induction, depending on the Dox dosage, induction can take up to a number of days, and reaching steady-state levels can take several more days (5). This is because the circulating levels of Dox in the system require some time for elimination. However, this system does provide tight control of gene expression, thereby minimizing the likelihood of leaky transgene expression.

Conversely, the rtTA or Tet-On system is more suitable for rapid induction of transgene expression upon Dox administration. Depending on the organ or tissue, transgene induction can be detected within 4 hours when Dox is supplied in the drinking water of bi-transgenic animals (15). This is particularly useful in cases when overexpression of the transgene is toxic to the animal. A drawback of the Tet-On system is that the commonly used Tet transactivator utilized in this system has a lower affinity for Dox; however, recent modifications have enhanced its sensitivity to Dox and diminished residual binding to TRE in the absence of Dox, thus improving the inherent leakiness (16).

Once Tet-Effector animals have been mated to the newly generated Tet-Responder mice to generate bi-transgenic animals, depending on the Tet-inducible system being employed (Tet-On

or Tet-Off), Dox should be administered accordingly. An important factor to consider when setting up matings and inducing transgene expression in the bi-transgenic animals is the experimental window under investigation. For example, to initiate expression during embryonic development and prior to weaning, Dox can be administered to the mother (for the Tet-on system). On the other hand, if using tTA Effector mice, Dox must be withdrawn to induce transgene expression. Another consideration to keep in mind is the nature of the keratinocyte-specific promoters driving the TetR, since their activity during embryonic skin development is variable and this will determine when the transgene is expressed (*see* **Note 2**).

Upon successful generation of bi-transgenic animals, transgene expression can be confirmed by performing Western blot and immunostaining techniques on skin sections using antibodies recognizing the epitope tag (**Fig. 20.3**).

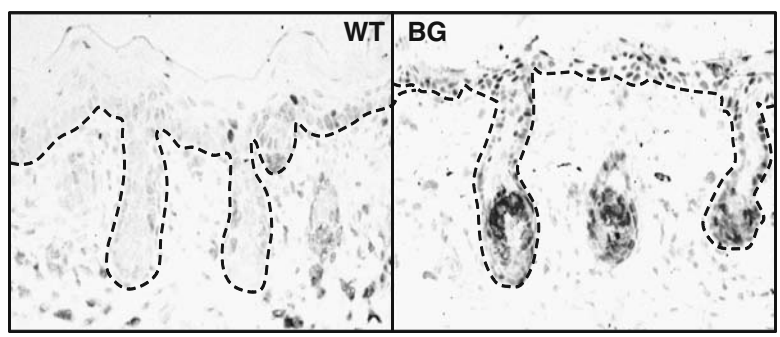

Fig. 20.3. Immunostaining to detect transgene expression in skin sections. Tet-Responder animals expressing an HA-epitope-tagged p63 fusion protein were mated with K5tTA animals to generate bi-transgenic animals (17). Transgene expression of p63 as detected by anti-HA antibodies is restricted primarily to the basal layer of the epidermis and outer root sheath of the hair follicle, where K5 promoter is normally expressed. Dashed lines demarcate dermal/epidermal junction.

A typical immunostaining protocol followed in the author's laboratory is described.

1. Deparaffinize the sections by gently shaking the slides in a glass coplin jar using histoclear for 3 min at RT. This can be repeated twice. Sections can then be rehydrated through a graded alcohol series. Gently shaking the slides in the coplin jar, add 95% alcohol for 3 min, followed by 70% alcohol for 3 min, and then 50% alcohol for 3 min. Slides can then be washed on the shaker in 1 × PBS for a total of three times at 5 min each.
2. Antigen retrieval solution:

   10 mM sodium citrate buffer (pH 6.0) containing 0.05% Tween-20.

   The solution can be stored at room temperature (RT).

3. Place the deparaffinized slides in the antigen retrieval solution with the sections facing up in a glass pyrex microwave safe dish. Cover the pyrex dish with Saran Wrap and poke tiny holes in the Saran Wrap to allow for ventilation. Microwave at maximum power for 20 min, then cool for 20 min at RT after removing the Saran Wrap.
4. Arrange the cooled slides in a coplin jar and rinse three times with 1 × PBS.
5. Suck off remaining PBS from the slides and circle the sections with a PAP pen, and place slides in a humidifying chamber.
6. Block using 20% Normal Goat Serum (NGS) with 0.1% Triton X-100 in PBS (blocking solution) for 60 min (or longer) at RT.
7. Add primary antibody by diluting the antibody in the 2% NGS. Incubate 2 hours (RT) or overnight at 4°C. Overnight is recommended if the transgene is weakly expressed.
8. Transfer slides to coplin jar and wash with 1 × PBS three times for 5 min each on a shaker.
9. Place slides back in the chamber and add secondary antibody diluted in 2% NGS. Incubate at RT for 45 min.
10. Wash the slides three times with 1 × PBS for 5 min on a shaker.
11. Rinse briefly in tap water and mount slides using Vectashield mounting medium for fluorescence with DAPI (cat# H-1200 Vector Laboratory).
12. Alternatively, slides can be blocked using 5% Bovine Serum Albumin (BSA) or 5% non-fat dry milk solutions.

3.5. Methods for Administering Doxycycline

Although there are several modes for administration of Dox for use in transgenic animals, the most commonly used method for delivery of Dox is through drinking water. An important parameter to consider is the amount of Dox required for effective transgene induction (*see* **Note 3**). Dox dosage is variable in that different promoter-specific Tet-Effector transgenic lines may require different amounts of Dox for induction of the transgene. Dosage levels should be consistent with the levels suggested by the research group who initially generated and characterized the Tet-Effector transgenic line (typically 1.125 mg/ml to 10 mg/ml). This information should be widely available.

In addition to Dox delivery in drinking water, Dox can also be administered through rodent chow (Bio-Serv; Frenchtown, NJ). Varying concentrations are available and should be consistent with the recommended dose corresponding to the Tet-Effector transgenic animals you are using for your cross-breeding. The only drawback to using Dox chow is that if multiple Tet-Effector animals will

be used, they may each require different amounts of Dox dosage. In this case, administering Dox through drinking water may be more sensible (*see* **Note 4** for important considerations).

Finally, the third most widely used alternative method of Dox delivery is through intraperitoneal (IP) injections. The main advantage of administering Dox using this method is that delivery and induction of the transgene are rapid. This form of delivery can be particularly useful in cases when the transgene is toxic to the animal. This mode of administration can provide transgene induction within 3 hours after delivery (10). Similar to the noted benefits of using Dox in drinking water, utilizing IP injections allows for increased variability in Dox dosage.

3.6. Mouse Strain Considerations

When generating transgenic animals, many factors must be taken into consideration. The genetic strain of the egg donor is an important parameter to consider due to differences in egg and offspring yields among different mouse strains. The following is a partial list of genetic strains commonly used for generating transgenic animals.

1) FVB mice are a commonly utilized strain of mice for the generation of transgenic animals due to their fully inbred genetic background. Use of this strain will ensure all founders to be genetically identical. This strain also provides reproductive advantages in that fertilized eggs contain large pronuclei facilitating the microinjection of DNA. In addition, FVB animals are highly fecund, generating large numbers of eggs and litters. These factors make this strain very efficient for making transgenics (18).
2) C57BL/6 is the most widely used inbred strain of mice. This strain has a good breeding performance, has a long life span, and is the most commonly used strain in biomedical research.

A second important factor to consider prior to microinjection is the mouse strain that will be utilized for cross-breeding in the future and whether the progeny will be analyzed on a pure or mixed genetic background. It is widely accepted that phenotypic differences between mouse genetic strains can influence the severity of the observed phenotype. For example, if your newly generated Tet-Responder transgenic animals will be mated to a pure FVB strain of Tet-Effector animals, you may want to keep the offspring on a pure FVB strain and may choose to use FVB donor eggs for microinjection. Maintaining the progeny on a pure background will reduce any observed variability. Conversely, if the progeny are bred on a mixed genetic strain, phenotypic variability between littermates is possible. However, one of the advantages of analyzing mice on a mixed strain is that the observed phenotype is more likely to represent the common phenotype across different genetic strains (19). If needed, transgenic mice on mixed backgrounds can be easily bred to generate a pure congenic strain by backcrossing for eight generations or more.

4. Notes

1) Successful generation of transgenic animals is dependent on a number of different variables including the purity and quality of the DNA. When purifying DNA for microinjection, keep in mind that CsCl preparations (preps.) are routinely suggested by many core facilities, which perform the transgene injections. In some cases, two rounds of CsCl preps. are requested. This is to ensure the highest quality and purity of DNA as the slightest traces of contaminating agents will affect successful generation of transgenic animals. If CsCl preps. are not feasible, we have found that DNA purification using the QIAfilter Plasmid Midi Kit works very effectively and provide highly pure DNA. If possible, endotoxin-free DNA is even a better option. After restriction digestion to eliminate the unnecessary plasmid backbone and gel electrophoresis, the excised band that will be injected can be purified using the QIAquick Gel Extraction Kit.

2) When planning experimental strategies one thing to consider is the relevant biological question and what cellular layer of the epidermis will be targeted for transgene expression. This will dictate which Tet-Effector animals will be utilized for mating to the newly generated Tet-Responder animals. A second parameter to consider is when to induce the transgene. If studying gene function during embryonic skin morphogenesis, then induction should occur during this developmental window. Conversely, if studying transgene function in adult skin, induction should occur in adult animals. For example, depending on the gene driving expression of the TetR, induction can occur as early as E8.5 (as seen with the K5/14 Tet-Effector animals) to as late as E16.5 (with the Involucrin Tet-Effector animals).

3) Optimal doxycycline dosage levels required to achieve maximal transgene expression vary between Tet-Effector transgenic mouse lines. Given this variability, it is wise to refer to the original report describing the initial characterization of the Tet-Effector animals. Generally, Dox dosage levels can range from 0.125 mg/ml to 10 mg/ml. When deciding on the dosage levels, it is important to consider the main goal of the research and the need to express the transgene at low or high levels. For example, in the case of a Tet-On system which is being controlled through Dox in drinking water, once animals are switched to regular water, animals kept on a low Dox dosage show a more significant decline of transgene expression as compared to animals on higher Dox dosages (12).

4) If administering Doxycycline to animals through drinking water, it is important to remember that Dox is light sensitive and requires storage in specially designed light-sensitive bottles when placed in the cages of mice. Alternatively, drinking bottles can be wrapped in aluminum foil to avoid exposure to light. However, this requires caution as mice can easily tear and claw at the foil and ingest it. In addition, the Dox drinking solution should be supplemented with 5% sucrose or Koolaid since Dox is not palatable and has a very bitter taste. If the solution is not supplemented and replenished appropriately, the animals might die of dehydration as reported in one study (20). Furthermore, to ensure the right concentration of Dox, the drinking solution needs to be made fresh every 2–3 days.

5) Mouse matings between Tet-Effector and Tet-Responder animals to generate bi-transgenic offspring should follow normal Mendelian frequencies. As basic as this may sound, careful attention should be paid to keep track of this as if you fail to identify bi-transgenic animals after a few litters, it may be due to the early postnatal lethality associated with expression of your transgene. This is particularly true if transgene expression is induced during the early stages of embryonic development. Given the fact that many of the promoters driving expression of the TetR in the skin are not restricted to the skin but are also expressed in various other stratified epithelial tissues such as the stomach and oral cavity, it is plausible that overexpression of the transgene in other tissues may be lethal. In such cases, induction during later stages of development, or at birth, or controlling the dosage of Dox may be required to circumvent this early lethality.

6) If you have positively identified a large number of transgenic founder lines and choose to distinguish the transgenic founder lines that express the transgene in a Tet-inducible fashion by isolating keratinocytes or fibroblasts, be sure to utilize cell culture reagents such as Fetal Bovine Serum (FBS) that are Tet-system approved and are free of tetracycline-derived contaminants.

7) To reduce the numbers of animals and minimize the breeding requirements that are associated with the bi-transgenic system, strategies that use co-injection or an integrated plasmid that harbors both components of the Tet-System can be utilized. For example, if plasmids bearing the Tet-Responder and the Tet-Effector units are co-injected into single-cell fertilized embryos, they typically co-integrate into the same site(s) of the genome. This will generate a single transgenic mouse that will respond to Dox. Alternatively, integrating all of the elements of the Dox-inducible system on a single

"all-in-one" vector is another way to avoid generating binary Dox-responsive transgenic animal model systems. Although these strategies eliminate the need to generate two independent transgenic lines and greatly facilitates mouse breeding, an inherent shortcoming with this strategy is the fact that the scope of inducible transgene expression is then limited to the specific Tet-Effector of choice and cannot be utilized for a different organ or tissue system if desired.

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Chapter 21

A Versatile Murine 3D Organotypic Model to Evaluate Aspects of Wound Healing and Epidermal Organization

Eve Kandyba, Malcolm Hodgins, and Patricia Martin

Abstract

Three-dimensional (3D) organotypic models are increasingly being used to study aspects of epidermal organisation and cutaneous wound-healing events. These are largely dependent on laborious histological analysis and immunohistochemical approaches. Here we outline a method for establishment of a versatile in vitro 3D organotypic skin equivalent that reflects murine epidermal organisation in vivo. The system is optically transparent and ideally suited to real-time analysis and integrated in situ imaging techniques. Moreover, the model permits the visualisation of epidermal regeneration following injury in real time, thereby facilitating avenues to explore distinctive modes of wound re-epithelialisation. The versatility of the model could help unravel molecular mechanisms underlying epidermal morphogenesis, assess novel therapeutic strategies and reduce animal experimentation in a non-invasive manner.

Key words: Organotypic model, Epidermal organization, Imaging, Connexin, Wound healing.

1. Introduction

Three-dimensional (3D) organotypic skin models, primarily human based, provide insights into developmental skin biology and epidermal differentiation events (1–3). Complementary use of in vivo transgenic and knockout mouse resources enable analysis of cellular events concerning epidermal morphogenesis (4, 5) and homeostasis; however, few in vitro murine organotypic skin models exist. Those available often employ fibroblast-incorporated collagen supports or a dead de-epidermised dermis (DED) (6, 7), which can impede the direct in situ visualisation of morphological events and require arduous histological processing. We address this problem and report a versatile and robust Transwell

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murine epidermal skin model, incorporating primary murine keratinocytes, which once raised to the air–liquid interface (ALI) stratifies and differentiates to produce a 3D organotypic skin equivalent that possesses morphological and differentiation characteristics typical of those observed in vivo. The transparent nature of the Transwell semi-permeable membranes permits visualisation of these complex morphological events in real time, negating the need for standard histological techniques, as epidermal differentiation events may be imaged in situ by whole-mount and 3D Z-stack reconstruction following single or dual-label indirect immunofluorescence (8). This versatile in vitro system provides the opportunity to examine aspects of integrated cellular behaviour and enables one to functionally probe, image and analyse the coordination of epidermal homeostasis. The model provides an excellent system to dissect the role of specific proteins in epidermal morphogenesis and wound-healing events and, in so doing, may dramatically reduce the need for live animals in such research.

2. Materials

2.1. Cell Isolation and Culture of Organotypic Model

1. Progenitor cell targeted (PCT) keratinocyte medium (Chemicon, Cambridge, UK) supplemented with penicillin/streptomycin (100 μg ml^{-1}, Lonza, Nottingham, UK). This medium was then referred to as keratinocyte growth medium (KGM).
2. Sterile Phosphate-buffered saline (PBS) without calcium and magnesium: (Product code: BE17-516F, Lonza)
3. Thin blade scissors
4. 2 × curved forceps
5. Large petri dishes (~10 cm diameter)
6. 0.25% solution of trypsin prepared by making a 1:10 dilution of neat trypsin solution (2.5% in Modified Hanks' BSS without calcium or magnesium; Cambrex) in sterile calcium- and magnesium-free PBS (Lonza)
7. Corning® 12 mm diameter Polyester Transwell Inserts (Sigma, Poole, Dorset)
8. Nylon 100 μm cell strainers (VWR, Lutterworth, UK)
9. 15 ml and 50 ml sterile centrifuge tubes
10. 70% methanol
11. Chelexed serum (see below)

2.2. Preparation of Chelexed Serum

1. Foetal bovine Serum (FBS) (Cambrex)
2. Corning® Chelex 100 sodium form resin (Sigma)

3. Distilled water
4. 1 M NaOH
5. 1 M HCl
6. Whatman filter paper (Fisher)
7. 2 × 250 ml beakers
8. Buchner funnel
9. pH machine
10. 0.22 μm filters (Millipore, UK)
11. 5 ml sterile plastic syringe (Fisher)
12. Sterile plastic bijous (Greiner, UK)

2.3. Confocal Immunofluorescence

1. 1 × phosphate-buffered saline solution (PBS), with calcium and magnesium (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4), autoclaved and stored at room temperature.
2. 0.1% Triton X-100 v/v (Fisher) solution in autoclaved PBS, with calcium and magnesium.
3. 5% w/v skimmed milk (Marvel) solution in autoclaved PBS, with calcium and magnesium.
4. Antibody Dilution Buffer: autoclaved PBS containing 0.1% Triton X-100 v/v and 5% skimmed milk v/v.
5. Appropriate primary antibody diluted in Antibody Diluting Buffer.
6. Appropriate secondary antibody conjugated with either Alexa488 or Alexa594 (Invitrogen) 1:500 dilution in Antibody Dilution Buffer
7. Microscope 24 × 32 mm glass cover slips (Fisher)
8. 100% methanol (ice-cold)
9. Nuclear counter stain: Diamidinophenylindole (DAPI, 10 μg/ml, Calbiochem) diluted in PBS.

2.4. Wound Assay

1. Sterile 1–200 μl pipette tips yellow
2. 200 μl pipette
3. Ruler (cleaned with 70% meths)
4. Digital camera and laptop

3. Methods

The optically transparent nature of the semi-permeable Transwell inserts permits the direct visualisation of cellular events in real time

under bright field or immunofluorescent conditions. This provides the model versatility over previous published organotypic skin models which employ collagen supports or the use of dead de-epidermidized dermis (DED) that can obstruct the direct visualisation or cellular and morphological events and, additionally, require arduous histological processing. The action of raising confluent mouse keratinocyte Transwell monolayers to the ALI induces epidermal stratification and differentiation over a period of 1–2 weeks (**Fig. 21.1**) and following whole-mount immunofluorescence displays a full spectrum of epidermal differentiation markers including Cytokeratin 10, filaggrin and loricrin (**Fig. 21.2**). The model is extremely versatile and has great potential to be utilised for the examination of a diverse range of epidermal proteins to elucidate their roles in epidermal organisation and morphogenesis. Moreover, the model also serves as a valuable wound-healing model where keratinocyte migration and epidermal re-epithelialisation can be examined in real time (**Fig. 21.3**) in a non-invasive manner.

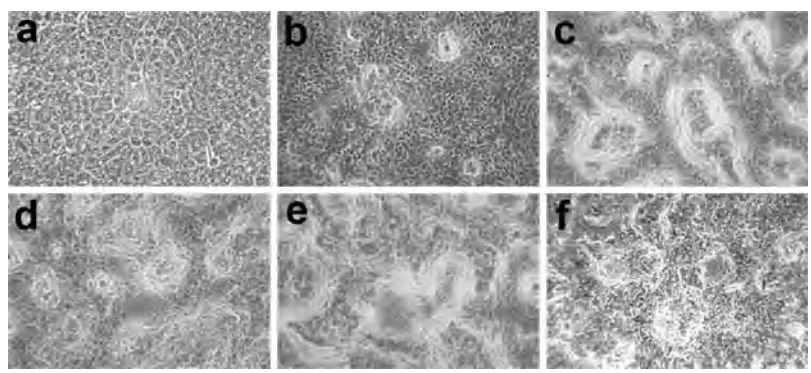

Fig. 21.1. By raising confluent primary mouse keratinocyte Transwell monolayers to the air–liquid interface (ALI) a complex 3D organotypic epidermis is formed. Primary keratinocyte monolayers **(a)**, when raised to the ALI, are induced to stratify **(b)** and differentiate **(c, d)** over a period of 7–10 days to form a complex 3D organotypic epidermis with large flattened cells in the upper layers **(e)** and evidence of cornification **(f)**.

3.1. For Preparation of 50 ml Chelexed Serum

1. Suspend 10 g chelex resin in 250 ml distilled water in a beaker and stir for 5 min. Decant the water and keep the resin in the beaker.
2. Add 250 ml distilled water and place on a stirrer. Using 1 M HCl (or 1 M NaOH as appropriate in the latter stages); pH the solution until the pH stabilises at 7.4 (approximately 8 hours as the pH will continue to fluctuate).
3. Using filter paper and a plastic funnel, filter the chelex resin and place in a clean beaker.

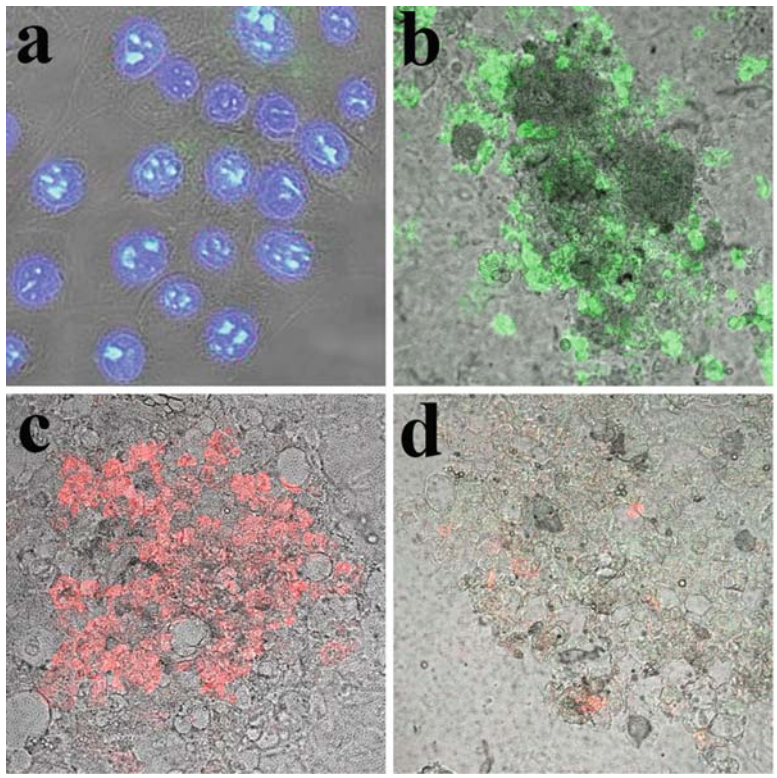

Fig. 21.2. Organotypic murine epidermis displays a differentiation profile similar to that found in vivo. Immunofluorescence results of day 7 ALI cultures revealed that Transwell organotypic epidermis expressed several differentiation markers typically found in vivo in mouse epidermis. **(a)** Basal, undifferentiated keratinocytes expressed the proliferation marker, Ki67 (turquoise, plus 4,6-diamidino-2-phenylindole staining illustrating cell nuclei, blue). **(b)** In the upper differentiating layers, cytokeratin 10 (green), a differentiation marker of suprabasal epidermal keratinocytes, was expressed and **(c)** filaggrin expression (red), a granular layer protein, was also evident in the upper terminally differentiating layers of organotypic cultures. **(d)** In the uppermost keratinocytes layers, the cornified envelope protein, loricrin (red), was also expressed.

4. Add 50 ml FBS to the chelex resin and stir at room temperature for no longer than 1 hour.
5. Decant the chelexed serum through filter paper in a Buchner funnel as before.
6. Under sterile conditions, filter sterilise the chelexed serum (using a 0.22 μm filter) and aliquot into plastic bijous.
7. Store at –20°C until required.

3.2. Primary Mouse Keratinocyte Isolation

1. Place sacrificed perinatal pups (less than 2 days old) in a large petri dish containing 70% methanol (v/v in distilled water).
2. Remove the tail and hind limbs (below the knee joint) and make an incision, posterior to anterior, along the dorsal surface just below the skin.

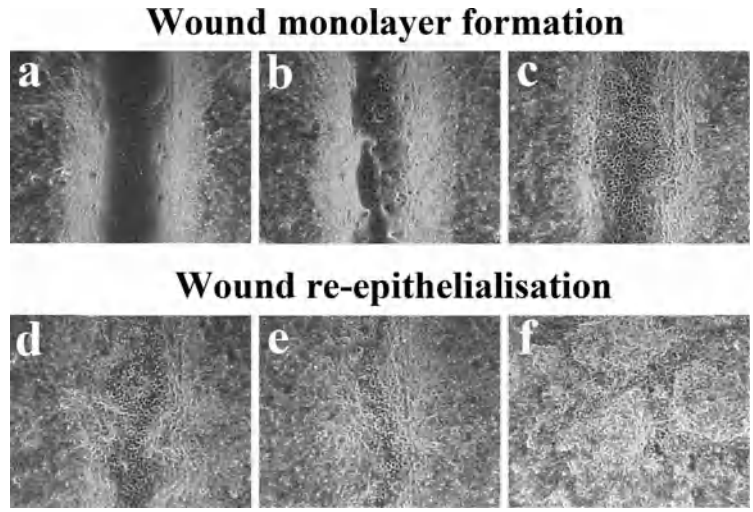

Fig. 21.3. Wounding promotes keratinocyte migration out of a stratified epidermis to form an intact monolayer which subsequently re-stratifies to form an intact organotypic epidermis. Using a simple scratch assay, organotypic epidermal cultures (ALI-4) were wounded by introducing a linear cut through the cells using a sterile 10-ml pipette tip **(a)**, and keratinocyte migration was monitored daily. Over the following 3 days post-wounding, keratinocyte migration was observed from both wound margins towards the centre of the wound **(b)**, with an intact keratinocyte monolayer formed by day 4 post-wounding **(c)**. Re-epithelialisation occurs over the following 4–5 days. By day 5 post-wounding **(d)** areas of stratification are observed in the centre of the wound bed, by day 6 post-wounding wound margins also begin to close the wound space and by day 8 post-wounding **(e)** re-epithelialisation is complete and produces a fully stratified organotypic epidermis.

3. Carefully peel the skin from each mouse with sterile curved forceps and lay flat in a sterile large petri dish, dermal side down.
4. Up to four skins can be placed in each petri dish.
5. Add 20 ml of 0.25% trypsin solution to each petri dish and detach the skins from the base of the dish using forceps and then float in solution for 48 hours at 4°C (*see* **Note 1**).
6. Incubate the skins at 37°C for at least 30 min before attempting to separate the epidermis from the dermis.
7. Carefully peel the epidermis apart (trying to maintain whole epidermal integrity) from the underlying dermal tissue and place in a sterile 15 ml centrifuge tube containing 5 ml KGM + 10% chelexed serum (with a maximum of four individual epidermis to each tube) (*see* **Note 2**).
8. Release cells manually from the epidermal tissue with gentle rocking in the hand for 10 min at room temperature.
9. Filter the resulting cell suspension through a 100 μm cell strainer into a sterile 50 ml centrifuge tube.

10. Then centrifuge at 300 × g for 5 min and, without dislodging the cell pellet, carefully remove and discard the supernatant. Resuspend the cell pellet at 1×10^6 cells/ml in KGM + 10% chelexed serum.

3.3. Preparation of Transwell Organotypic Model

1. Carefully seed 300 µl epidermal cell suspension directly into the upper chamber of a 12 mm Transwell insert with no medium in the lower chamber.
2. Leave the cells to adhere overnight, at 37°C with 5% CO_2 ensuring the cells are not moved during this time (*see* **Note 3**).
3. The following day, wash the cells twice in calcium-free PBS and then add 500 µl KGM to both the upper and lower chambers.
4. Wash the cells daily in calcium-free PBS before replacing 500 µl KGM in both the upper and lower chambers until a confluent monolayer of cells is obtained.
5. At confluence, remove KGM from both the lower and upper chambers and add just enough KGM to the lower chamber so that medium just touches the underside of the Transwell membrane (approx 200 µl) to expose the cells to the ALI (*see* **Notes 4 and 5**)
6. The initial day of raising the cells to the ALI is named ALI-0 with each day thereafter ALI-1, ALI-2, etc. (*see* **Notes 6 and 7**).
7. Culture cells for up to 2 weeks with daily lower chamber medium changes.

3.4. Whole-Mount Immunofluorescence of Organotypic Cultures

1. Remove medium from the organotypic cultures and add 500 µl ice-cold methanol to both the upper and lower Transwell chambers for 10 min at 4°C.
2. Carefully remove the methanol from both chambers and add 500 µl PBS to the upper and lower chambers. At this point the cells can be stored at 4°C until required for immunofluorescent staining.
3. When immunofluorescence is ready to be performed, remove the PBS from the upper and lower Transwell chambers and add 500 µl 0.1% Triton X-100 solution to the upper Transwell chamber for 30 min at room temperature to permeabilise the cells. (*see* **Note 8**).
4. Remove the Triton X-100 solution and replace with 500 µl 5% skimmed milk (blocking solution) for 20 min at room temperature.
5. Carefully remove the blocking solution and replace with at least 250 µl of primary antibody solution for 1.5 hours at room temperature.

6. Remove the primary antibody and wash the cells three times in PBS for 10 min with gentle rocking.
7. Remove the PBS and replace with at least 250 μl of the appropriate fluorescent secondary antibody for 45 min in the dark at room temperature (*see***Note 9**).
8. Then wash the cultures three times in PBS for 10 min with gentle rocking.
9. If required, counterstain the cell nuclei with DAPI for 5 min at room temperature in the dark.
10. Wash the cells in PBS for 5 min with gentle rocking in the dark.
11. Replace 500 μl of PBS in the upper Transwell chamber and then the immunolabelled cells can be stored at 4°C in the dark until required for analysis.
12. To view the cells under confocal microscopy, place an individual Transwell (containing PBS) upon a clean (24 × 32 mm) glass coverslip and treat in the same manner as a microscope slide.
13. Examples of immunofluorescent characterisation of the organotypic model for epidermal differentiation markers are shown in **Fig. 21.2**.

3.5. Organotypic Epidermis Wound Assay

1. Perform wound assay experiments on cultures at ALI-3 or ALI-4 when organotypic stratification is readily observed.
2. To create a wound through the organotypic culture, an alcohol-wiped sterile ruler is used for guidance and by then applying gentle pressure onto the Transwell membrane with a sterile yellow pipette tip, a straight wound is created through the organotypic culture.
3. Perform daily lower chamber medium changes as described above.
4. Approximately 4 days after wounding, primary mouse keratinocytes will migrate out from the basal layer of the organotypic culture and form a monolayer between the two wound margins.
5. Epidermal re-epithelialisation will then occur over a period of 4–5 days to form a fully stratified organotypic epidermis (**Fig. 21.3**).

4. Notes

1. Incubation in trypsin solution for 48 hours improves the number of keratinocytes harvested from newborn skin epidermis and improves Transwell attachment rates.

2. Chelexed serum, in which Ca^{2+} has been removed, must be used when harvesting keratinocytes from newborn skin epidermis as this helps ensure cells are maintained in an undifferentiated state similar to that of the basal layer in vivo.
3. It is very important that in the initial 24 hours following epidermal cell suspension being seeded onto the Transwell inserts the cultures are not moved as this can affect keratinocyte attachment rates.
4. Once raised to the ALI it is critical that the upper surface of the organotypic cultures is kept as dry as possible. This can be achieved by aspirating any excess medium from the inside edge of the upper Transwell chamber at regular intervals twice a day.
5. When raising the cultures to the ALI the volume of KGM to be added to the lower chamber is important and may vary slightly. It is essential that just enough medium is added so that it just touches the underside of the Transwell membrane. Adding too much medium can cause leakage through the membrane resulting in the cultures getting wet which will impede subsequent stratification and differentiation.
6. Medium changes must be performed daily once raised to the ALI.
7. When replacing growth medium it is important to remove the used medium gently from the organotypic cultures by aspirating from the inside edge of the Transwell insert.
8. To facilitate primary and secondary antibody penetration of the organotypic cultures ensure 0.1% Triton X-100 is included in the antibody dilution buffer.
9. When required to incubate samples in the dark, wrap the Transwell plate in aluminium foil.

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Chapter 22

Optical and Biochemical Dissection of Connexin and Disease-Linked Connexin Mutants in 3D Organotypic Epidermis

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Abstract

The epidermis is a complex tissue composed principally of differentiated keratinocytes that form a keratinized stratified squamous epithelium. The gap junction proteins, connexins (Cx), are differentially expressed throughout the stratified layers of the epidermis and their exquisite regulation appears to govern the delicate balance between cell proliferation and differentiation in normal skin homeostasis and in wound healing. In the last 10 years, germ line mutations in the genes encoding five connexin family members have been linked to various types of skin diseases that appear to offset the balance between keratinocyte differentiation and proliferation. Consequently, in order to determine how these connexin gene mutations manifest as skin disease, disease-linked mutants must be expressed in 3D organotypic epidermis reference models that attempt to mimic the human condition. Given the complexity of organotypic epidermis, confocal optical and biochemical dissection of connexin or disease-linked connexin mutants within the regenerated epidermal layer is required. The procedures necessary to assess the architectural characteristics of genetically modified organotypic epidermis and its state of differentiation will be described in this chapter.

Key words: Connexins, Connexin mutants, Differentiation, Epidermis, Keratinocytes, Confocal microscopy.

1. Introduction

The large connexin family of gap junction proteins has up to 10 of its members expressed within the mammalian epidermis (1). Interestingly, the expression pattern of these connexins is linked to the degree of keratinocytes differentiation and, at the same state of differentiation, most keratinocytes co-express two or more connexins (2). Selective deficiency or knockdown of connexins has demonstrated that at least some connexin family members are necessary for

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epidermal differentiation and the maintenance of healthy skin (3–5). In addition, autosomal dominant and/or recessive germ line mutations in the genes encoding Cx26, Cx30, Cx30.3, Cx31, and Cx43 have been linked to various skin diseases that tend to reflect a dysregulation of the balance between keratinocytes proliferation and differentiation (6). To study the fate of keratinocytes that express any of these mutants, it is first necessary to characterize the connexin constituents of reference models that are chosen to mimic the epidermis. Second, it is important to determine the differentiation capacity of such a reference model and the effect of epidermal differentiation on connexin expression patterns. Finally, it is imperative to be able to genetically alter the connexin content or express disease-linked connexin mutants in the keratinocyte reference model to evaluate their potential role in epidermal differentiation. In all cases, confocal microscopy can be used to determine the connexin distribution in the multiple vital layers and the overall architecture of the epidermis, while a biochemical assessment of protein markers, indicative of the state of keratinocyte differentiation, can be utilized to determine the consequence of cells harboring abnormal connexin levels or disease-linked mutants. In the present chapter, we will use the example of rat epidermal keratinocytes (REKs) that possess the ability to differentiate into a complete organotypic epidermis with two to four vital layers and a thick cornified layer when grown at a liquid–air interface (**Fig. 22.1**). However, the techniques described below should be applicable to any keratinocyte source that retains the ability to differentiate into organotypic cultures and are amenable to genetic manipulation.

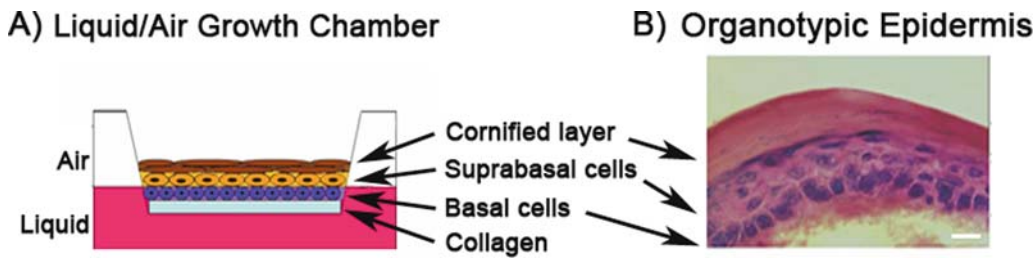

Fig. 22.1. Organotypic model of REK differentiation. **(A)** Rat epidermal keratinocytes (REKs) can be grown at a liquid–air interface that supports 3D differentiation into cell layers that mimics the epidermis (basal, suprabasal, and cornified layers). **(B)** Hematoxylin and eosin staining of 2-week-grown organotypic epidermis clearly shows that REKs differentiated into three to four vital layers and a thick cornified layer. (Reproduced from (13) with permission from Taylor and Francis.)

2. Materials

2.1. Cell Culture and Lysis

1. Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Burlington, ON, Canada) supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT).

2. Trypsin solution (0.25 %) from Invitrogen.
3. Rat epidermal keratinocytes (REKs) were a generous gift from Dr. Vincent Hascall. These cells were spontaneously immortalized from newborn rat epidermal keratinocytes and originally described by Baden and Kubilus (7).
4. Solution of Hygromycin B 50 mg/ml in PBS (Invitrogen).
5. Hanks' balanced salt solution 10 × containing phenol red (Sigma-Aldrich), HEPES buffer solution 1 M (Invitrogen), and 1 M NaOH solution.
6. Collagen type I rat tail (BD Biosciences Bedford, MA) and cell culture inserts 3.0 μm pore size (#353092, Falcon, VWR International, Mississauga, ON, Canada).
7. Phosphate-buffered saline (PBS): Prepare 10 × stock with 1.37 M NaCl, 27 mM KCl, 18 mM KH_2PO_4, and 100 mM Na_2HPO_4. Adjust pH to 7.4 with HCl. Dilute one part of 10 × PBS with nine parts of water as a working solution.
8. 2 × lysis buffer: 300 mM NaCl, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 1 % Nonidet P-40, 2 % Triton X-100. Store at 4°C. Prior to use for lysis of organotypic epidermis, add sodium orthovanadate and sodium fluoride to a desired volume of 2 × lysis buffer in order to obtain a final concentration of 1 mM each. Also freshly add proteases inhibitors (Roche Diagnostics, Mannhein, Germany) to the 2 × lysis buffer: one protease inhibitor cocktail tablet per 10 ml.
9. Fresh solutions of sodium orthovanadate (Sigma-Aldrich, Oakville, ON, Canada) and sodium fluoride (EM Science, Gibbstown, NJ) are dissolved at 100 mM in water.

2.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating buffer (4 ×): 1.5 M Tris-HCl, pH 8.8, 0.4% (w/v) sodium dodecyl sulfate (SDS).
2. Stacking buffer (4 ×): 0.5 M Tris-HCl, pH 6.8, 0.4% (w/v) SDS.
3. 30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide stock solution (37.5:1) (National Diagnostics, Atlanta, GA) and N,N,N,N′-tetramethyl-ethylenediamine (TEMED; National Diagnostics).
4. Ammonium persulfate (APS; Caledon, Georgetown, ON, Canada): prepare a 10% (w/v) solution in water (dissolve 1 g of APS in 10 ml of water). Solution is stable for 2 weeks at 4°C.
5. 4 × SDS-PAGE sample buffer: 200 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, and 40% (v/v) glycerol. Store at room temperature. Before using for samples preparation, freshly add 50 μl of 2-mercapto-ethanol (EM Science) to 450 μl of 4 × loading buffer.

6. Running buffer (10 ×): 0.25 M Tris, 1.92 M glycine, 1% (w/v) SDS. Store at room temperature. Dilute one part of 10 × running buffer with nine parts of water as a working solution.
7. Pre-stained molecular weight standards: Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, CA).

2.3. Western Blotting for Connexins and Differentiation Markers

1. Transfer buffer: 25 mM Tris, 190 mM glycine, 20% (v/v) methanol. Store at 4°C.
2. BioTrace™ NT Nitrocellulose membrane (Pall Corporation, East Hills, NY).
3. PBS-T: Phosphate-buffered saline with 0.05% (v/v) Tween 20 (Caledon).
4. Fisherbrand thick chromatography paper (05-714-4, Fisher, Nepean, ON, Canada).
5. Blocking buffer: 5 % (w/v) Blotto, nonfat dry milk (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS-T.
6. Primary antibodies: anti-Cx43 at a dilution of 1/1000 (C6219, Sigma-Aldrich), anti-Cx26 at a dilution of 1/500 (71-0500, Zymed Laboratories Inc. (Invitrogen)), anti-keratin10 at a dilution of 1/1000 (AB-2 MS-611-P1, NeoMarkers, Fermont, CA), anti-keratin14 at a dilution of 1/1000 (RB-9020-P1, NeoMarkers), anti-loricrin at a dilution of 1/1000 (PRB-145P, Covance (Cederlane, Hornby, ON, Canada)), anti-involucrin at a dilution of 1/1000 (PRB-140C, Covance), anti-β-actin at a dilution of 1/5000 (clone AC-15, Sigma-Aldrich), and anti-Hsc70 at a dilution of 1/1000 (SPA-815, Stressgen Bioreagents Corp (Cederlane)). The primary antibodies dilutions are prepared in the blocking buffer.
7. Infrared fluorescent-labeled secondary antibodies: IRDye 800 anti-rabbit or anti-mouse (Rockland Immunochemicals, Gilbertsville, PA) and Alexa-680 anti-rabbit or anti-mouse (Invitrogen). The secondary antibodies dilutions (1/5000) are prepared in the blocking buffer.

2.4. Preparation of Sections from Paraffin-Embedded Organotypic Epidermis and Hematoxylin/Eosin Labeling

1. Bouin's fixative solution (750 ml of picric acid (Sigma-Aldrich), 250 ml 37% formalin (VWR), 50 ml glacial acetic acid (VWR)).
2. Anhydrous ethyl alcohol (Commercial Alcohols Inc., Toronto, ON, Canada). Prepare 30%, 50%, 70%, and 95% ethanol solutions by diluting 100% ethanol with distilled water.
3. Xylene solution (Caledon).
4. Tissue processing/embedding cassettes (M490.5 Simport Histosette, Beloeil, QC, Canada).

5. Harris Hematoxylin, acidified (Lerner Laboratories) (Fisher).
6. Eosin Y, alcoholic (Lerner Laboratories) (Fisher).
7. Acid ethanol: 1 ml concentrated HCl diluted in 400 ml of 70% ethanol.
8. Paraplast® regular BioChemika (Sigma-Aldrich).
9. Feather Microtome blades (S35) (08310E, Surgipath Medical Industries Inc., Richmond, IL).
10. Surgipath Xtra slides (00200, Surgipath) and micro cover glass 22 × 40 mm (48393 048, VWR).
11. Cytoseal™ 60 (Fisher).

2.5. Preparation of Cryosections and Immunolabeling for Confocal Microscopy Imaging of Intact or Cryosections of Organotypic Epidermis

1. O.C.T. Compound (Tissue-Tek®, Sakura Finetek U.S.A. Inc., Torrance, CA). Store at room temperature.
2. Fisherbrand Superfrost®/Plus Microscope Slides (12-550-15, Fisher) and micro cover glass 22 × 40 mm (VWR International).
3. Formaldehyde solution 37% (w/w) (Fisher): Make a working solution 3.7% (v/v) in PBS. Store at room temperature.
4. Blocking solution: 2% (w/v) bovine serum albumin (BSA, Sigma-Aldrich), 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS.
5. Primary antibodies: Anti-Cx43 at a dilution of 1/500 (Sigma-Aldrich), anti-Cx26 at a dilution of 1/50 (Zymed Laboratories Inc.). The dilutions of primary antibodies are prepared in the blocking solution.
6. Secondary anti-rabbit antibodies, conjugated to Texas red (Jackson ImmunoResearch Laboratories, West Grove, PA) (at a dilution of 1/200) or Alexa Fluor 488 (Invitrogen, Burlington, ON, Canada) (1/500), are diluted in the blocking solution.
7. Nuclear stain: Hoechst 33342 (Molecular Probes (Invitrogen)) diluted 1/1000 in water.
8. Mounting medium: Vectashield (Vector Laboratories Inc., Burlington, ON, Canada).

3. Methods

Numerous effective and efficient procedures (transfection, infection, and electroporation) for introducing cDNAs and/or RNAis into mammalian cells, including keratinocytes, have been described elsewhere. For this reason, we will focus our detailed protocol descriptions on characterizing the connexin constituents

of reference models of the epidermis and evaluating the effect of connexin manipulation on the architecture and the differentiation status of the organotypic epidermis derived from these genetically modified keratinocytes.

3.1. Culture and Genetic Engineering of REKs

1. Wild-type or genetically engineered REKs grown in F25 flasks or in 60-mm dishes are sub-cultured when approaching confluence by treating with trypsin/EDTA solution. A 1:5 dilution of the cell number at the time of sub-culturing should provide cultures that are approaching confluence after 24 h.
2. Cx43-targeted shRNA constructs together with a control vector containing a nonsense sequence were designed and used to make infectious viral supernatants as we previously described (5, 8). REKS were infected and then cultured in selection medium containing 50 μg/ml hygromycin (5, 9). Antibiotic resistance cells should be passed at least three times prior to experimentation and the stable reduction of Cx43 (or any targeted molecule) expression should be assessed by Western blotting (*see* **Note 1**). This assessment should be preformed routinely at later cell passages to ensure continued knockdown of the targeted molecule.
3. Constructs containing cDNA for GFP-tagged or untagged wild-type human Cx, as well as disease-linked mutants, were generated within the AP2 replication-defective retroviral vector as we previously described (9–12). The overexpression of these Cx variants is continuously verified by Western blotting and/or immunofluorescence microscopy prior to any further experimentation (*see* **Note 1**).

3.2. Growth of Organotypic Epidermis

1. The volumes of reagents necessary for four collagen gels are 4.25 ml of type I collagen, 0.5 ml of 10 × Hanks' balanced solution 10 × , and 50 μl of 1.0 M HEPES (*see* **Note 2**). All reagents must be kept on ice and sterile.
2. While working in a laminar flow hood, very gently mix together the collagen, Hank's buffer, and HEPES solutions in a cold conical tube (15 ml) on ice. Mixing is continued until a homogenous mixture is obtained while avoiding bubbling. The mixture is always kept on ice during this process to avoid collagen polymerization.
3. At this point, the color of the mixture is likely to be yellow, indicating that it is too acidic. Add 50 μl of 1.0 M NaOH into the mixture using a pipette. The final color needs to be orange-pink to ensure an appropriate pH has been reached. If the mixture is still yellow after the first addition of NaOH, keep adding 25 μl aliquots of 1.0 M NaOH and mix until the appropriate color is reached (*see* **Note 3**).

4. Place inserts into a 6-well plate. Add 1.0 ml of collagen mixture into each insert chamber to cover the insert surface. Let the collagen polymerize at least 2 hours, to overnight, at 37°C in the cell culture incubator.
5. Once the gels have polymerized, briefly wash the collagen inserts twice with REK media and aspirate. Add 2 ml of REK media to the top and bottom chambers of each insert and place in the incubator at 37°C until ready to use.
6. REKs should be close to confluency in 60 mm dishes prior to harvesting and re-culturing on collagen inserts. To prepare the cells for the assay, first remove the media from the cells, rinse the cells once with either trypsin/EDTA or sterile PBS, aspirate, and add 1 ml of trypsin/EDTA per dish. Incubate at 37°C until the cells are detached from the dish. Add 4 ml of media (DMEM high glucose containing 10% FBS) per dish to neutralize the trypsin. The cells are then transferred in sterile conical tubes and counted using a hemacytometer.
7. Aspirate the media from the collagen insert. Add 2 ml of media to the bottom chamber of each well, and the cells (5×10^5 cells in a total volume of 2 ml) into the top chamber of each collagen insert. Place the plates into the incubator to allow the cells to grow.
8. Visualize the cells under the light microscope everyday. Once the cells reached confluency, gently aspirate the media from the upper chamber to expose the cells to air and allow them to differentiate at the air–liquid interface. Cells typically take 14 days to differentiate. The media in the lower chamber should be changed (1.5–2 ml/well) every day or every 2 days (*see* **Note 4**).

3.3. Preparation of Organotypic Epidermis Samples for Assessment of Connexin and Differentiation Markers Levels by Western Blotting

1. After 14 days of growth, the organotypic epidermis should be harvested. In order to detach the organotypic epidermis from the insert, use a scalpel or a razor blade and gently pass it around the edges of the organotypic epidermis. In some cases it may be necessary to add a small volume of PBS on top of the organotypic epidermis to facilitate its detachment. If paraffin-embedding sections are to be preformed, the organotypic epidermis can stay attached to the filter to facilitate its manipulation (*see* **Section 3.6**). If not, gently transfer the organotypic epidermis into a dish that contains PBS. Using a scalpel or a razor blade to gently cut the organotypic epidermis into half. Transfer one half into a graduated 1.5 ml tube that is kept on ice (or put directly at –80°C for later usage) and keep the other half in PBS for cryosections and/or paraffin embedding (*see* **Sections 3.6** and **3.8**).
2. Evaluate the volume displaced by the piece of organotypic epidermis into the graduated tube and add the same volume

of 2 × lysis buffer. If the samples were frozen, add the 2 × lysis buffer and let the samples thaw on ice. Sonicate on ice, three times for 10 seconds each round (based on using the 60 Sonic Transmembrator set at 8, Fisher) or until the lysate becomes clear. Make sure that the lysate stays cold during sonication and avoid aeration.

3. Centrifuge the lysate for 10 minutes at 10,000 × g at 4°C to remove cell debris and unsolubilized material. Transfer the supernatant into a 1.5 ml tube.
4. The protein content of the supernatant (whole organotypic epidermis lysate) is determined using the BCA™ Protein Assay Kit (Pierce).
5. The samples for SDS-PAGE are prepared by mixing 40 μg of organotypic epidermis lysate with 4 × Laemmli buffer into a labeled 1.5 ml tube where water is added to obtain a final volume of 20–30 μl.
6. The tubes are closed and boiled for 5 min. After cooling to room temperature, the samples are ready for separation by SDS-PAGE.

3.4. SDS-PAGE

1. These instructions assume the use of Mini 2-D Protein system (Bio-Rad Laboratories Inc).
2. Prepare a 1.5-mm thick 8% gel for the detection of keratin, involucrin, or loricrin by mixing 3.2 ml of acrylamide/bis solution, 3.0 ml of 4 × separating buffer, 5.8 ml water, 120 μl ammonium persulfate solution, and 7.2 μl TEMED. Prepare a 1.5-mm thick 12% gel for the detection of Cx43 and Cx26 by mixing 4.8 ml of acrylamide/bis solution, 3.0 ml of 4 × separating buffer, 4.2 ml water, 120 μl ammonium persulfate solution, and 7.2 μl TEMED. Pour the gels, leaving space for a stacking gel, and overlay with water. The gel should be polymerized in approximately 30 min.
3. Remove the water and rinse the top of the gel twice with water. Remove as much water as you can before pouring the stacking gel.
4. Prepare the stacking gel by mixing 1.0 ml of acrylamide/bis solution, 1.5 ml of 4 × stacking buffer, 3.5 ml water, 60 μl ammonium persulfate solution, and 3.6 μl TEMED. The stacking gel should be polymerized in approximately 30 min.
5. Prepare the running buffer by mixing 100 ml of the 10 × running buffer with 900 ml of water in a measuring cylinder.
6. Once the stacking gel has polymerized, gently remove the comb and rinse the wells twice with water. Install the gels in the electrophoresis unit.

7. Add the running buffer to the upper and lower chambers of the gel unit and load 20–30 μl of each sample to the well. Include one well for pre-stained molecular weight markers (4 μl/well).
8. Assemble the lid of the gel unit and connect to the power supply. The gel is run at 100 V and the migration is stopped when the dye front (blue) reaches the bottom of the gel.

3.5. Western Blotting for Connexins and Differentiation Markers

1. The organotypic epidermis lysates that have been separated by SDS-PAGE are then transferred to a nitrocellulose membrane. The directions listed below assume the use of a semi-dry transfer system (Trans-Blot SD Semi-dry Transfer Cell, Bio-Rad).
2. Prepare the transfer buffer by mixing 100 ml of 10 × transfer buffer and 200 ml of methanol with 800 ml of water in a measuring cylinder.
3. Prepare a tray that is large enough to lay out the nitrocellulose membrane and six sheets of thick chromatography paper. Add some transfer buffer and submerge the nitrocellulose membrane (cut just a bit larger than the separating gel) and six thick chromatography paper sheets cut the same size of the nitrocellulose membrane.
4. The gel unit is disconnected from the power supply and disassembled. The stacking gel is removed and discarded. One corner of the separated gel can be cut in order to track its orientation and for its identification if many gels were transferred. Keep the gel submerged in a tray that contains transfer buffer.
5. Three sheets of chromatography paper are successively layered on top of each other. The membrane is laid on top of the papers and the separating gel is then laid on top of the nitrocellulose membrane. Finally, three additional sheets of chromatography paper are layered on top of the gel. Make sure to remove any bubble that could be trapped.
6. Place the lid on top of the transfer system and connect to the power supply. Transfer at 15 V for 30 min.
7. Once the transfer is complete, cut the shape of the gel into the membrane (including the cut corner). The gel, filter papers, and the excess nitrocellulose membrane are then discarded. The pre-stained molecular weight markers should be clearly visible on the nitrocellulose membrane.
8. The nitrocellulose membrane is then incubated in 15–20 ml blocking buffer for 30 min at room temperature on a rocking platform.
9. The blocking buffer is removed and the membrane is incubated with 15 ml of a dilution of the primary

antibody in the blocking buffer overnight at 4°C on a rocking platform. We mainly use keratin10, keratin14, loricrin, and involucrin as differentiation markers for Western blot analysis and a mouse skin lysate is used as a positive control.

10. The primary antibody is then removed and the membrane is washed three times 5 min each with 10–15 ml of PBS-T.
11. The appropriate fluorescent-labeled anti-rabbit or anti-mouse secondary antibody is prepared at a dilution of 1/5000 in the blocking buffer and 15 ml is added to the blot for 1 h at room temperature on a rocking platform (*see* **Note 5**). Protect the membrane from light during this step and during the following washes until it has been scanned.
12. The secondary antibody is then removed and the membrane is washed three times for 5 min each with 10–15 ml PBS-T.
13. After the final wash, the membrane is now ready to scan in the appropriate channels using the Li-COR Infrared Imaging system.
14. Once exposed for antibody binding, the membrane can be re-probed with an antibody against β-actin (5), or Hsc70 (9), as an internal loading control, by repeating the procedures above (*see* **Note 6**). Whole lysates from organotypic epidermis contain high levels of collagen which can lead to some variation in sample loading (*see* **Note 7**). An example of Western blotting results for Cx43, Cx26, and the various differentiation markers is shown in **Fig. 22.2**.
15. The expressions of connexins and the various differentiation markers are quantified by densitometry using the Li-COR Odyssey Imaging System and are then normalized to the amount of the internal control protein (β-actin or Hsc70) for each sample. There is some variability from one experiment to another in terms of the growth of the organotypic epidermis, thickness of the cornified layer, and the amount of matrix that is analyzed with organotypic sample. For this reason, averaging many repeats is required in order to obtain quantitative results (*see* **Fig. 22.2F**).

3.6. Histological Staining for Organotypic Epidermis Architecture Assessment

1. In order to analyze the epidermal structure, sections of paraffin-embedded organotypic epidermis are stained with hematoxylin and eosin (H/E). While H/E staining can be done on organotypic cryosections (*see* **Fig. 22.2A, B**), best results are obtained using paraffin-embedding sections which allow a more detailed architectural assessment (*see* **Fig. 22.3**).

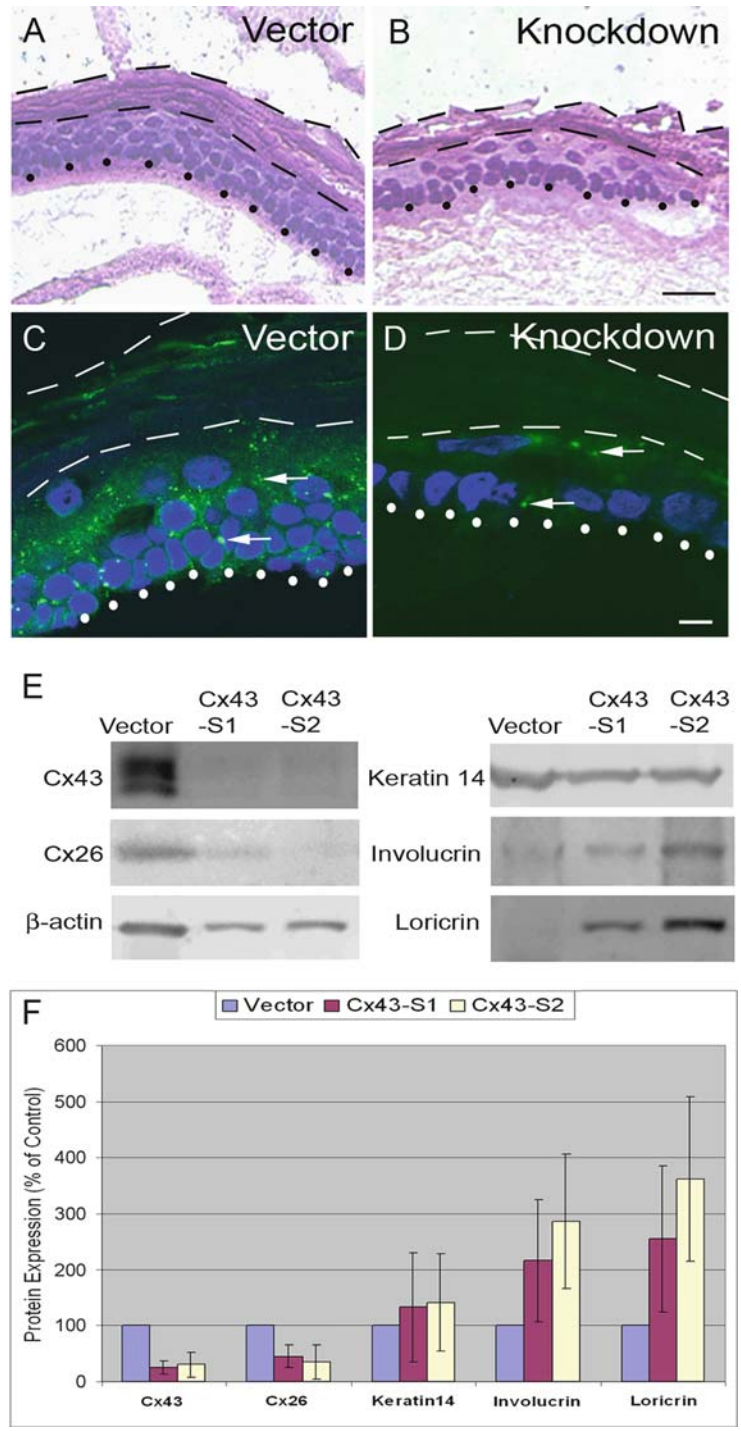

Fig. 22.2. Effect of reducing Cx43 expression on REK differentiation: assessment of the organotypic epidermis architecture and differentiation markers expression**.** Hematoxylin and eosin staining revealed that in wild-type epidermis, a thick cornified layer (between dashed lines) and vital layers (dotted line to first dashed line) are evident **(A)**, while these layers are both reduced when Cx43 expression is knocked down **(B)**. *Bar* = 50 μm. Cx43

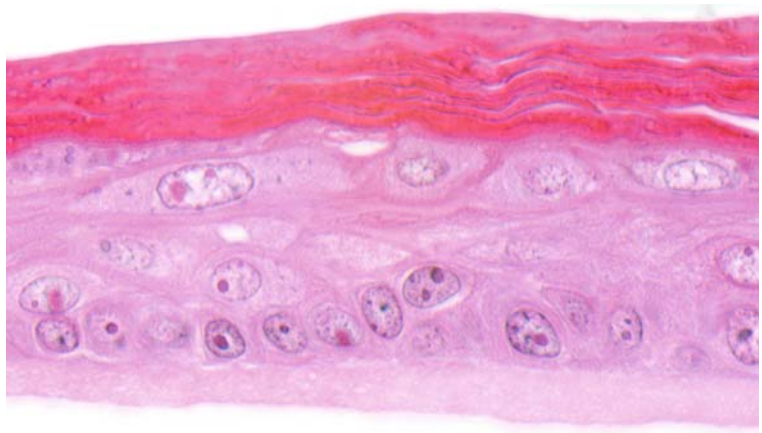

Fig. 22.3. Architecture of wild-type REKs organotypic epidermis as revealed by hematoxylin and eosin staining of paraffin-embedded sections**.**Hematoxylin and eosin staining of paraffin-embedded sections of organotypic epidermis allow a more detailed assessment of the epidermal architecture compared to the staining obtained using cryosections (*see* **Fig. 22.2**). In using paraffin sections, it is possible to observe the phenotypic progression from an organized basal cell layer to large, squamous and spinosum-like cells with strong circumscribed eosinophilic staining.

2. One-quarter of each organotypic epidermis should be enough for paraffin embedding. After 14 days of growth, the filter insert containing the organotypic epidermis is removed from the tissue culture well insert using tweezers.
3. A sharp probe is used to pierce the filter membrane and circumscribe around the organotypic culture through the filter. This will release the bottom filter membrane and the attached organotypic culture from the rest of the plastic filter insert. Take care not to disturb the organotypic culture at this point since it is easy to crease the filter membrane when removing the membrane from the rest of the insert. Removing the filter membrane with the organotypic culture provides a firm substrate to cut samples from and minimizes the disturbances caused from transferring the organotypic culture.

Fig. 22.2 (continued) immunofluorescence (green) of cryosectioned organotypic epidermis revealed abundant Cx43 throughout the vital layer of the control **(C)**, a situation not evident in the Cx43-knockdown organotypic epidermis **(D)**. *Blue*, nuclei; *Bar* = 20 μm. Western blot **(E)** and densitometric analysis **(F)** of Cx43-reduced organotypic cultures revealed a notable decrease in Cx26 expression and an increase in loricrin and involucrin compared to wild-type cultures. Please note that in this case the defect in epidermal differentiation is reflected by an increase, and not a decrease, of the differentiation markers loricrin and involucrin which might indicate an immature and fragile cornified envelope. (Reproduced from (5) with permission from the American Society for Biochemistry and Molecular Biology.)

4. Using a sharp pair of scissors, cut a 0.5 cm sample from the organotypic culture.
5. The sample is fixed by gently placing in a 15 ml tube filled with Bouin's fixative solution at 4°C and incubating overnight.
6. The next day, the Bouin's solution is aspirated and the organotypic sample is incubated in 30% ethanol for 30 min at room temperature. The ethanol is then aspirated and fresh 30% ethanol is added for another 30 min. During each aspiration cycle, be careful not to disturb the organotypic sample.
7. The ethanol solution is aspirated and the organotypic sample is dehydrated by two incubations with 50% ethanol for 30 min, followed by two incubations with 70% ethanol for another 30 min.
8. Using forceps, the organotypic sample is transferred to a properly labeled tissue processing/embedding cassette and immersed in 70% ethanol. Samples can be stored in 70% ethanol for short term, but prolonged incubation is not advised as samples will become brittle. At this stage, proceed with the paraffin wax infiltration (*see* **Note 8**).
9. Samples are first submerged in 95% ethanol for 1 h at room temperature and then incubated in fresh 95% ethanol for 90 min. This is followed by two changes of 100% ethanol for 1 h and an additional change of 100% ethanol for 90 min.
10. Samples are immersed into xylene for 1 h at room temperature and then into fresh xylene solution for another 90 min at 40°C.
11. Finally, the organotypic epidermis samples are submerged in Paraplast for 1 h at 60°C and then in two changes of Paraplast for 90 min at 60°C. Paraffin wax infiltration can be enhanced under vacuum and can be automated using a tissue processor machine such as a Leica Microsystems ASP300 Tissue processor. If not, an incubator containing trays of melted paraffin can be set at the appropriate temperature.
12. At this point, samples can be embedded into wax molds with the aid of a tissue-embedding machine (Leica EG1150H paraffin-embedding station). Place a paraffin-embedding mold (Tissue-Tek base metal molds) with a deep enough well to accommodate the size of your organotypic sample on the hot "work area." Remove your organotypic sample with forceps from the tissue cassette and place it on the hot "work area."
13. Add hot Paraplast wax to the paraffin-embedding mold and place the mold onto the refrigerated cold work area.
14. Quickly use forceps to grab the base of your organotypic sample on each edge and transfer the organotypic sample to the middle of the cooling paraffin-embedding mold. Orient

the sample vertically/on edge for coronal cross sections or flat for horizontal cross sections and wait until the organotypic sample maintains its orientation.

15. Place the base of the tissue cassette on top of the paraffin-embedding mold and add more wax to the top of the tissue cassette base until the wax touches the tissue cassette.
16. Place the paraffin-embedding mold along with the adjoining tissue cassette base onto a cooled surface to allow the paraffin wax to solidify.
17. After the mold has set, remove the paraffin-embedding mold and collect the embedded samples for tissue sectioning.
18. Section the embedded organotypic samples at a thickness of 5 μm using a microtome equipped with Feather Microtome blades (S35). Surgipath Xtra slides are also recommended for optimum results.
19. Prior to hematoxylin/eosin staining, incubate your slides at 60°C for 30 min.
20. Place the paraffin section slides in a slides holder and deparaffinize sections in three changes of xylene (3 min each). Remove the excess xylene.
21. Transfer slides to 100% ethanol (three times, 3 min each) followed by once through 95% ethanol and 70% ethanol for 3 min each and rehydration in water for 5 min.
22. Remove the excess water from the slides and incubate them in the hematoxylin solution for 5 min. Rinse with water (until the water is clear) and leave the slides in tap water for 5 min.
23. Quickly dip the slides (8–12 times) in acid ethanol to destain.
24. Remove excess acid ethanol from the slides and incubate them in eosin solution for 5 min.
25. Remove excess eosin and transfer slides to 95% ethanol (three times, 3 min each). Then transfer slides through 100% ethanol (three times, 3 min each).
26. Remove excess ethanol and transfer slides into xylene (three times, 5 min each change).
27. Finally, samples are mounted with micro cover glass using Cytoseal® 60 and allowed to dry overnight in the fume hood. Slides are then ready for imaging.

3.7. Imaging of the Histological Staining to Assess the Organotypic Epidermis Architecture

1. At least five random areas of H&E-stained organotypic epidermis sections are imaged with a $\times 40$ or $\times 63$ oil objective lens mounted on a Zeiss (Thornweed, NY) axioscope microscope workstation equipped with a Sony (Tokyo, Japan) PowerHAD camera and Axio Vision LE software (Carl Zeiss, Jena, Germany).

2. Using this software, the thicknesses of the total, vital, and cornified layers can be measured from five random areas. The thickness of three regions within each image is then measured (15 measurements/sample). These measurements need to be obtained from at least three separate preparations of organotypic cultures prior to any statistical analysis. An example of histological staining of organotypic epidermis is shown in **Fig. 22.2**, which illustrates the alteration of the epidermal architecture of the Cx43 knockdown organotypic epidermis compared to its control.

3.8. Immunolabeling of Connexins Using Organotypic Epidermis Cryosections

1. In order to characterize connexins localization in REK organotypic epidermis, samples are cryosectioned and immunofluorescently labeled.
2. One-quarter of the organotypic epidermis is kept flat in a dish in PBS and cut in small rectangular pieces (~10 mm long and 5 mm wide) using a razor blade or a sharp scalpel.
3. These pieces are embedded in optimal cutting temperature compound (O.C.T.) on dry ice. Place the pieces with the long side down in O.C.T. compound.
4. The resulting embedded organotypic sections are placed on a small piece of cork (the piece should be just a bit bigger than the square piece of embedded organotypic epidermis) using few drops of O.C.T. compound for their subsequent sectioning. Embedded organotypic epidermis is then kept at –80°C.
5. Section blocks are cut at a thickness of 10–12 μm using a cryostat and mounted on Superfrost PLUS slides.
6. Cryosections are fixed in 3.7% formaldehyde at room temperature for 1 h.
7. Nonspecific labeling is blocked by incubating the sections in a solution of 2% BSA in PBS for 30 min.
8. Organotypic epidermis sections are then incubated in primary antibodies (e.g., anti-Cx43 (1/500) or anti-Cx26 (1/50) in 2% BSA in PBS) for 1 h. Untagged connexins (endogenous or overexpressed mutants) need to be immunolabeled using appropriate primary and secondary antibodies in order to be visualized, while the GFP-tagged connexins can be visualized directly by fluorescent microscopy. In both cases, nuclei need to be labeled with Hoechst 33342.
9. Samples are washed three times (5 min each wash) in PBS.
10. Sections are then incubated in secondary antibodies (Alexa 488 anti-rabbit 1/500 in 2% BSA in PBS) for 1 h.
11. After this incubation, the samples are washed three times with PBS (5 min each wash) and then incubated for 10 min with Hoechst 33342 (1/1000 in water).

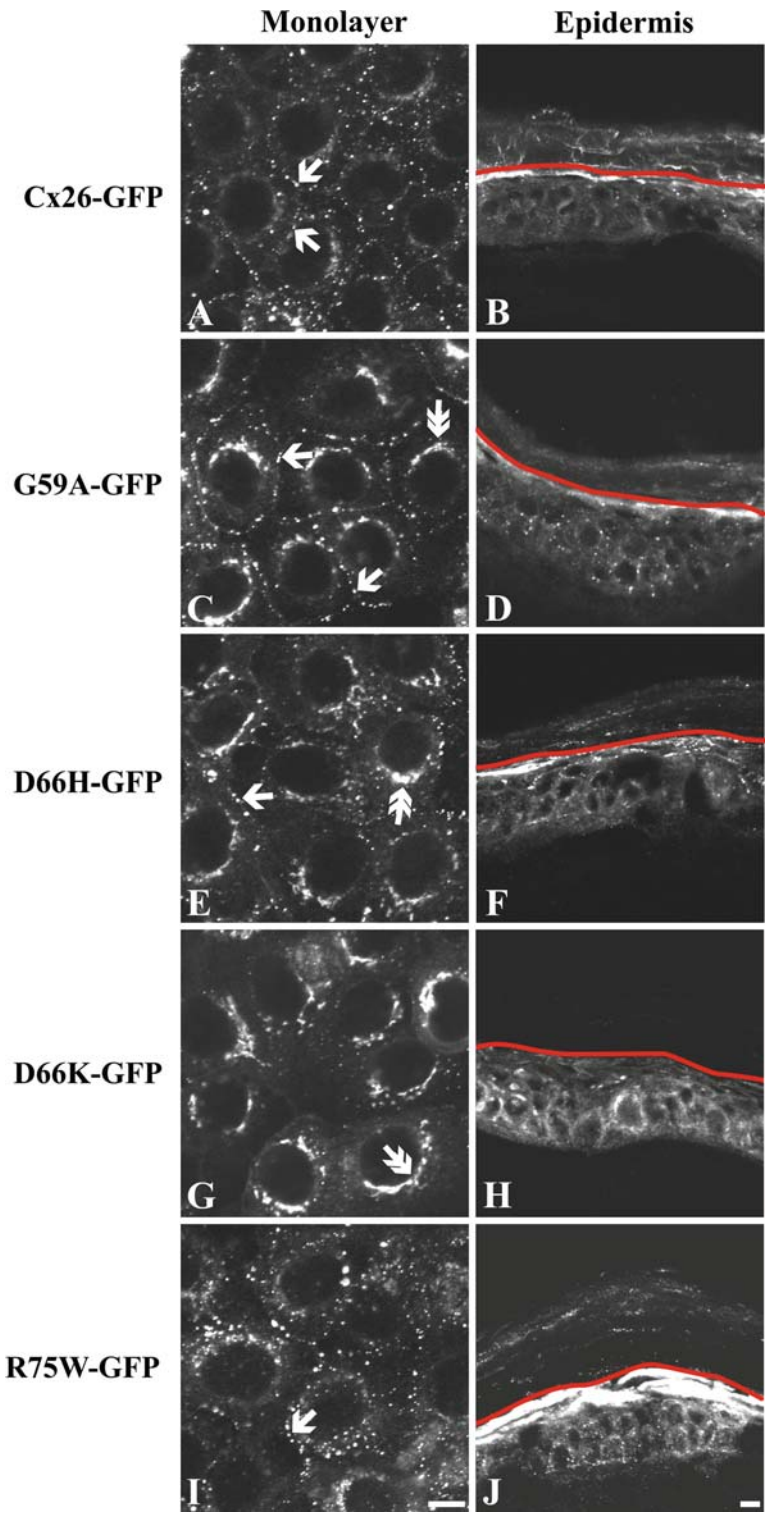

Fig. 22.4. REKs expressing wild-type or mutant Cx26 formed a stratified and cornified epidermis. REKs expressing GFP-tagged wild-type or mutant Cx26 variants were grown

12. Organotypic sections are washed three times with PBS (5 min each) and then two times with water (5 min each).
13. Finally, samples are mounted in Vectashield on glass slides and are ready for confocal microscopy imaging.

3.9. Imaging of Organotypic Epidermis Cryosections

1. Immunolabeled cryosections of organotypic epidermis are imaged using a 63 × oil objective lens mounted on a Zeiss LSM 510 META system (Carl Zeiss).
2. The following laser and filter settings are used: Texas Red is excited with a 543 nm helium-neon laser line and collected on a spectral analysis detector configured to accept 600–650 nm wavelengths; GFP and Alexa 488 are excited with a 488 nm argon laser line and captured after passage through a band pass 500–550 filter; and Hoechst 33342 is detected using a Chameleon multiphoton laser set to 730 nm with fluorescent emissions collected after passage through a 400–450 band pass filter.
3. The Zeiss LSM 510 META software is used to overlay the fluorescent images. An example of immunolabeling of endogenous Cx43 in organotypic epidermis cryosections is shown in **Fig. 22.2**, and an example of GFP-tagged wild-type or mutant Cx26 is shown in **Fig. 22.4**.

3.10. Immunolabeling of Connexins in Intact Organotypic Epidermis

1. Two-week cultures of organotypic epidermis are peeled off the collagen gel as an intact epidermis.
2. Epidermis is fixed overnight in 3.7% formaldehyde in PBS at room temperature.
3. Samples rinsed three times in PBS are immunolabeled as described in **Section 3.8** except that incubations are 2 h each for primary and secondary antibodies. Untagged connexins or connexin mutants need to be immunolabeled in order to be visualized, while the GFP-tagged connexins can be visualized directly by fluorescent microscopy (*see* **Section 3.9**). In both cases, nuclei need to be labeled with Hoechst 33342.

Fig. 22.4 (continued) in monolayer or organotypic epidermis. In monolayer, wild-type and some mutant variants of Cx26 (G59A, D66H, R75W) are detected at the cell surface in punctuate structure reminiscent of gap junction plaques (arrows) **(A, C, E, I)**. G59A and D66H exhibit partial intracellular localization reminiscent of the Golgi apparatus (double arrows) **(C, E)**, while D66K is almost exclusively localized in the Golgi apparatus **(G)**. Interestingly, cryosections prepared from organotypic cultures revealed a stratified and cornified epidermis regardless of the overexpression of Cx26 and Cx26 mutants **(B, D, F, H, J)**, and their different subcellular localization. *Red line* indicates the boundary between the vital cellular layers and the cornified layer, *Bar* = 10 μm. (Reproduced from (9) with permission from Springer.)

4. Following immunolabeling, samples are mounted in Vectashield and are ready for imaging. Concave microslides can be used to facilitate the mounting since the intact organotypic epidermis samples are thicker than sections.

3.11. Imaging of Intact Organotypic Epidermis

1. For imaging intact organotypic epidermis, a 40× water objective lens mounted on a Zeiss LSM 510 Meta system is used to generate z-stacks. The optical sections of intact organotypic epidermis acquired using this technique allow for the close analysis of the subcellular localization characteristics of wild-type and mutants connexins (either GFP-tagged or labeled untagged connexins). An example of analysis of immunolabeled endogenous Cx43 and Cx26 in optical sections of intact organotypic epidermis is shown in **Fig. 22.5**, while an example of the spatial localization of GFP-tagged wild-type or mutant Cx26 is shown in **Fig. 22.6**.
2. The laser and filter settings used are the same as those used for imaging of organotypic epidermis cryosections (**Section 3.9**).
3. Motor steps of 1 μm are used for optical sectioning of epidermis with the first image of a z-stack set just below the detection of nuclei stained with Hoechst 33342.
4. Acquired image sections are reconstructed in 3D using software built into the Zeiss LSM 510 META software package.
5. Presentation of optical slices depicting labeling patterns can be presented in gallery format (**Figs. 22.5** and **22.6**) or combined into 3D composites suitable for rotational analysis and viewing from all *x*, *y*, and *z* planes.

4. Notes

1. Genetically engineered cells should be monitored by Western blotting and/or immunofluorescence microscopy (in the case of GFP (or other fluorescent protein)-tagged molecules) for the overexpression of GFP-tagged connexins or mutants, as well as for the knockdown of connexins or other molecules of interest. For any reason, if the engineered cells do not sufficiently express the appropriate level of the molecules of interest, these cells should not be used.
2. If more collagen gel is required, multiply the volumes of all solutions accordingly (and use larger sterile conical tubes) in order to keep the same final concentration of collagen.

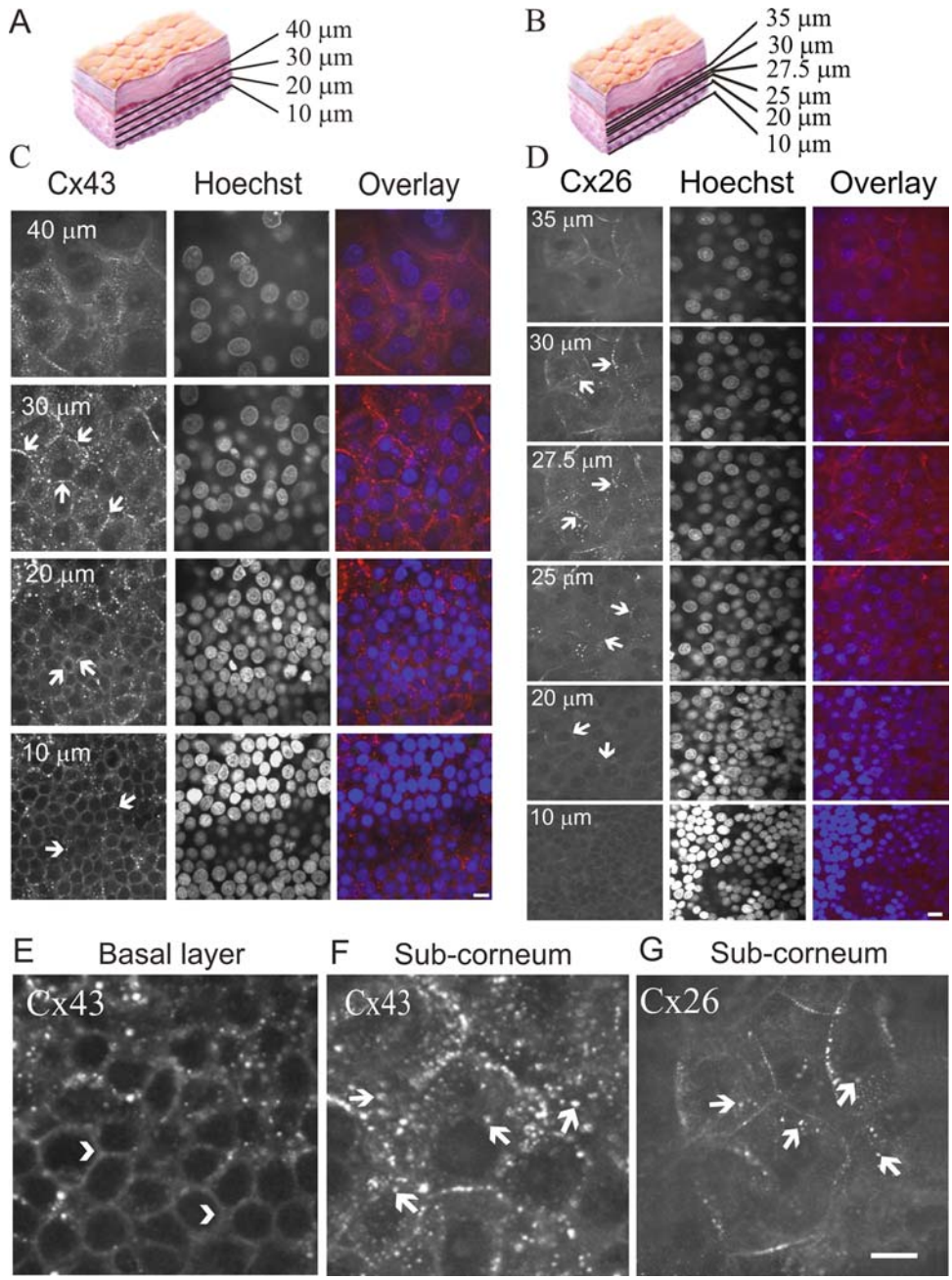

Fig. 22.5. Optical sectioning of intact organotypic epidermis for visualization of the spatial localization of endogenous Cx43 and Cx26. The schematic of the organotypic epidermis depicts the relative position of the optical slices for Cx43 **(A)** and Cx26 **(B)** immunolabeled epidermis. Optical sectioning of immunolabeled 3D intact organotypic epidermis revealed that Cx43 is present throughout the basal and suprabasal cells **(C, arrows)**. Cx43 was diffusely localized to, or near, the cell surface **(E, arrowheads)** and was also observed as punctuate gap junction plaques in basal keratinocytes **(C, E)**. Cx43 was more readily observed as punctuate gap junctions in suprabasal cells where intracellular vesicles also became apparent **(F, arrows)**. Cx26 was not detected in basal cells (**D**, 10 μm) but was prominent as punctate gap junction plaques in suprabasal cells **(D, arrows)**. Cx26 was detected in intracellular vesicles as cells approach the cornified layer **(G, arrows)**. *Blue*, nuclei; *Bar* = 20 μm. (Reproduced from (13) with permission from Taylor and Francis.)

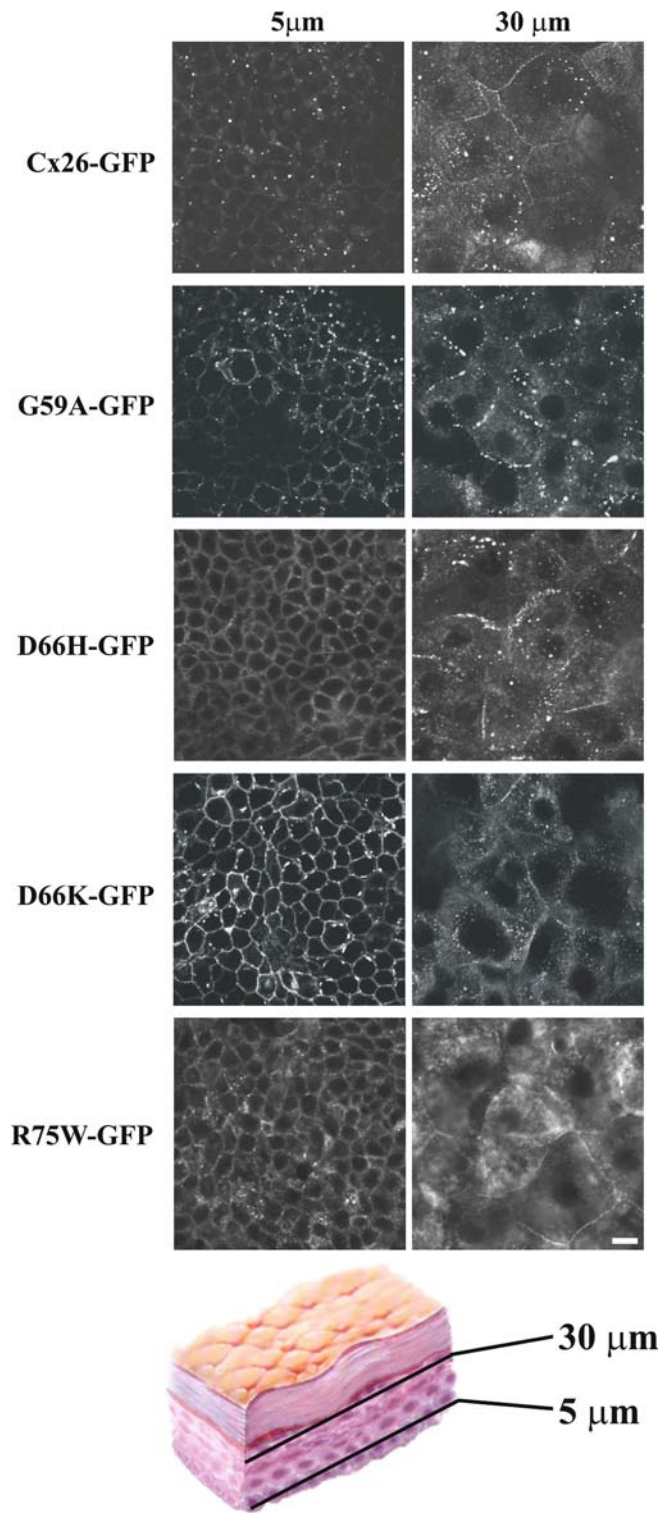

Fig. 22.6. Optical sectioning of intact organotypic epidermis can reveal differential intracellular spatial localization in 2D and 3D cultures: Example of Cx26 mutants. Optical sections of organotypic epidermis expressing Cx26 variants were acquired by confocal microscopy. Optical slices from positions 5 (basal layer) and 30 μm (suprabasal layer) are noted by

3. If too much NaOH is added to the collagen mixture, it will become bright pink indicating that it is too basic. If this happens, small volumes of diluted HCl or collagen can be added and mixed into the mixture until it becomes orange-pink.
4. If during epidermis differentiation the media becomes yellow after a single day, then the media has to be changed at single day intervals for all wells within an experimental set.
5. Antibody binding can also be detected with horseradish peroxidase (HRP)-conjugated secondary antibodies with an appropriate substrate.
6. Since the fluorescent signal on the membrane is often difficult to fully strip and to avoid any confusion with respect to the origin of the bands detected after re-probing, the species of the primary antibody that is used to detect the internal control protein must be different from the one used in the first blotting procedure.
7. If genetic manipulation of REKs, such as reduction of Cx43 expression (**Fig. 22.2**), leads to a defect in differentiation and in a thinner organotypic epidermis, the proportion of collagen becomes higher in that sample and can result in some variation in samples loading (seen by variation of the level of β-actin or Hsc70). In these cases, the expression levels of the various differentiation markers are still normalized to the level of the loading control protein.
8. If a Pathology Core Facility is available in your idea, at this stage samples can be submitted for the paraffin wax infiltration/tissue processing steps.

Acknowledgments

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Fig. 22.6 (continued) the schematic of the epidermis. Gap junction-like plaques were observed in the basal cells when wild-type Cx26-GFP was expressed. Importantly, there were increased incidences of intracellular structures in suprabasal cells immediately below the cornified layer for all Cx26 variants. These structures likely represent internalized connexins en route to degradation as the cells in this region undergo keratinization. Interestingly, the distribution pattern of some Cx26 variants (D66H, D66K, R75W) was distinctly different from what was found in monolayer REK cultures (*see* **Fig. 22.4**), suggesting that 3D architecture of organotypic epidermis, possibly in conjunction with the onset of the expression of other connexins or molecules, alters the cellular distribution profile of some connexin mutants. *Bar* = 10 μm. (Reproduced from (9) with permission from Springer.)

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Chapter 23

Cytokine Release in Tissue-Engineered Epidermal Equivalents After Prolonged Mechanical Loading

Lisette H. Cornelissen, Debbie Bronneberg, Susan Gibbs, Carlijn V.C. Bouten, and Cees W.J. Oomens

Abstract

Prolonged mechanical loading of soft tissues may result in degeneration of these tissues, resulting in formation of pressure ulcers. The risk assessment of individuals might be improved by including measurements of the tissue response to mechanical loading. Cytokines, which are released by the top layer of the skin upon chemical irritation, might be used to determine the epidermal response to mechanical loading.

This chapter describes methods to measure the release of cytokines IL-1α, IL-1RA, and IL-8 from tissue-engineered epidermal equivalents in response to sustained mechanical loading. A custom-built loading device was used to apply load to the epidermal equivalents and the cytokines were measured using an enzyme-linked immunosorbent assay.

Key words: Interleukin-1α, Interleukin-1RA, Interleukin-8, Mechanical loading, Pressure ulcers, Enzyme-linked immunosorbent assay (ELISA).

1. Introduction

Sustained mechanical loading of soft tissues, such as the skin and muscle tissue, might result in degeneration of these soft tissues, resulting in the formation of pressure ulcers. The use of risk score lists, currently used in clinical institutions, often results in inappropriate assessment of the risk of individuals for the development of pressure ulcers (1). These risk score lists are based on patient features that can easily be recorded by nursing staff in clinical settings. However, none of these scales incorporate a measure to indicate the tissue response to applied mechanical loading.

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Several cytokines are released by keratinocytes upon irritation of the epidermis. Spiekstra et al. (2) demonstrated that chemical irritation to the irritant sodium lauryl sulfate (SLS) of human skin equivalents resulted in an increased release of cytokines such as interleukin-1α (IL-1α), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α). Furthermore, the release of IL-1α and interleukin-1 receptor antagonist (IL-1RA) was shown to be increased after mechanical loading of keratinocyte monolayers (3, 4).

We propose that these signaling molecules can also be used to assess the epidermal tissue response after mechanical loading. A custom-built loading device was developed (5, 6) to apply load to tissue-engineered equivalents. After load removal, cytokine values were determined in the culture supernatant, which is explained in detail in the current chapter. It was shown that IL-1α, IL-1RA, and IL-8 were increasingly released upon sustained mechanical loading (7).

2. Materials

2.1. Mechanical Loading of the EpiDerm Cultures

1. EpiDerm cultures (EPI-200, MatTek Corporation, Ashland, MA, USA)
2. Hydrocortisone free maintenance medium (EPI-100-MM-HCF, MatTek Corporation, Ashland, MA, USA)
3. Custom-built bottom plate (**Fig. 23.1a**)
4. Custom-built positioning rings (**Fig. 23.1b**)
5. Custom-built lid for vertical positioning of the indenters (**Fig. 23.1c**)
6. Custom-built indenters and additional weights (**Fig. 23.1d**)
7. Custom-built cover lid of the loading device (**Fig. 23.1e**)
8. Formaldehyde (Cat. No. 1.03999.1000, Merck, The Netherlands). Be careful, this chemical is toxic and corrosive.

2.2. ELISA for Determination of IL-1α, IL-1RA, and IL-8

1. $Na_2CO_3 \cdot 10H_2O$ (Cat. No. 1.06391.1000, Merck, The Netherlands). Be careful, this substance is an irritant.
2. $NaHCO_3$ (Cat. No. 1.06329.1000, Merck, The Netherlands)
3. PBS (Cat. No. P4417, Sigma, Belgium)
4. Tween 20 (Cat. No. 8.22184.0500, Merck, The Netherlands)
5. Bovine Serum Albumin (Cat. No. 10735086001, Roche, The Netherlands)
6. Monoclonal antibodies

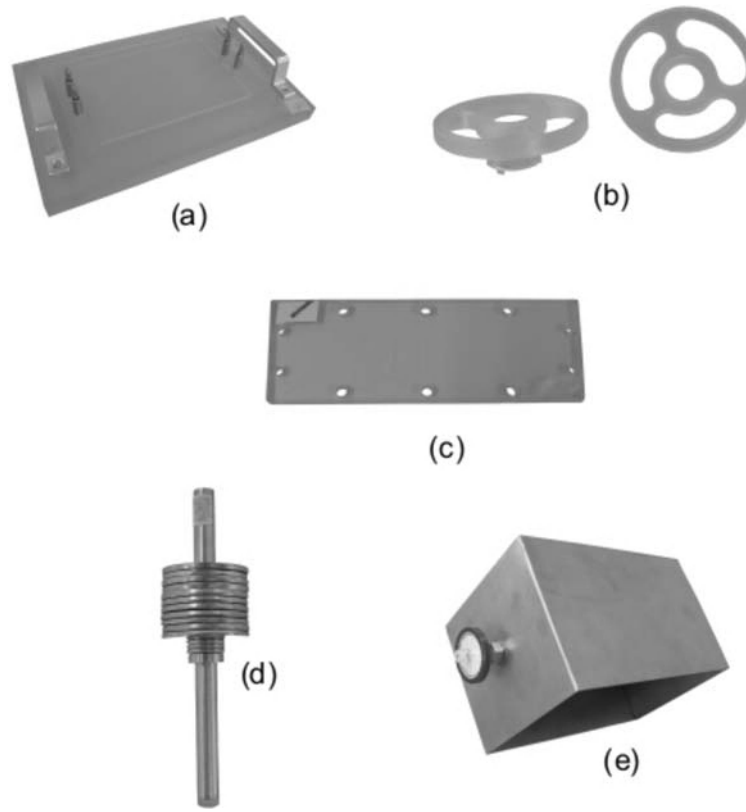

Fig. 23.1. Different parts of the custom-built loading device: The bottom plate of the loading device in which the 6-well plate perfectly fits **(a)**. Two position rings of which one is placed at the top of a cell culture insert **(b)**. The lid for vertical positioning of the indenters **(c)**. Indenters with rings to apply additional weight **(d)**. Lid with air filters to cover the total experimental set-up **(e)**.

a. IL-1RA stock1 (MAB280, R&D systems, United Kingdom) is dissolved at 1 mg/ml in PBS and stored in single-use aliquots at –30°C
b. IL-1α stock1 (MAB200, R&D systems, United Kingdom) is dissolved at 500 μg/ml in PBS and stored in single-use aliquots at –30°C
c. IL-8 stock1 (M9318, red tubes, Sanquin Reagents, The Netherlands) is stored at –30°C

7. Recombinant antibody
   a. IL-1RA stock2 (280-RA-010, R&D systems, United Kingdom) is dissolved at 100 ng/ml in PBS with 0.1% BSA and stored in single-use aliquots at –30°C
   b. IL-1α stock2 (200-LA-002, R&D systems, United Kingdom) is dissolved at 100 ng/ml in PBS with 0.1% BSA and stored in single-use aliquots at –30°C

c. IL-8 stock2 (M9318, black tubes, Sanquin Reagents, The Netherlands) is stored at –30°C

8. Biotinylated antibody
   a. IL-1RA stock3 (BAF280, R&D systems, United Kingdom) is dissolved at 50 μg/ml in PBS with 0.1% BSA and stored in single-use aliquots at –30°C
   b. IL-1α stock3 (BAF200, R&D systems, United Kingdom) is dissolved at 50 μg/ml in PBS with 0.1% BSA and stored in single-use aliquots at –30°C
   c. IL-8 stock3 (M9318, yellow tubes, Sanquin Reagents, The Netherlands) is stored at –30°C
9. Streptavidine-HRP (M2032, Sanquin Reagents, The Netherlands), store in single-use aliquots at –30°C
10. Sodium acetate trihydrate (Cat. No. S7670, Sigma, Belgium)
11. 1 M hydrochloric acid (Cat. No. 1.00316.1000, Merck, The Netherlands). Be careful, this fluid is corrosive.
12. O-phenylene diamine diHCl (Cat. No. 1953791, MP-Biomedicals, USA), this substance is light sensitive, store in the dark at 4°C. Be careful, this substance is very toxic.
13. 3% H_2O_2 solution (Cat. No. 16407071, Added Pharma, The Netherlands)
14. 3 M sulfuric acid (Cat. No. 1.00716.1000, Merck, The Netherlands). Be careful, this fluid is corrosive.

2.3. Histological Analysis: Hematoxylin/ Eosin Staining

1. Xylene (Cat. No. 1.08685.1000, Merck, The Netherlands). Be careful, this chemical is harmful.
2. 100% Ethanol (Cat. No. 1.00986.1000, Merck, The Netherlands) and diluted to 96% and 70%. Be careful, this chemical is highly flammable.
3. Mayer's hematoxylin solution (MHS16, Sigma, Belgium). Be careful, this chemical is an irritant.
4. Aqueous eosin Y solution (HT110-2-16, Sigma, Belgium).
5. Glacial acetic acid (Cat. No. 1.00056.2500, Merck, The Netherlands). Be careful, this chemical is corrosive.
6. Entellan (Cat. No. 1.07961.0500, Merck, The Netherlands). Be careful, this chemical is harmful.

3. Methods

In vitro experiments can be used to study the tissue response to load application without interference by other external factors, since several experimental conditions, such as temperature and

humidity, can be controlled. In this protocol, commercially available EpiDerm cultures were used. These epidermal equivalents provide a representative model to evaluate the release of biochemical markers, since the general morphology of the EpiDerm cultures is comparable to that of normal human epidermis. The load was applied to the EpiDerm cultures using a custom-built loading device (developed at the University of Technology, Eindhoven (6)), which was developed in such a way that the cultures could be maintained under sterile conditions during the experiments. The cytokine values were measured using an enzyme-linked immunosorbent assay (developed at the VU university medical centre, Amsterdam, adapted from the protocols of Sanquin (The Netherlands) and R&D systems (United Kingdom)).

3.1. Mechanical Loading of the EpiDerm Cultures

Day 1

1. Upon receipt, the EpiDerm cultures were transferred to 6-well plates under sterile conditions (*see* **Notes 1 and 2**). All wells were filled with 900 µl hydrocortisone free maintenance medium. The cultures were incubated overnight at 37°C and 5% CO_2.

Day 2

2. Add 900 µl fresh maintenance medium to all wells of the plates that will be used in the experiments. Incubate this medium at 37°C.
3. After overnight incubation, transfer each EpiDerm culture into a well with medium at 37°C using sterile forceps and incubate the plates.
4. Place a filled 6-well plate in the bottom plate of the loading device and position the cultures in the middle of the device (**Fig. 23.2a**). Place the positioning rings on top of the cultures to position them exactly in the center of the well (**Fig. 23.2b**).
5. Place a lid containing six holes for the indenters above the cultures in the loading device (**Fig. 23.2c**).
6. Put the indenters with the additional weights on top of the samples (**Fig. 23.2d**). Be careful that the indenters do not fall on top of the cultures to prevent impact loading (*see* **Note 3**).
7. Place the outer cover over the loading device (**Fig. 23.2e**) and place air filters on the lid.
8. Place the loading devices in an incubator at 37°C and 5% CO_2 and set the timer.
9. After loading time is completed, mix the medium with a pipette and make sure that all medium beneath the culture insert is also mixed.
10. Collect the medium and store it in tubes at –80°C prior to further analysis of the cytokines (*see* **Note 4**).

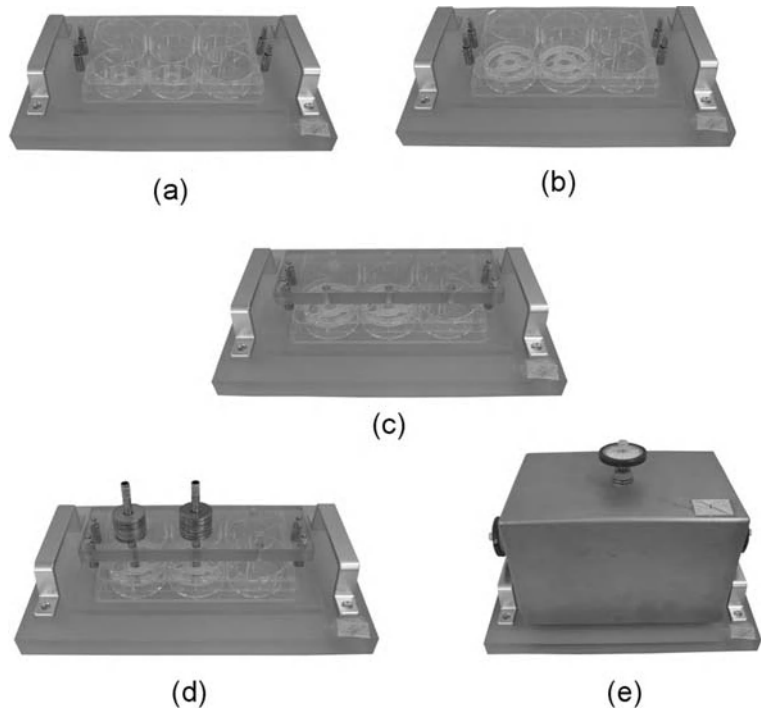

Fig. 23.2. The assembling of the loading device. A 6-well plate is placed in the bottom plate and the EpiDerm cultures are transferred into the wells **(a)**. Accordingly, the positioning rings are placed on top of the cultures **(b)** after which the lid is positioned on top of that **(c)**. The indenters are placed in the holes of the lid, atop the cultures **(d)**. Finally, the total set-up is covered with a lid on which air filters are placed **(e)**.

11. Wash the EpiDerm cultures twice in PBS and place in 4% formaldehyde fixative for at least 24 hours, after which they can be placed in the tissue processor. Accordingly, paraffin slices are cut at 5 μm for histological analysis.

3.2. ELISA for Determination of IL-1α, IL-1RA, and IL-8

Day 1

1. Prepare buffer A: dissolve 1.41 g $Na_2CO_3 \cdot 10H_2O$ in 50 ml MQ water.
2. Prepare buffer B: dissolve 0.42 g $NaHCO_3$ in 50 ml MQ water.
3. Prepare for each cytokine 10 ml coating buffer, consisting of 0.1 M sodium carbonate/bicarbonate (pH 9.6). For this, add 2.8 ml of buffer A to 7.2 ml of buffer B. Store both buffer A and buffer B at 4°C.
4. Prepare three coating solutions by adding each monoclonal antibody (25 μl IL-1RA stock1, 40 μl IL-1α stock1, or 50 μl IL-8 stock1) to 10 ml coating buffer, resulting in final concentrations of 2.0 μg/ml IL-1α and 2.5 μg/ml IL-1RA (For IL-8, *see* **Note 5**).

5. Pipette 100 µl of the appropriate coating solution into each well of a 96-well plate (coat at least two lanes per plate for standard solutions and one lane per plate for dilution buffer). Incubate the plates overnight at room temperature in the dark. Cover the plates with parafilm.
6. Prepare 5 l wash buffer of PBS with 0.005% Tween.

Day 2

7. Prepare 500 ml block buffer of PBS with 0.5% BSA, store this buffer at 4°C.
8. After overnight incubation: shake off excess liquid and be sure that the plates are as dry as possible. Pipette 140 µl block buffer to each well and cover the plates with parafilm. Incubate 1 hour at room temperature in the dark (longer incubation times will not affect results).
9. Prepare 150 ml dilution buffer of PBS with 0.5% BSA and 0.005% Tween, store this buffer at 4°C.
10. Dilute the appropriate recombinant antibody in dilution buffer to obtain the standard solutions: add 20 µl IL-1RA stock2 to 480 µl dilution buffer, add 5 µl IL-1α stock2 to 495 µl dilution buffer, and add 75 µl IL-8 stock2 to 425 µl dilution buffer. Prepare serial dilutions of these solutions to obtain the following series of standard solutions:

    IL-1RA: 4000-2000-1000-500-250-125-62.5-31.25 pg/ml

    IL-1α: 1000-500-250-125-62.5-31.25-15.93-7.81 pg/ml

    IL-8: 1500-750-375-187.5-93.75-46.88-23.44-11.72 pg/ml
11. Prepare sample dilutions to a concentration that is in the regime of the standard curve. If the expected concentration is unknown, measure sample dilutions of 1, 10, and 100 times.
12. After incubation is complete: shake off excess liquid and dry the plates. Add 100 µl of the samples to each well off the right plate. Add the standard solutions to two columns of the 96-well plate to have a duplicate measurement of the standard curve. Add 100 µl dilution buffer to a column as negative control. Incubate the plates 1 hour at room temperature in the dark. Cover the plates with parafilm.
13. Dilute the appropriate biotinylated antibody in dilution buffer. For this, add 40 µl IL-1RA stock3 to 10 ml dilution buffer, add 2.5 µl IL-1α stock3 to 10 ml dilution buffer and add 100 µl IL-8 stock3 to 10 ml dilution buffer, resulting in final concentrations of 12.5 ng/ml IL-1α and 200 ng/ml IL-1RA (for IL-8, *see* **Note 5**).
14. After incubations are complete, wash plates four times with wash buffer. Shake off excess liquid, dry the plates, and add

100 µl of the appropriate biotinylated antibody to each well of the right plate. Cover the plates and incubate 1 hour at room temperature in the dark.

15. Prepare conjugate solution by adding 5 µl Streptavidine poly-HRP stock (1 mg/ml) to 50 ml dilution buffer.
16. After incubations are complete, wash the plates four times with wash buffer. Shake off excess liquid and be sure that the plates are as dry as possible. Add 100 µl conjugate solution to each well. Cover the plates with parafilm and incubate 30 minutes at room temperature in the dark.
17. Prepare 0.11 M acetate buffer and adjust the pH by the addition of 1 M HCl to pH 5.5. Store this buffer at 4°C. Do not store the acetate buffer longer than 2 weeks.
18. Prepare substrate solution by adding 0.010 g O-phenylene diamine diHCl to 50 ml acetate buffer and add 500 µl 3% H_2O_2 (prepare this substrate solution always fresh).
19. Prepare stop solution by adding 40 ml 3 M H_2SO_4 to 20 ml H_2O. Store this solution at room temperature.
20. After incubations are complete, wash plates four times with wash buffer. Shake off excess liquid and be sure that the plates are as dry as possible. Add 100 µl substrate solution to each well and incubate until the 5th standard for IL-1α and IL-8 and the 2nd standard for IL-1RA from the highest concentration begin to develop yellow color (*see* **Note 6**).
21. Stop the reaction by adding 50 µl stop solution to each well. Color will turn orange.
22. Measure optical density at 490 nm by use of the ELISA reader and use the standard curves to determine sample concentrations.

3.3. Histological Analysis: Hematoxylin/ Eosin Staining

1. Dewax and hydrate the tissue slides by placing them subsequently in the following solutions:
   a. Two times 5 minutes in xylene
   b. Three times 2 minutes in 100% ethanol
   c. One time 2 minutes in 96% ethanol
   d. One time 2 minutes in 70% ethanol
   e. One time 2 minutes in MilliQ water
2. Stain the slides by placing them 10 minutes in Mayer's hematoxylin solution.
3. Wash the slides in slow running tap water for 5 minutes.
4. Add 1 ml glacial acetic acid to 200 ml eosin Y solution to acidify this solution.
5. Stain in acidified aqueous eosin Y solution for 3 minutes.
6. Dehydrate, mount, and cover the sections

a. 10 dips in 70% ethanol
b. 10 dips in 96% ethanol
c. Three times 10 dips in 100% ethanol
d. Two times 3 minutes xylene
e. Put some drops Entellan on top of the sections and cover the slides with coverslip and let them dry in the fume hood (*see* **Note 7**).
f. An example of H&E colored EpiDerm culture is given in **Fig. 23.3**.

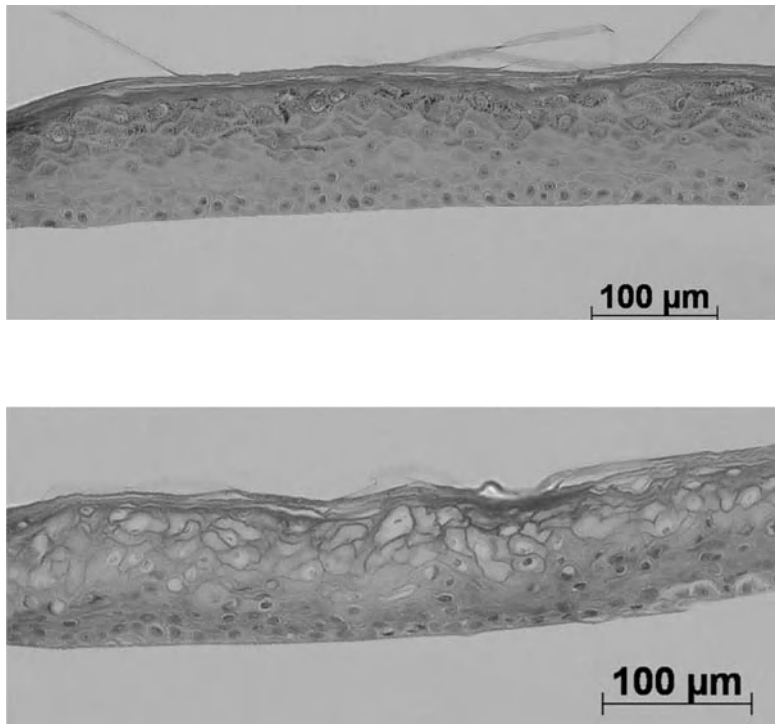

Fig. 23.3. Tissue sections colored with hematoxylin/eosin staining. In the left picture, a control tissue sample is depicted whereas the tissue in the right picture is loaded with 150 mmHg for 24 hours.

4. Notes

1. The EpiDerm cultures should be treated with care and always under sterile conditions. The cytokine release that is investigated is part of the initial steps of an inflammatory response. The effect of load application on cytokine release is investigated and therefore, cytokine release due to other infections should be omitted.
2. There is some variability between different batches of EpiDerm cultures. It is therefore important that at least three different batches are tested to take this into account.

3. Placing the indenters at the top surface of the EpiDerm culture should be performed very carefully. If the indenter falls on top of the cultures, this might result in impact loading, which probably induces additional damage and thereby additional release of cytokines.
4. The cytokine release from the cultured epidermis in these experiments is determined in the medium that surrounded the EpiDerm culture. By developing numerical models that describe this loading experiment, more insight into the cytokine concentrations inside the culture can be obtained.
5. The concentrations of the monoclonal antibody and biotinylated antibody for IL-8 might vary among batches and are therefore not explicitly given. They can be used after dilution as indicated in the protocol.
6. The color reaction of the Elisa should be stopped when the 2nd or 5th well in the standard column changes color, depending on the cytokine that is measured. It is very important to prevent coloring in the column with dilution buffer. If that column does also color, there is too much background coloring and the results of the assay should not be trusted.
7. After H&E staining, the slides have to be covered by a coverslip. Be careful that in this procedure formation of air bubbles between the slides and the coverslip is avoided.

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Chapter 24

Three-Dimensional Human Tissue Models of Wounded Skin

Christophe Egles, Jonathan A. Garlick, and Yulia Shamis

Abstract

Human skin equivalents (HSEs) are in vitro tissues in which a fully differentiated, stratified squamous epithelium is grown at an air–liquid interface on a Type I collagen gel harboring human dermal fibroblasts. HSEs now provide experimental human tissue models to study factors that direct re-epithelialization and epithelial–mesenchymal cross-talk following wounding. This chapter describes the fabrication of HSEs from human keratinocytes and fibroblasts and how HSEs can be modified to characterize the response of the human epithelium during wound repair. The protocols outlined first describe techniques for the generation of human tissues that closely approximate the architectural features, differentiation, and growth of human skin. This will be followed by a description of a protocol that enables HSEs to be adapted to monitor their response following wounding. These engineered human tissues provide powerful tools to study biological process in tissues that mimic the healing of human skin and of the epithelial tissue.

Key words: Organotypic culture, Three-Dimensional model, Human skin, Wound repair, Fibroblasts.

1. Introduction

1.1. Fabrication of Three-Dimensional Model of Human Skin

The development and application of tissue-engineered models that mimic human skin, known as human skin equivalents (HSEs), provide in vivo-like tissues to study epidermal biology (1, 2). We first describe methods for constructing models of human epidermis that mimic the three-dimensional tissue architecture and behavior of normal human skin. Construction of a multi-layered epithelium is accomplished by growing skin keratinocytes on the surface of a Type I collagen gel that is populated with dermal fibroblasts (**Fig. 24.1**). Following several days during which tissue constructs are immersed in medium, HSEs are grown at an air–liquid interface, so that tissues

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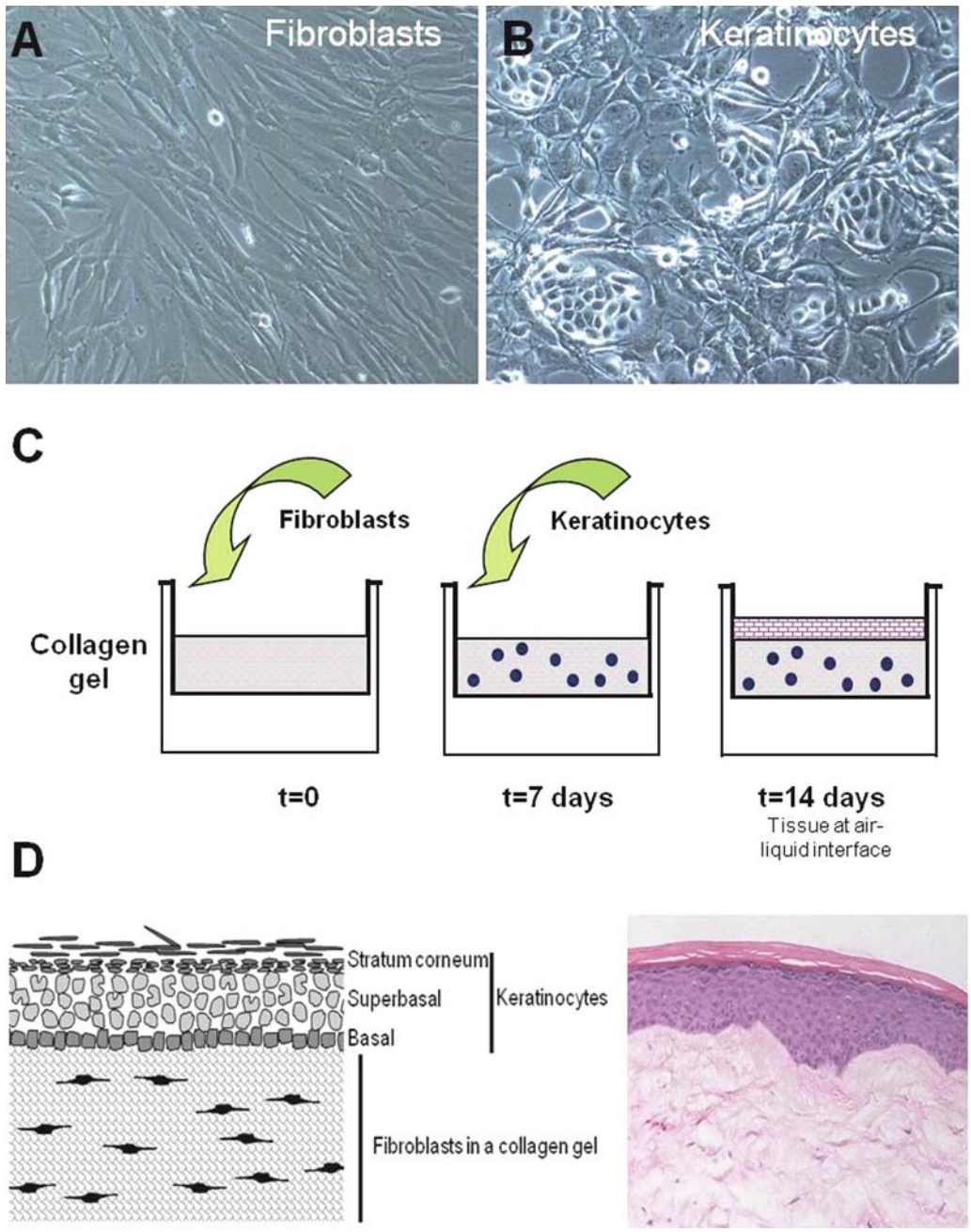

Fig. 24.1. The three-dimensional culture technique. Cultures of human dermal fibroblasts **(A)** and human keratinocytes **(B)** are cultivated several days prior to the construction of the HSEs. Fibroblasts should be incorporated into collagen gels when they have a high number at dividing cells. To achieve this, cells are grown to confluence **(A)**, split at 9:10 ratio and used the following day to construct collagen gels. To limit the number of differentiated cells and to increase cellular growth fraction, colonies should be relatively small in size **(B)**. To fabricate tissues **(C)** fibroblasts are mixed with type I collagen and allowed to contract for 1 week prior to seeding keratinocytes. After culturing the HSEs at the air–liquid interface, a fully stratified epidermis is formed demonstrating all layers present in normal human epithelium **(D)**.

can fully recapitulate the in vivo-like morphologic and biochemical processes of human skin. These HSEs are amenable to manipulation of medium, substrate conditions and cellular constituents to create novel microenvironments that mimic a variety of wound conditions in cutaneous tissues.

1.2. Fabrication of Wound Healing Model

The second part of this chapter will describe how HSEs can be adapted to characterize their response during wound re-epithelialization. This protocol describes the fabrication of HSEs that are wounded and undergo re-epithelialization as keratinocytes reestablish epithelial integrity (**Fig. 24.2**). In vitro studies of wound re-epithelialization have often been limited by their inability to simulate wound repair as it occurs in humans. For example, "scratch" wound models using two-dimensional monolayer, keratinocyte cultures demonstrate very limited stratification, partial differentiation, and hyperproliferative growth. These cultures are helpful in studying keratinocyte migration in response to wounding, but have been of limited use in studying the complex nature of keratinocyte response and epithelial–mesenchymal cross-talk during wound repair, as monolayer cultures do not provide the tissue complexity needed to study the in vivo wound response.

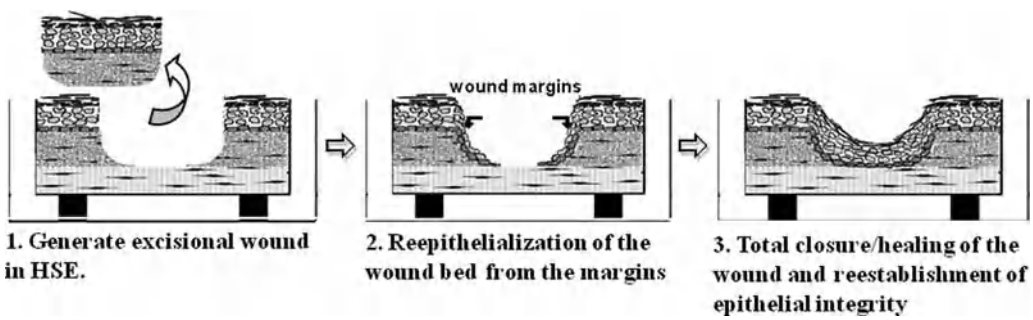

Fig. 24.2. Diagram of the wounding of skin-equivalent cultures. (1) An excisional wound is created by removing the central portion of an HSE. (2) This tissue is layered onto a freshly contracted Type I collagen gel and migrating keratinocytes from the edge of the wound move across the wound bed to repopulate it. (3) Complete re-epithelialization leads to reestablishment of epithelial integrity as keratinocytes reconstitute a fully stratified epithelium.

HSEs can be adapted to study wound repair in human epithelium in a manner that simulates the chronology of events that occur during re-epithelialization in human skin (3, 4). These HSEs enable direct determination of the phenotypic response parameters of a wounded epithelium including cell proliferation, migration, differentiation, growth-factor response, and protease expression of epithelial and stromal cells. This wound repair model demonstrates the utility of HSEs in studying phenotypic responses that are characteristic of the switch from a normal to a regenerative epithelium upon wound re-epithelialization. This protocol

describes construction of tissues that allow monitoring of the response of HSEs during re-epithelialization of wounded human skin, from the onset of keratinocyte activation and ending upon restoration of epithelial integrity. Using these protocols, HSEs are fabricated as described above and are wounded 7 days after keratinocytes are seeded onto the contracted collagen gel. One week before wounding these tissues, an additional collagen gel is fabricated that serves as a substrate onto which the wounded tissue will be transferred to monitor re-epithelialization.

1.3. Modification of Fibroblasts and Incorporation into Collagen Gels

Specialized adaptations of this HSE model that allow direct study of the role of modified fibroblasts and stromal substrate are also described. In fact, HSEs allow multiple manipulations of several components of the tissue fabrication process: the modification of the composition of the culture medium, the nature of the scaffold material, and the type of cells incorporated in the HSEs. As an example, in this protocol we describe the incorporation of phenotypically modified dermal fibroblasts and how this modification was linked to the response of wounded HSEs.

We have previously published a study using intact and wounded HSEs to test the capacity of fibroblasts passaged extensively or exposed to ECM composed of either normal collagen (NC) or denaturated collagen (DC) to modulate the phenotype of these tissues (5). We have demonstrated that dermal fibroblasts grown after extended passage on DC and incorporated into HSEs were able to modify the properties of the adjacent surface epithelium by increasing the proliferation of basal keratinocytes and significantly shortened the time needed for wounded HSEs to undergo complete re-epithelialization (**Fig. 24.3**). This study demonstrated that cell modifications mediated by cross-talk between dermal fibroblasts in the ECM microenvironment hold tremendous potential for novel therapeutic applications and for understanding mechanisms through which paracrine interactions between fibroblasts and keratinocytes may accelerate wound repair and reestablish tissue homeostasis.

2. Materials

2.1. Medium Components

1. Water (*see* **Note 1**)
2. 0.5 M EDTA, pH 8.0 (Invitrogen, Carlsbad, CA, cat.# 15575)
3. PBS (Invitrogen, Carlsbad, CA, cat.#14190)
4. 0.25% Trypsin (Invitrogen, Carlsbad, CA, cat.# 15050)
5. 5 mM EDTA; add 5 ml of 0.5 M EDTA to 500 ml PBS
6. 10% EDTA/PBS; mix 50 ml of 5 mM EDTA with 450 ml PBS

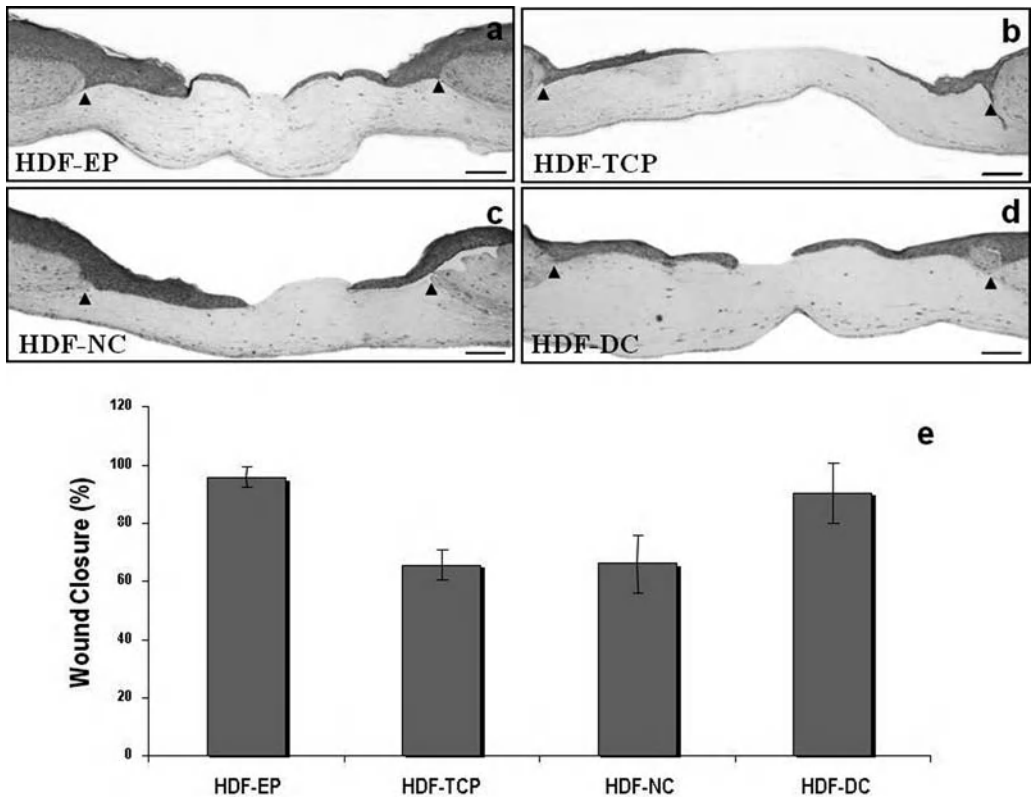

Fig. 24.3. Fibroblasts modified by growth on denatured collagen accelerate the rate of re-epithelialization of wounded HSEs.

Panels **(a–d)** illustrate the morphology of skin-equivalent cultures fabricated using human dermal fibroblasts early passaged (less than 7 passages on plastic culture plates) named HDF-EP cells **(a)** or passaged extensively (more than 15 passages) on plastic culture plates HDF-TCP **(b)**, on plastic plates coated with normal collagen HDF-NC **(c)**, and on plastic plates coated with heat-denaturated collagen HDF-DC **(d)** cells 48 hours after wounding. Arrows demarcate the initial wound edges. The progress of the wound re-epithelialization was determined by comparing the cultures immediately after wounding (time zero) with those seen 48 hours later for each condition expressed graphically as the percentage of wound closure **(e)**. Bar = 100 μm.

7. 0.1% Trypsin – mix 50 ml 0.25% trypsin with 75 ml PBS
8. 50% Trypsin/EDTA – mix 50 ml of 0.1% trypsin with 50 ml of 5 mM EDTA
9. 100× HEPES (800 mM) – dissolve 47.24 g in 250 ml ddH_2O, store at –20°C for up to 1 year (Sigma, St. Louis, MO, cat.# H-4034)
10. 100× Adenine (18 mM) – dissolve 0.972 g in 2.4 ml 4 N NaOH, q.s. to 400 ml with ddH_2O, store at –20°C for up to 1 year (MP biomedicals, Solon, OH, cat.# 100190)
11. 500× Hydrocortisone (0.25 mg/ml) – dissolve 0.0538 g in 200 ml ddH_2O, store at –20°C for up to 1 year (Sigma, St. Louis, MO, cat.# H-4881)

12. 1,000 × Cholera toxin (10^{-7} M) – dissolve 9 ng/ml in ddH_2O, store at –20°C for up to 1 year (Sigma, St. Louis, MO, cat.# C-8052)
13. 1,000 × EGF (10 μg/ml) – dissolve 10 μg/ml in 0.1% BSA, store at –20°C for up to 1 year (Austral Biological, San Ramon, CA, cat.# GF-010-9)
14. 1,000 × Insulin (5 mg/ml) – dissolve 50 mg in 10 ml of 0.005 N HCl, store at –20°C for up to 1 year (Sigma, St. Louis, MO, cat.# I-2643)
15. 10 nM triiodothyronine (T3) – add 1 ml T3 to 99 ml ddH_2O for 500 × stock (Sigma, cat # T-5516)
16. 2 μM progesterone – dissolve 1 mg in 1 ml absolute ethanol, add 14.7 ml ddH_2O, dilute 1 ml in 100 ml DMEM for 1,000 × stock (Sigma, St. Louis, MO, cat.# P-8783)
17. Chelated BCS – Chelate serum by adding 10 g CHELEX 100 (Sigma, St. Louis, MO, cat.# C-7901) to 100 ml serum and stirring for 3 h at 4°C, then filter through Whatman paper, then through a sterile filter.
18. Transferrin (5 mg/ml) – (BioSource cat. # 352-020, 200 ml)
19. 500 × PES – contains O-phosphorylethanolamine (0.01 mM final), ethanolamine (10 μM final), and selenium (10 μg/ml final) (BioSource, cat.# P02-45-100)

2.1.1. 010 medium

1. 43 g DME powder (JRH Biosciences, this is a special order medium base that is prepared in bulk) contains no glucose and no $CaCl_2$
2. 5 L ddH_2O
3. 0.5 g $MgSO_4$
4. 18.5 g $NaHCO_3$

2.1.2. Keratinocyte Culture Medium

1. 338 ml DME medium (Invitrogen, Carlsbad, CA, cat.# 11885)
2. 112 ml F12 medium (Invitrogen, Carlsbad, CA, cat.# 11765)
3. 25 ml FBS (Hyclone, Logan, UT, cat.# SH30071.03) (5% final)
4. 5 ml 18 mM adenine (0.18 mM final)
5. 3.4 ml 100 × penicillin/streptomycin (Invitrogen, Carlsbad, CA, cat.# 15140-122)
6. 5 ml 800 mM HEPES (8 mM final)
7. 1 ml 0.25 mg/ml hydrocortisone (0.5 μg/ml final)
8. 0.5 ml 10^{-7} M cholera toxin (10^{-10} M final) (*see* **Note 2**)

9. 0.5 ml 10 μg/ml EGF (10 ng/ml final)
10. 0.5 ml 5 mg/ml insulin (5 μg/ml final)
    Store up to 2 weeks at 4°C.

2.1.3. Fibroblast Culture Medium

1. 500 ml DME medium (Invitrogen, Carlsbad, CA, cat.#11885)
2. 55.6 ml FBS (Hyclone, Logan, UT, cat.# SH30071.03) (10% final)
3. 5.6 ml 800 mM HEPES (8 mM final)
4. 3.4 ml 100 × Penicillin/Streptomycin (Invitrogen, Carlsbad, CA, cat.#15140-122)
   Store up to 2 weeks at 4°C.

2.1.4. 3T3 Medium

1. 500 ml DME medium (Invitrogen, Carlsbad, CA, cat.# 11885)
2. 55.6 ml Bovine Calf Serum (Hyclone, Logan, UT, cat.# SH30072.03) (10% final)
3. 3.4 ml 100 × Penicillin/Streptomycin (Invitrogen, Carlsbad, CA, cat.#15140-122)

2.2. Fabrication of Tridimensional Tissues

2.2.1. Human Skin Equivalents

1. 6-well tissue culture tray with 3 μm porous polycarbonate membrane inserts (Organogenesis, Canton, MA, cat.# MS-10-305)
2. Bovine tendon Type I collagen (Organogenesis, cat.# 200-055)
3. Human foreskin fibroblasts
4. Fibroblast culture medium (see Reagents and Solutions)
5. Trypsin/ethylenediaminetetraacetic acid (EDTA)
6. Human neonatal foreskin keratinocytes
7. Phosphate-buffered saline (PBS)
8. PBS/EDTA
9. Centrifuge (1,000–2,000 RPM range)

2.2.2. Fabrication of Three-Dimensional Wound Healing Model of Human Skin

1. 6-well tissue culture tray with 3 μm porous polycarbonate membrane inserts (Organogenesis, Canton, MA, cat.# MS-10-305)
2. 14 cm stainless steel dermatological punch (Delasco, Council Bluffs, IA, cat.# KP-14)
3. Dental mirror for transfer of wounded culture
4. Sterile scalpel with #22 blade

3. Methods

3.1. Fabrication of Collagen Matrix with Dermal Fibroblasts

1. Culture human foreskin fibroblasts (HFFs) in monolayer culture so that they are almost confluent one day before incorporation into the collagen.
2. The day before incorporation, passage fibroblasts at a 9:10 split ratio so they will be mitotically active the next day when incorporated into the collagen gel.
3. Passaging cells from a confluent plate ensures a higher fraction of actively dividing cells upon incorporation into collagen gels. A 9:10 passage is performed by resuspending the trypsinized cells in 10 ml of medium and adding 9 ml of the cell suspension to a new plate.
4. The following day, the collagen matrix is prepared by fabricating successive layers of acellular and cellular collagen onto the polycarbonate membrane. Prepare the acellular collagen as a mixture that is cooled on ice to prevent premature gelation (*see* **Note 3**). To do this, pipettes should be chilled at –20°C for 15 min before use to prevent warming of collagen when it is mixed. Avoid air bubbles when mixing. Collagen should be a straw-yellow to light pink color to ensure optimal gelation. If the color is bright yellow, add a single drop of sodium bicarbonate and triturate until a straw-yellow color is seen.
5. Add 1 ml of acellular collagen to each insert. Ensure that the matrix coats the entire bottom surface of the insert and allow it to gel at room temperature for 20 min. Do not move the tray while it is undergoing gelation. The color will turn pink when the collagen has fully gelled.
6. Trypsinize, count, and resuspend the fibroblasts to a final concentration of 3×10^5 cells/ml. A total of 5×10^5 fibroblasts will be used per 6-well tray.
7. Prepare the cellular collagen as a mixture that is cooled on ice (*see* **Note 3**). Fibroblasts should be added last after collagen has been neutralized so that the cells will not be damaged by the alkaline pH that exists before neutralization. Resuspend the cell/collagen suspension by gentle trituration to evenly incorporate fibroblasts into the collagen gel.
8. Gently triturate the cellular matrix and add 3 ml into each insert on top of the gelled acellular collagen matrix. Gently transfer the mixture to the incubator for 30 min.
9. When the cellular matrix has turned pink and is completely gelled (usually less than 30 min), feed the gels with 12 ml of fibroblast medium by adding 10 ml of medium to the well around the insert and 2 ml of medium directly onto the insert.

10. Gels are then incubated for 5–7 days to allow complete gel contraction.
11. During the first few days, the sides of the gel contract and will form a plateau in the center. Gels are stable between 5 and 10 days after initial construction.

3.2. Addition of Keratinocytes to the Surface of Contracted Collagen Gels

1. Normal human keratinocytes are cultured on a feeder layer of mitotically inactivated mouse 3T3 fibroblasts. Keratinocytes should be grown to no more than 50% confluence to minimize the number of differentiated cells seeded onto the collagen gel. Alternatively, keratinocytes can be grown in monolayer culture in low calcium and serum-free medium (*see* **Note 4**).
2. Remove the 3T3 feeder cells from the culture by incubating the plates in PBS/EDTA for 5 min at 37°C. 3T3's can then be displaced by gentle pipetting so that keratinocytes will remain attached. It is important not to allow the cultures to incubate for an excessive time in PBS/EDTA, as the keratinocytes may detach from the plate as well. As soon as the 3T3's have begun to detach, replace the PBS/EDTA with PBS, gently rinse the plate three times with PBS until all 3T3's have been completely removed. PBS is then removed, leaving only keratinocyte colonies attached to the plate.
3. Trypsinize the keratinocytes with trypsin/EDTA (0.05%) for 5 min at 37°C to obtain a single cell suspension. Remove the detached cells into a 15 ml tube containing keratinocyte medium (to neutralize the trypsin) and count them. The desired number of cells is dispensed into a 15 ml tube from the tube with the trypsinized cells. Cells are then centrifuged 2,000 $\times$ *g* for 5 min and resuspended in a volume so that a total of 5×10^5 keratinocytes can be used per insert.
4. Remove all fibroblast medium from the trays with the contracted collagen 20 min before seeding keratinocytes so that keratinocytes can be seeded onto a moist collagen gel. Keratinocytes should be seeded directly onto the contracted collagen gels in an aliquot of 50 µl containing 5×10^5 cells. To modify the nature of the substrate on which keratinocytes are seeded, de-epidermalized dermis or coated polycarbonate inserts can be applied directly on top of the contracted collagen gels at this point (*see* **Note 5**).
5. Resuspend keratinocytes in a volume of Epidermalization I medium to a final concentration of 500,000 cells/50 µl. Carefully add the 50 µl of the cell suspension to the center

of the contracted collagen gel (or onto the center of the intervening substrate placed on the collagen gel). Do not move the tray for 15 min to allow the keratinocytes to attach. Constructs are then incubated at 37°C for 30–60 min. without any medium to allow the keratinocytes to fully adhere.

6. Add 12 ml of Epidermalization I medium to each insert by adding 10 ml to the bottom of the well and 2 ml gently into the insert on top of the keratinocytes. Incubate at 37°C.
7. Cultures are fed with medium every 2 days as follows:
   a) Epidermalization I medium – 12 ml per well for the first 2 days.
   b) Epidermalization II medium – 12 ml per well for the next 2 days.
   c) Cornification medium – At this point, cultures are raised to the air–liquid interface by adding 7 ml per well to the bottom of the well so that the insert just contacts the medium. Aspirate medium from the inside of the insert so that tissues can be grown at the air–liquid interface. Additional feedings with Cornification medium are done every 2 days until termination of the experiment.

3.3. Fabrication of Three-Dimensional Wound Healing Model of Human Skin

1. HSEs to be wounded are first generated as described above. This protocol requires that an HSE with normal, primary keratinocytes and a second contracted collagen gel (onto which the wounded epithelium will be transferred) will first be simultaneously constructed.

3.3.1. Tissue Wounding

2. Aspirate all medium from the HSE after 7–10 days of culture. Remove the insert from the tray and place it upside down in a sterile dish. Using a scalpel, cut away the insert membrane, and place the culture in a sterile dish right side up.
3. Trim the culture with the scalpel by cutting around the raised, mesa-like region to remove the part of the collagen gel not covered by keratinocytes. This will facilitate the removal and transfer of the wounded tissue from the membrane.
4. Cultures can be wounded with either an incisional or excisional wound. An incisional wound can be generated by incising tissues with a scalpel in a way that will allow the wound edges to be separated to generate an elliptical wound.
5. An excisional wound can be generated using an elliptical dermatological punch that completely penetrates the center of the tissue through the epidermis, collagen, and membrane. The excised tissue can be fixed and preserved for H&E staining in 10% formalin.

6. Use forceps to gently lift the edge of the wounded tissue by separating the collagen gel from the membrane. Drag the tissue onto a dental mirror while leaving the membrane behind. The transfer may be easier if the mirror is moistened with medium.
7. Unfold any wrinkles in the culture by gently moving the tissue back and forth on the mirror using the forceps. Once the culture is smooth, pull one side of the culture slightly over the edge of the mirror.
8. Carry the mirror directly over the second contracted collagen matrix so that the edge of the mirror and wounded tissue are in contact with the matrix. Slide the tissue onto the second collagen gel by teasing it gently with a closed forceps as the mirror is slowly pulled away, leaving the culture on the contracted collagen gel.
9. Using the forceps, tease apart the tissue wounded by incision to create an elliptical space that should be 2–3 mm at its greatest width. Smooth the tissue with the forceps to ensure that it is completely free of any folds or wrinkles.
10. Maintain the tissue at an air–liquid interface by adding 8 ml of Epidermalization II medium beneath the insert during re-epithelialization, change the medium every 2 days until the end of the experiment.

3.3.2. Modification of Fibroblasts and Incorporation into Collagen Gels

1. Phenotypically modified Human dermal fibroblasts. Cells were seeded at a density of 5×10^4 cells/ml and cultures were sequentially passaged when cell density reached confluence. Passage 8 HDFs (EP HDF) were maintained for 12 additional passages on tissue culture plastic (HDF-TCP), heated-denatured collagen films (HDF-DC), or native collagen films (HDF-NC).
2. Type I collagen (Roche, cat. #1179179) was dissolved at 5 mg/ml in 0.1% acetic acid and denatured by incubation at 50°C for 12 hours. These conditions were chosen based on the complete denaturation of collagen following thermal transition at 45°C.
3. To prepare collagen films for cell passage, 1.5 ml of collagen solution (0.5 mg/ml) was added to 35-mm tissue culture plates (Corning) and dried under vacuum.

3.4. Tissue Harvesting and Embedding for Morphological and Immunohistochemical Analysis

1. Remove medium from inserts and gently rinse tissues twice in PBS.
2. Cut away the insert membrane from the plastic insert at its base using a scalpel.

3. Bisect the culture and place one-half in a tissue processing cassette and immediately immerse in 10% formalin. Tissues are very thin and thus only require a short fixation (1 hour) before paraffin processing.
4. The other half of the tissue should be placed in a 2 M sucrose solution prepared in water. Tissue should be soaked in sucrose at 4°C for at least 1 hour, but for not more than 24 hours. Sucrose replaces water in the hydrated collagen gel and protects against freezing damage during embedding and processing.
5. To embed sucrose-soaked tissue for frozen preservation, make a small mold with aluminum foil using the cap or bottom of a small bottle (roughly 2 cm in diameter). Fill three-fourths of the mold with embedding medium.
6. Gently remove the tissue from the sucrose using narrow-tipped forceps, making sure to keep the tissue on the nylon insert membrane. Gently touch the membrane side of the tissue to a kimwipe to remove excess sucrose.
7. Place the tissue in the embedding medium and allow the tissue to soak for 20–30 min at room temperature.
8. Place a metal rack inside a Styrofoam box and fill it with liquid nitrogen to a height just under the top of the metal rack. Place the aluminum foil mold on top of the rack and stand the tissue inside to an upright position. The liquid nitrogen vapors will freeze the embedding medium and tissue in about 5 min. The tissue can then be stored at –80°C.
9. For histological analysis of wounded HSE: Process the tissue as described above, making sure to bisect the tissue perpendicular to the long axis of the wound (i.e., along the greatest width of the wound). Mount the tissue for sectioning en face so that the greatest width of the wound is sectioned first. It is essential to capture the first few sections as these will be the most informative sections of the wound.

3.5. Anticipated Results

Several points regarding keratinocyte behavior in HSEs should be mentioned. The first concerns the length of time during which cultures can be maintained at the air–liquid interface. In our experience, cultures can be kept at this interface for up to 17 days. After this time, the surface layer of the epithelium becomes excessively thickened due to a failure to desquamate. As a result, lower layers of the epithelium become compressed and the longevity of cultures is limited. A second and related issue concerns the growth potential of keratinocytes in HSEs. While HSEs demonstrate a basal level of proliferation that is greater than that of human skin, it has been shown that these cultures have a tremendous potential for cell growth and are very responsive to external growth stimuli.

It should be kept in mind that although keratinocytes grown in HSEs share many morphologic and biochemical features with in vivo skin, there are differences in tissue phenotype. For example, integrin receptors not normally expressed in skin may be constitutively expressed in keratinocytes grown in HSEs. This may be the result of HSEs being somewhat deficient in barrier function.

3.6. Time Considerations

Construction of HSEs requires approximately 3–4 weeks from the time HFF cells are seeded in monolayer culture until three-dimensional tissues are fully mature. HFF cells should be confluent 2 days before construction of the collagen gels. At that time, cells should be passaged at a 9:10 ratio to provide cells with a growth stimulus before incorporation into collagen gels. Complete contraction of the collagen gels requires 7–10 days, during which time the human keratinocyte cell cultures are initiated and expanded. The keratinocytes will need another week of culture before the HSEs are ready for wounding.

4. Notes

1. Milli-Q water should be used for preparation of all medium and supplements, and all solutions should be filtered through a 0.22 μm filter for sterility.
2. Cholera toxin is very toxic. Use appropriate precautions when handling stock solutions.
3. Fabrication of the collagen gel requires that all components be kept on ice until the gel mixture is placed into the insert. This will ensure that collagen will not prematurely precipitate from these solutions. Plastic pipettes used for collagen should be chilled before use.

   The amounts listed are for a single 6-well tray scale accordingly for a smaller or larger number of inserts.

   0.6 ml 10 × MEM (Minimum essential medium with Earle's salts) (Cambrex, Walkersville, MD, cat.# 12-684F)

   54 μl 200 mM L-Glutamine (Invitrogen, Carlsbad, CA, cat.# 25030-081)

   0.68 ml FBS (Hyclone, Logan, UT, cat.# SH30071.01)

   187 μl 71.2 mg/ml $NaHCO_3$ (Cambrex, Walkersville, MD, cat.# 17-605E)

   5 ml Bovine Type I Collagen (Organogenesis, Canton, MA, cat.# 200-055) Fresh and used immediately. It should not be stored once mixed.

4. Keratinocyte proliferation and a high growth fraction are the most critical factors in the successful fabrication of HSEs. Most keratinocytes seeded onto HSE cultures will adhere to the connective substrate, but only replicating cells will grow after seeding. Keratinocytes that underwent a commitment to terminal differentiation while still in submerged culture will also attach to the substrate, but will not undergo further proliferation to form a well-stratified HSE. It is therefore important to grow keratinocytes so that a high growth fraction is present when monolayer cultures are seeded onto the contracted collagel gel of the HSEs. This can be accomplished by growing keratinocytes as small colonies at high clonal density in submerged cultures on 3T3 feeder layers, so that terminal differentiation will be minimized and the fraction of replicating cells will be maximized. Keratinocyte strains can be tested by screening them using a colony efficiency assay to determine those strains with the highest colony forming efficiency providing optimal morphologic differentiation and tissue architecture of HSEs.

   In addition, the protocols described can be modified to allow growth of epithelial tissues on a variety of connective tissue substrates. Each of these interfaces presents an ECM that can be tailored to answer specific experimental questions. For example, tissues grown on the de-epidermalized dermis serve as an interface on which the rapid assembly of structured basement membrane occurs and tissue morphology is optimized (1). Alternatively, cultures can be grown directly on polycarbonate membranes coated with specific ECM proteins to directly study the effect of these proteins on cellular phenotype in these tissues (6).

5. We have found some variability in the degree to which fibroblast strains support keratinocyte growth after their incorporation into collagen gels. It appears that fibroblast support of HSE organization and growth is directly related to the degree to which fibroblasts are able to contract the collagen gel. In general, fibroblast strains demonstrating more shrinkage of the collagen gel before adding keratinocytes are better able to support keratinocyte growth. This parameter may be used to screen fibroblast strains for optimal growth support when initiating HSEs.

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Chapter 25

In Vivo Transplantation of Genetically Modified Mouse Embryonic Epidermis

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Abstract

Situations in which epidermal mutant mice display early lethality after birth are rather frequent. This condition precludes any kind of analysis of adult or even newborn mice tissues. We propose the in vivo embryonic skin transplantation as an alternative to solve this problem. This method allows the generation of a stable epidermal tissue that mimics the specific mutant adult epidermis. It also reproduces the phenotypic consequences and susceptibility to tumorigenesis, enabling multiple studies. Moreover, different recipient mice can help in determining problems such as different inflammation processes or the contribution of dermal cells to the different phenotypes.

Key words: Embryonic epidermis/skin, Epidermal transplantation, Graft, Mouse.

1. Introduction

The genetically manipulated mouse strains lead to early lethality in many cases, which precludes the analysis of adult phenotypes in skin or tumor susceptibility. This can be solved if embryonic (17.5–18.5 days *postcoitus,* dpc) skin is transplanted onto recipient mice.

In this chapter we describe a skin grafting method that allows fetal epidermis isolated from 17.5 to 18.5 dpc mutant mice, to develop in the recipient adult mice in a histologically similar manner to the process in vivo. The method allows "long-term" *in vivo* studies in epidermal biology with these genetically modified mutant mice.

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These techniques, described here in detail, should be of interest to investigators currently producing transgenic or null mice with epidermal defects, as they provide a powerful tool to make analysis in adult mice (1), hair follicle morphogenesis (2), and tumor susceptibility (3), otherwise impossible to perform due to the early lethality.

The method has clear advantages: it is a highly successful method; if properly performed, embryonic grafted tissues attach to dermis, survive, proliferate, and differentiate in almost 100% of the cases. The procedure allows graft-take monitoring and reproduces the mutant skin phenotype (also relative to skin appendages such as hair follicles and glands). Experiments with embryonic skin engrafted mice can be performed at selected time points after transplantations for periods of over 3–4 months. Engrafted embryonic epidermis is suitable for any kind of experiment for cell and molecular biology. Moreover different recipient mice can help in determining problems such as different inflammation processes or the contribution of dermal cells to the different phenotypes.

2. Materials

1. Pregnant mice at defined gestational days: 17.5 or 18.5 dpc
2. Dissecting instruments. A sterile surgical kit including small scissors and fine forceps (at least two fine pairs)
3. Dulbecco's modified Eagle's medium, DMEM, containing 10% fetal bovine serum, FBS (Biowhittaker, Inc., Walkersville, MD)
4. Plastic Petri dishes
5. PBS, ethanol 70%, betadine
6. 6.8 weeks old immunodeficient mice
7. Avertin 2.5%
8. Animal clipper
9. Thermal pad
10. Razor blade
11. Surgical silk suture
12. Silver sulfadiazine cream (1%; Flammazine Cerio®)
13. Laminar flow hood and sterile mouse cages
14. CO_2 chamber

3. Methods

3.1. Donors

1. Check for vaginal plugs in the morning. Take the appearance of vaginal plug as day 0.5 of gestation (0.5 dpc).
2. Sacrifice pregnant mice at 17.5–18.5 dpc in a CO_2 chamber.
3. Work in a laminar flow hood. Open the abdominal cavity, hold the uterine horn. Cut the connecting membranous tissues while lifting up the uterus. Cut the uterus and transfer it to a dish containing PBS. Cut the muscle layer of the uterus and release embryos (**Fig. 25.1A**); embryos are wrapped in extraembryonic membranes. Remove the extraembryonic membranes with fine forceps as in a "nipping" action, release the embryo. Use fine scissors to cut off the placenta; embryos are removed and killed with a CO_2 chamber.

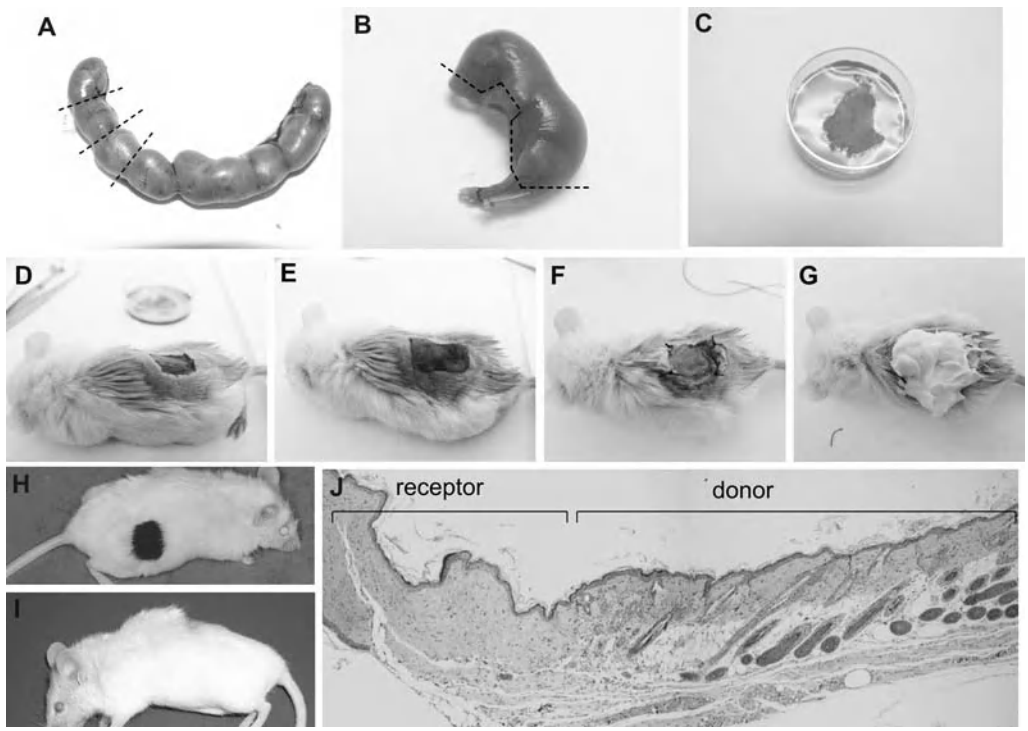

Fig. 25.1. **(A)** Intact uterus dissected cutting across the cervix and the utero-tubal junctions. Dashed lines denote the cutting line in the uterus between each individual embryo. **(B)** Cutting line in a mouse embryo. **(C)** The skin tissue with the epidermal side up, cut out from a mouse embryo at 17.5 dpc, floating on DMEM+10% FBS. **(D)** Graft bed prepared on the dorsal skin of the recipient mouse. **(E)** The graft is placed onto its site, i.e., the graft bed in the recipient mouse. **(F)** Suture of graft and host edges. **(G)** Flammazine-covered area of transplant. **(H)** Donor skin 2 months after graft distinguished by coat color. **(I)** Differentially oriented hairs of the donor when donor and recipient are of the same coat color. **(J)** Histological appearance of a control transplanted skin 2 months after graft.

4. Transfer an embryo into a 35-mm Petri dish. Cut the tip of the tail (3.4 mm) and use it for DNA extraction and genotyping.
5. Sequentially wash the embryo with (1) ethanol 70%, (2) betadine, (3) PBS 1 ×.
6. Cut the back skin with small scissors and fine forceps along a line shown in **Fig. 25.1B**. Carefully tear the skin tissue away from the muscle layer and subcutaneous fat holding with the forceps in one hand and gently lifting with the blade of a small scissors. Avoid breaking the skin tissue. Ideally, you should obtain a piece of fetal skin that consists of almost the entire integument (*see* **Notes 1 and 2**).
7. Spread the fetal skin as much as possible (epidermal side up) on a sterile plastic Petri dish (**Fig. 25.1C**).
8. Add the medium little by little (DMEM containing 10% FBS). Do not cover the tissues with medium. Avoid the tissues being dried.
9. Distinguish (marking the dish with a permanent marker) in the piece of skin between the head and the tail (*see* below, *see* **Note 3**).
10. Leave the skin tissues floating in the medium. They can be stored overnight at 4°C.

3.2. Fast Genotyping

1. The embryos can be genotyped while the tissue skin is stored at 4°C on a Petri dish. There is enough time for accurate genotyping.
2. Isolate DNA following Laird's protocol (4). About 2.3 hours of continuous agitation in lysis buffer at 55°C is enough for a 3 mm embryonic tail biopsy. Carry out genotyping by PCR as usual.
3. Once genotyped, only the desired genotyped skin tissues are placed into the graft beds of adult recipient mice

3.3. Recipients

1. About 6.8 weeks old SCID mice (NOD-CB17-*Prkdc*^{scid}/J or any other immunosuppressed strain. *See* **Note 3**).
2. All work with SCID receptors is performed in a sterile field. Do the transplantations in a laminar flow hood.
3. Anesthetize with avertin 2.5% (15.17 μl/g weight). The mouse should usually be fully sedated after 5 min, and for 45 min, which provides enough time to perform this technique. Preferably work on a thermal pad.
4. Shave carefully the back area for the transplantation. Run ethanol 70% and betadine over the shaved area.

3.4. Surgical Transplantations

1. Prepare the graft bed, excising with a razor blade a full thickness skin specimen approximately equal in shape and a little bit smaller in size than the fetal skin graft (**Fig. 25.1D**; *see* **Note 4**).
2. In the case that the embryonic donor skin is of an unknown coat color, or equal to the receptor, place the donor skin in a tail-to-head position respect to the adult recipient (the tail of the donor located in the head of the recipient and the head of the donor located in the tail of the recipient) (*see* **Note 5**).
3. The embryonic tissue is very easily dried up during the process. Avoid the tissues being dried. Keep the tissue permanently humid. Moisten with PBS or medium at any time.
4. Graft and host skin edges are ligated using surgical silk suture (**Fig. 25.1F**).

   As embryonic tissue is very thin, be careful when penetrating with the needle point. Tie knots firmly. Cut the ends of the suture as short as possible.
5. Fully cover the transplantation area with flammazine cream (alternative vaseline can be used) as the only protective dressing (no gauzes or bandages) directly to the freshly transplanted tissue (**Fig. 25.1G**; *see* **Note 6**).
6. Administer a broad spectrum antibiotic such as ciprofloxacin (10 mg/kg i.p.).

3.5. Post-grafting Care

1. Preferably keep the sedated recipients on a thermal pad until awake to prevent hypothermia after anesthesia. Transplanted animals are maintained in isolators with individually ventilated cages (IVC).
2. Check recipients and graft sites daily for the first 10 days. It is not necessary to remove the suture knots.
3. Cover the transplantation area with additional flammazine cream 1–2 days post-grafting only if necessary

4. Notes

1. When handling embryonic skin tissues, it is important not to pinch with forceps or tear them. Damage to the embryonic skin tissues hinders ulterior suturing, graft take, and diminishes the size and success of the grafting

2. As the donor graft keeps its original size, or rather slightly withdraws, isolate as much embryonic epidermis as possible to ensure a successful graft with enough material for ulterior analysis.
3. The choice of a particular immunodeficient strain as a receptor can help to solve different problems relative to immunology, infectious diseases, different inflammation processes, or the contribution of dermal cells to the different phenotypes. There is an increasing number of different immunosuppressed strains available at http://www.jaxmice.jax.org
4. The fit of the graft in size and shape to the graft bed in the receptor is important. Any open space between the graft and the edges of the wound performed in the receptor provokes the re-epithelialization of the recipient skin tissue. Make sure that the size of the graft bed is slightly smaller than that of the graft so that the graft can be well adapted to the margins (**Fig. 25.1E**). Tie firmly the knots within the edge of the margin and the donor edge. Tie extra knots, if necessary.
5. Take care of the "tail-to-head epidermal orientation" of the donor skin in a head-to-tail position of the recipient. As eventually donor skin becomes indistinguishable from receptor this is a very useful trick to follow-up the donor skin as you can recognize the different orientation of the donor hairs. It is particularly recommended when donor skin is of unknown coat color, donor and receptor are of the same mutant color, or in control animals in which no phenotype or no difference from receptors is expected (**Fig. 25.1H**, **I**).
6. If grafts are air exposed for more than 24 h, transepidermal water loss through the graft will result in desiccation and loss of transplants. Silver sulfadiazine cream (1%; flammazine) is not only efficient in avoiding transepidermal water loss but also represents a broad-spectrum topical antibiotic.

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Chapter 26

A Transplant Model for Human Epidermal Skin Regeneration

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Abstract

This protocol describes a technically simple in vivo assay of long-term skin regeneration in human skin, providing a reliable method for epidermal tissue reconstitution using small numbers of several types of epithelial cells, from epithelial cell lines to primary epithelial stem cells and transplanted with or without prior culture. The model relies on the repopulation of devitalized rat tracheas by human keratinocyte suspensions following subcutaneous transplantation into immunodeficient mice. Here, we describe complete optimization and characterization of this model for robust regeneration of epithelium from cell suspensions of a limited number of primary human keratinocytes.

Key words: Human skin regeneration, Keratinocytes, Epithelial stem cells, Rat tracheas, In vivo transplants.

1. Introduction

The epidermis forms the outer protective layers of the skin and is a rapidly renewing tissue undergoing constant regeneration. The regenerative capacities of epithelial populations have been demonstrated for many years with the development of cultured keratinocytes derived from the epidermis, used to produce autologous grafts that regenerate an epidermis over a full-thickness wound (1–3). However, skin grafts can still fail, in part due to a lack of knowledge of the long-term cellular and molecular biology of epithelial regeneration, particularly that of the epidermal stem cells and their committed progeny. A long-term epithelialization system called the "rat trachea" (RT) model has been developed to allow the study of the regenerative potential of keratinocytes. This assay was modified from a method originally developed to evaluate

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carcinogen-induced neoplastic progression in rat tracheal cell lines (4) and was used more recently to demonstrate hair follicle reconstitution (5). We specifically developed this assay to study the growth and differentiation properties of freshly isolated keratinocytes from human and mouse skin (6–8) including the epithelial stem cell-enriched population (Keratinocyte Stem Cells – KSC) and their progeny (Transit Amplifying – TA, and Early Differentiating – ED keratinocytes). This in vivo assay has several key advantages: (i) the reconstructed epithelium presents a well-defined, stratified and differentiated 3D architecture; (ii) the tissues can be easily maintained, harvested and studied; (iii) the model allows the study of skin regeneration using small numbers of rare cells; and (iv) co-transplantation of selected cellular components (e.g., cultured or primary keratinocytes; specific dermal subpopulations; and/or particular molecular regulators including chemokines, growth factors and components of the extracellular matrix). Here, we describe how to implant freshly isolated human neonatal keratinocytes into the RT system to grow a differentiated human epithelium in vivo in a murine recipient.

2. Materials

2.1. Preparation of the RTs

For the development of this protocol, the rat tracheas were purchased from the Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia. They were aseptically removed from 200 to 250 g Sprague–Dawley rats. The RT were collected in batches of 100 and stored frozen at –80°C until further processing. The RT were prepared as described below, at least 1 day prior to seeding the keratinocytes.

Set up the following in a biohazard laminar flow hood:

1. Sterile PBS^{+++} (without calcium and magnesium) containing penicillin (final concentration 12 mg mL^{-1}), gentamicin (final concentration 160 mg mL^{-1}) and fluconazole (final concentration 6 mg mL^{-1}). Filter sterilize the solution through a 0.22 μm filter. Keep cold.
2. DMEM (Gibco).
3. Three large Petri dishes (10 cm diameter), one containing PBS^{+++}, one with DMEM, and one empty.
4. Sterilized instruments: one pair of small dissecting scissors (ProSciTech), hemostats (forceps with clamping arm and serrated tips), dissection forceps (ProSciTech), 18G 1½″ drawing-up needle (Terumo), surgical McKenzie 6″ clip applying forceps (Surgipro, USA), medium titanium ligating clips (LT200, Ethicon Endo-Surgery, USA), sterile 10 mL

syringe (Termo), 1.5 cm long microbore polytetrafluoroethylene (PTFE) tubing (Cole-Parmer Instrument Company, USA) all prepared and autoclaved in advance.

2.2. Implanted Cells

For the preparation of the implanted cells, the following materials are required:

1. Gilson pipettes P1000 and P200 with suitable sterile-filtered tips.
2. Trypan blue solution: 0.4% (Sigma).
3. Trypsin-EDTA 0.05% (pH 7.0) without Phenol Red (Thermo Electron Corp.).
4. Epilife supplemented with HKGS supplement (Cascade Biologics) (*see* **Note 1**).
5. Trypsin inhibitor: 0.1 g of soybean trypsin inhibitor (Sigma), 0.5 g of BSA (tissue culture grade) in 500 mL of DMEM. Filter sterilize through a 0.22 μm filter. Pre-warm to 37°C.
6. Sterile PBS (without calcium or magnesium).
7. Several sterile 1.5 mL Eppendorf tubes.

2.2.1. Support Cells

The support cells are irradiated p0 or p1 neonatal human keratinocytes. Plan for 5×10^5 support cells per RT. The following equipment is required:

1. Caesium gamma irradiator (Nordion Gammacell 40 Irradiator).

2.2.2. Fresh Keratinocytes

For the freshly isolated primary keratinocytes, plan for between 1×10^3 and 0.5×10^6 keratinocytes per RT.

2.3. Skin Sample Dissection

About 2–3-week-old neonatal healthy human foreskins. Consent must be obtained and the project approved by an Institutional human ethics committee. The dissection of the epithelium is performed in a biohazard laminar flow hood. Set up the following:

1. 70% ethanol.
2. PBS^{+++} (as previously described in **Section 1.1**), keep cold.
3. Several 10 cm Petri dishes.
4. Autoclaved dissecting forceps and scalpel holder with sterile blades (ProSciTech, Australia).
5. Dispase solution: Neutral Dispase II (Roche Diagnostics) at $4\,mg\,mL^{-1}$ in PBS. Dissolve in PBS and filter sterilize by passing through a 0.22 μm filter just prior to use.

2.4. Keratinocyte Isolation

Set up the following in a biohazard laminar flow hood:

1. Several inverted 10 cm Petri dishes containing cold PBS^{+++}. Keep on ice.

2. Autoclaved dissecting forceps and scalpel holder with sterile blades (ProSciTech, Australia).
3. Trypan blue solution, trypsin-EDTA, trypsin inhibitor and Epilife (all as previously described in **Section 1.2**).
4. Sterile plastic disposable pipettes (Livingston, Transfer Pipettes).

2.5. Inoculation of the Keratinocytes into the RT

Set up the following in a biohazard laminar flow hood:

1. Transplant medium: 1:1 mix of Epilife (Cascade Biologic) with 1 volume of DMEM (Gibco) supplemented with 10% bovine serum, 20 ng mL^{-1} murine epidermal growth factor (EGF) from the murine submaxillary gland (Sigma Cat. E4127), 400 ng mL^{-1} hydrocortisone (Sigma, St Louis, MO, USA), 10 ng mL^{-1} cholera toxin (Sigma), 6 mg mL^{-1} penicillin, 80 mg mL^{-1} gentamycin (Life Technologies, USA) and 6 mg mL^{-1} Diflucan (Pfizer). This complex medium is stable for 6 months at 4°C. 30 μL of transplantation medium is required per RT.
2. Sterile drawing-up needle fixed on a sterile 10 mL syringe (as previously described) filled with cold PBS^{+++}.
3. Gilson 200 μL pipette with elongated sterile tips (Prot/Elec Tips-Bulk Bio-Rad).
4. Sterilized instruments: hemostats, dissection forceps (ProSciTech), surgical McKenzie 6″ clip applying forceps (Surgipro, USA), medium titanium ligating clips (LT200, Ethicon Endo-Surgery, USA).
5. Several sterile 15 mL yellow cap tubes.

2.6. In Vivo *Transplantation*

About 6–8-week-old female SCID mice. The use of nude mice has also been published for engraftment of human epithelium (9).

2.6.1. Recipient Mice

2.6.2. Surgical Procedure

Set up the following in a biohazard laminar flow hood:

1. 50 mL Falcon tube containing a sterile gauze.
2. Isofluorane (200 μL per mouse).
3. Anaesthetic solution containing 2 mg mL^{-1} Xylazine (Bayer Australia, Pymble, Australia) and 10 mg mL^{-1} Ketamine (Parnell Laboratories, Sydney, NSW, Australia) prepared in PBS.
4. Cotton pads soaked with 70% ethanol.
5. Sterile instruments: medium operating size scissors blunt/blunt (ProSciTech), two dissecting forceps (ProSciTech), one autoclip wound clip applier (BD, Australia), one box of 9 mm sterile autoclips (one clip per mouse) (BD, Australia), one autoclip wound clip remover (BD, Australia).

2.6.3. Harvesting the Grafts

For the harvesting of the grafts from the murine recipients, the following materials are required:

1. CO_2 euthanasia chamber.
2. Operating scissors, dissecting forceps, and scalpel holder with sterile blades (ProSciTech, Australia).
3. Several 10 cm Petri dishes.
4. Clean PBS.
5. Decalcifying solution: 5 mM EDTA in PBS, pH 8. Dissolve 6.26 g of NaCl + 80 g EDTA, adjust the pH to 8 and the volume to 250 mL with water.
6. Fixative: 4% (w/v) buffered formalin.
7. Tissue-Tek O.C.T. Compounds (Sakura, USA).
8. Eppendorf tubes.
9. Rotating wheel at 4°C.

3. Methods

The preparation of the RT, the isolation of each cell type, the inoculation of cells into the RT and grafting into the recipient mice have to be performed under sterile conditions in a laminar flow hood using autoclaved or sterile instruments.

3.1. Preparation of the Tracheas

The RTs were purchased in batches of 100 and stored frozen at –80°C until further processing.

1. Thaw tracheas and process individually as follows.
2. In a large inverted Petri dish, excess connective tissue surrounding the tracheas is carefully trimmed away using scissors and dissecting forceps (**Fig. 26.1a**).
3. A piece of 1.5 cm long PTFE tubing is then applied against each trachea to hold the lumen open after transplantation as described below.
4. The PTFE tubing and the trachea are clamped together with a haemostat at one end of the trachea.
5. The free end of the trachea is carefully stretched with a second haemostat along the PTFE tubing to obtain a length of about 1 cm and then clamped again.
6. The PTFE tubing and stretched RT are then secured together with LT400 Ligaclips (**Fig. 26.1b**, c) using the surgical McKenzie 6″ clip applying forceps.
7. Using a large scalpel, the larynx (top part of the trachea) and the bronchi (bottom part of the trachea) are trimmed off (**Fig. 26.1d**).

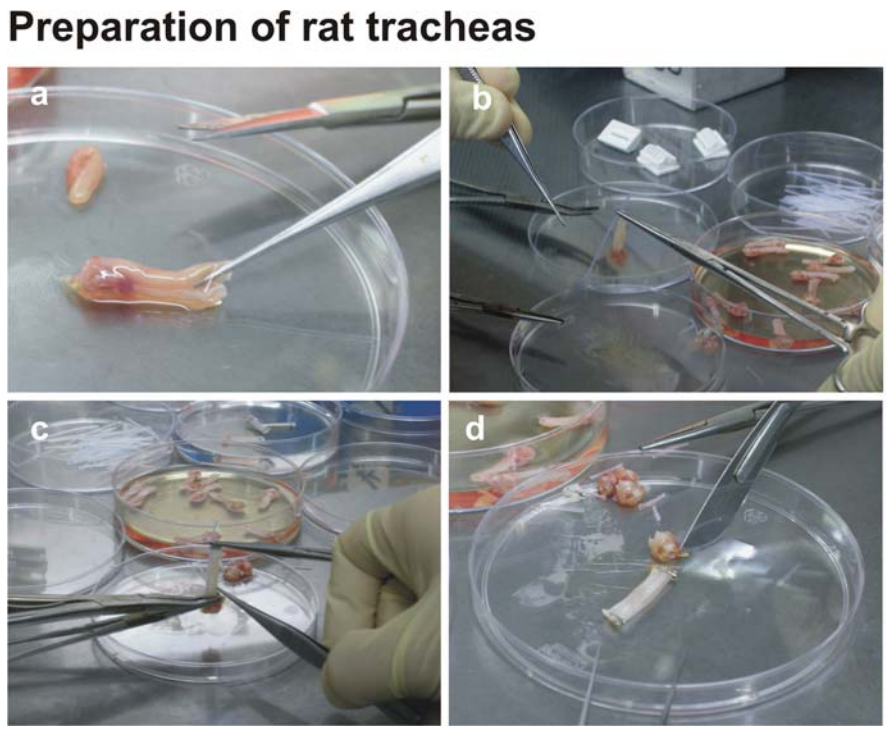

Fig. 26.1. Preparation of rat tracheas prior to transplantation. Rat tracheas are individually trimmed of connective tissue **(a)** and stretched onto pieces of sterile PTFE tubing secured in place with hemostats **(b, c**). The ends of the rat tracheas are then sealed and clipped onto the tubing with LT400 ligaclips and excess tissue is trimmed **(d),** following which the trachea is freeze-thawed to remove native epithelia.

8. The tracheas are then devitalized via three cycles of freeze-thawing (–70°C and 37°C) to detach the native tracheal epithelium.
9. The tracheas are nicked with a pair of scissors at one end and flushed to remove cellular debris within the lumen as follows.
10. Clamp one extremity of the RT above the upper clip with a haemostat clamping both the PTFE tubing and the trachea (the RT must face the experimenter).
11. Maintaining the RT in this position, make a 2 mm deep incision just beneath the upper clip on the RT. This incision will allow pipetting of cells into the RT and should be as close as possible to the upper clip of the RT as shown in **Fig. 26.2**.
12. Holding the trachea with a pair of forceps, use a drawing-up needle fixed onto a 10 mL syringe to wash the inside of the RT with multiple flushes of cold PBS^{+++}, aspirating all remaining liquid from the lumen.

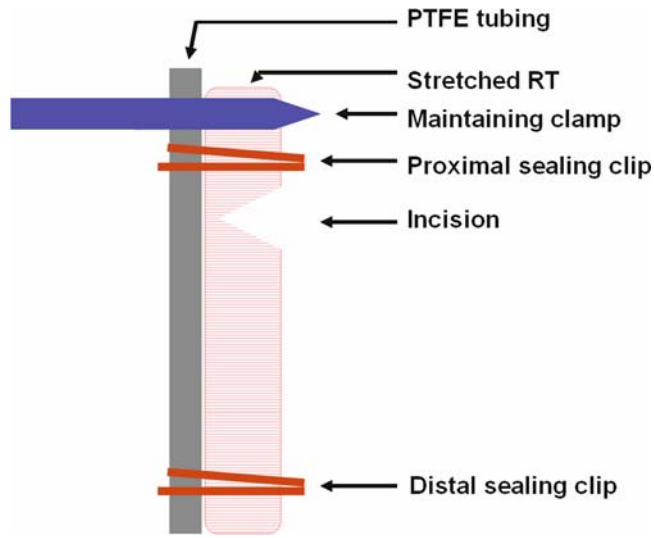

Fig. 26.2. Schematic representation of placement of sealing clips and incision in the rat trachea to permit flushing and inoculation of cells to be transplanted. The PTFE tubing is clipped onto the distal and proximal extremities of individually stretched tracheas. A 2 mm deep incision is made beneath the upper clip on the sealed RT as shown and the lumen of the trachea flushed to remove cellular debris prior to inoculating test cells.

13. The stretched and secured trachea are immersed in DMEM for ~30 seconds to test for leaks – any infiltration of pink liquid into the trachea indicates a puncture in the wall of the trachea. The location of a hole responsible for a leak can be difficult to correct depending on its position. If the leaks are close to the clips, fix another clip directly behind the existing clip and test again for leakiness of the RT. If this fails, discard leaky trachea and use another. Importantly, all tracheas in a batch must be a minimum of 0.5 cm in length to accommodate the 30 µL cell suspension.
14. The cleaned, secured, and non-leaky RT can then be stored in PBS^{+++} frozen at –80°C for 1 year, at –20°C for 6 months or 1 week in the fridge.

3.2. Preparation of the Cells

Further details can be found in (10).

Process the skin sample rapidly after surgical excision.

3.2.1. Fresh Human Keratinocytes

1. Using forceps, rinse the skin in 70% ethanol and immediately wash thoroughly in PBS^{+++}.

2. Place the skin into a Petri dish and remove excess connective and fat tissue from dermal side by trimming using the scalpel with new blade, scissors, and forceps.
3. Rinse the skin thoroughly with PBS^{+++}.
4. Place the skin onto another sterile inverted lid and cut into small pieces of approximately 2 mm × 2 mm using a new scalpel blade.
5. Transfer the skin pieces into a 50 mL Corning tube containing the Dispase solution.
6. Incubate overnight at 4°C with gentle agitation on a rotating platform, allowing the enzyme to cleave the skin at the epidermal–dermal junction.
7. Place the skin pieces into a Petri dish lid, and holding the dermal tissue with a pair of forceps, gently peel away the epidermis from the dermis with another pair of forceps.
8. Place the epidermal sheets in a separate Petri dish containing cold PBS – do not allow to dry.
9. Using sterile forceps transfer the epithelial sheets into a 50 mL Corning tube and add 10 mL of trypsin-EDTA. (Trypsin-EDTA is pre-warmed in a 37°C water bath.)
10. Trypsinize epidermal sheets by pipetting up and down vigorously using a plastic transfer pipette for precisely 5 min.
11. Quench the reaction by adding an equal volume of pre-warmed soybean trypsin inhibitor. Mix gently.

    From this step on, keep the cells on ice to preserve optimal viability.
12. Filter the cell suspension through a 70 μm cell strainer into a 50 mL Falcon tube.
13. Centrifuge the epithelial suspension at 1400 rpm for 5 min at 4°C and resuspend the pellet in 5 mL of cold Epilife medium, count the cells, and assess their viability with Trypan Blue. A viability exceeding 80% is needed for successful transplants. This will constitute the cellular stock solution of freshly isolated keratinocytes.

Keratinocytes can now be directly inoculated into the RT. However, the keratinocytes can also be labelled with antibodies and separated by FACS into keratinocyte stem cells and their progeny prior to introduction into RTs. Immunostaining and purification methods have been previously described: please refer to (10).

3.2.2. Support Cells

The support cells are irradiated primary keratinocytes at passage 1 or 2. They provide soluble factors necessary to sustain the process of skin regeneration and constitute a cellular scaffold for the test epithelial cells that facilitates their spatial organization inside the RT.

1. Prior to harvest, the support cells are fed with fresh Epilife medium twice weekly until they are 80% confluent.
2. The support cells are then irradiated in situ at a dose of 15 Gy gamma rays.
3. The irradiated cells are trypsinized, pooled, and resuspended in cold Epilife. This constitutes the cellular stock of support cells.

3.2.3. Transplanted Keratinocytes

Numbers of fresh keratinocytes from 10^3 up to 5×10^5 cells per RT have been used successfully. These keratinocytes are routinely mixed with 5×10^5 irradiated support cells per RT. The desired number of grafted cells is resuspended in 30 μL of transplantation medium per RT. In calculating the required number of cells, allow for an extra sample (*see* **Note 2**).

1. Pre-chill a 1.5 mL sterile Eppendorf tube.
2. Add the desired quantities of support cells and freshly isolated keratinocytes from both cellular stock solutions.
3. Mix gently by flicking the tube – do not vortex.
4. Pellet the cells by centrifuging at 400 g at 4°C for 5 min.
5. Resuspend in the calculated volume of pre-chilled transplantation medium and keep the cells on ice.

3.3. Inoculation of the Keratinocytes into the RT

1. RTs are thawed on ice if frozen.
2. Hold the RT with the haemostat with the incision facing the experimenter, and wash the inside of the RT with several flushes of PBS introduced through the incision using a blunt-ended needle attached to a syringe.
3. Aspirate any remaining PBS from the tracheal lumen.
4. To inoculate the cells, pipette the cell suspension up and down in the Eppendorf tube to obtain a uniform cell suspension – do not vortex. Aspirate 30 μL for inoculation, avoiding bubbles.
5. Place the pipette tip inside the RT, at the base, and slowly pipette the cell suspension into the lumen of the RT. The pinkish colour of the medium, visible through the translucent tissue of the RT, indicates the meniscus of the cell suspension. If a bubble is trapped within the RT lumen, remove the cell mixture by gentle aspiration and repeat the inoculation.
6. When 30 μL of cellular solution is inoculated, seal the upper part of the RT with a clip as close as possible to the meniscus. It is important to orient the top clip parallel to the bottom one to minimize discomfort to the recipient animal, after subcutaneous implantation of the RT. The inoculated RT sealed at both ends is approximately 1 cm long.

7. Keep the inoculated RTs horizontal in 15 mL yellow cap tubes containing cold PBS^{+++} on ice until transplantation into the recipient animals. Storing filled tracheas vertically may lead to cell pelleting and hence uneven coverage of the lumen (*see* **Note 3**).

3.4. Grafting the Transplants into the Mice

1. Inject 250 µL of Ketamine/Xylazine cocktail intraperitoneally to anesthetize mice. For each mouse, apply 2 drops (approximately 300 µL) of isofluorane to sterile gauze in a 50 mL Falcon tube. Delicately place the snout of the mouse into the tube until the mouse is deeply sedated, as evidenced by pinching the hind foot-pad until there is no reaction.
2. Sterilize the back of the anaesthetized mouse from the bottom of the ears to the middle of the back with 70% ethanol.
3. With large forceps, pinch part of the skin located between the two scapula and pull upwards by roughly 2 cm: this facilitates the separation of the skin from the underlying viscera. Keep the skin stretched with the forceps.
4. Using sterile scissors make a 1 cm long incision.
5. Insert the scissors into the incision and open/close the blades several times in order to enlarge the open space between the upper flanks. The space created under the skin should be roughly 3 cm × 3 cm square. Keep the skin around the opening stretched.
6. Using sterile forceps, insert 2 RTs deep into the subcutaneous space created.
7. To close the cavity, use the forceps to bring the skin surrounding the incision back together and apply a Michel wound clip. The final position of the implants is shown in **Fig. 26.3**. The closing clip must be fixed deep enough in the skin margin to allow the closure of the wound (~0.5 cm), without restricting the normal movements of the mouse's head.
8. For the well-being of the mouse, remove the clip 1 week after implantation, using the clip remover.

3.5. Harvesting the Transplants

Several time points can be chosen from 2 to 12 weeks post-implantation depending on the number of cells inoculated.

1. Sacrifice the recipient mice by asphyxiation with CO_2, since cervical dislocation may damage RTs lodged near the neck.
2. Recover the RTs by cutting the skin away from the body around the rat tracheas with scissors.
3. Typically, the implanted RTs are amalgamated in a single plug of connective tissue containing fat and blood vessels enveloping the grafts. Using forceps, scissors and a scalpel, carefully cut the tissue surrounding the plug and immerse the plug into an inverted Petri dish lid containing cold PBS.

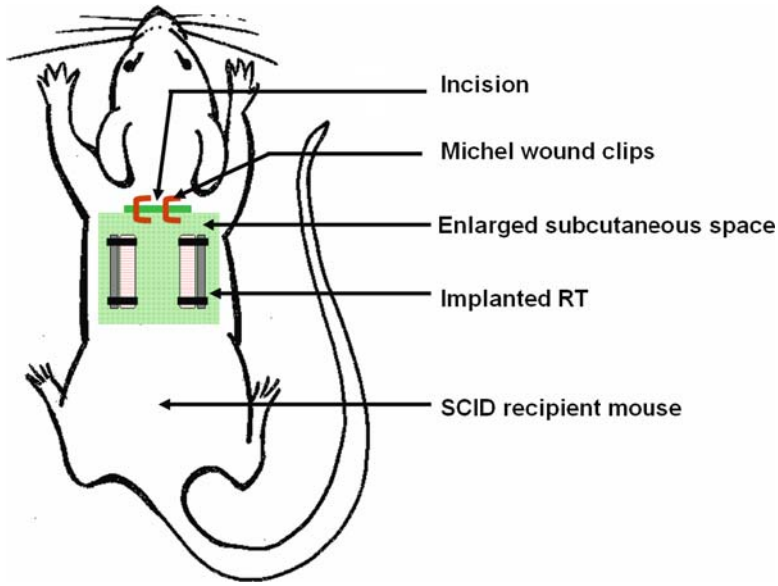

Fig. 26.3. Schematic representation of the placement of inoculated tracheas onto SCID mice. Two rat tracheas are implanted per mouse in a subcutaneous space on the dorsal surface of the mouse by making an incision as shown between the upper flanks of the mouse and freeing the skin from the underlying tissue. After insertion of the rat tracheas, the cavity is closed with two Michel wound clips placed as shown.

4. Using forceps and a scalpel, delicately separate and clean the surface of the tracheas by trimming the connective tissues, taking care not to crush the RT.
5. Fix the cleaned RTs in 4% (w/v) buffered formalin for 2 h at room temperature.
6. Cut the RTs in half at the middle and inspect the lumen macroscopically. The presence of a white cotton-like layer covering the inside of the RT cartilage often indicates a successfully reconstituted epithelium.
7. Decalcify one half of each RT in 1 mL of decalcifying solution for 24 h at 4°C, on a rotating wheel. Wash the sample in PBS and embed in OCT, for cryosections and immunostaining.
8. Fix the other half for a further 18 h at 4°C on a rotating wheel, then decalcify and process for paraffin embedding. When embedding, carefully orientate the lumen of the RT perpendicular to the section plane to allow transverse histological sectioning of the samples. At early time points of transplants performed with small numbers of cells, epithelialization may be restricted to small regions of the RT lumen. It is therefore recommended that serial sectioning and histological analysis of the entire RT is performed on selected sections spaced at regular intervals through the lumen.

The samples can be analysed using traditional histological or immunostaining techniques. We have used the RT transplant model to investigate long-term tissue reconstitution (10 weeks) obtained from human foreskin stem cells via H&E staining as illustrated in **Fig. 26.4a**,b. We have also performed serial transplantation experiments by harvesting the epithelial cells lining the lumen (**Fig. 26.4c** – using dispase as described for foreskin in **Section 3.2.1**) and performing secondary transplants (**Fig. 26.4d**). Importantly, the newly reconstituted epithelium has been shown to exhibit many features of normal epidermal differentiation (11) as evidenced by appropriate expression of biochemical markers of keratinocyte proliferation and differentiation. EM studies demonstrate the formation of hemidesmosomes, basement membrane assembly at the tracheal:epithelial junction while confirming immunohistochemical evidence for sequential keratinocyte differentiation through successive layers (11). Murine

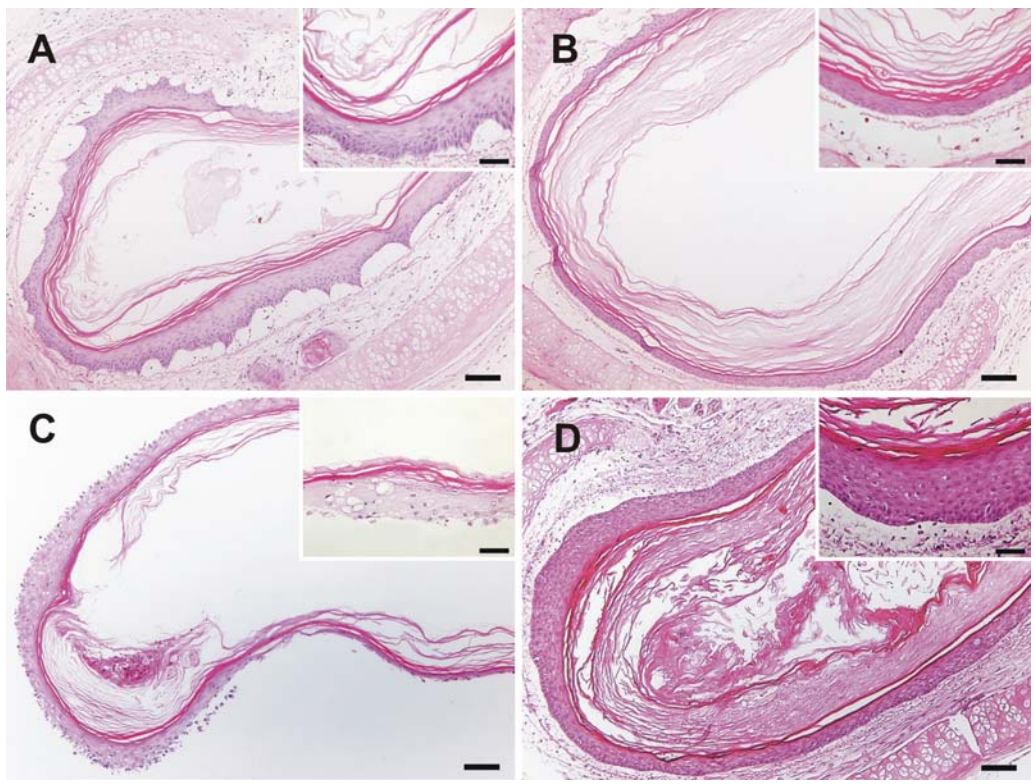

Fig. 26.4. Histological sections of human epidermis stained with haematoxylin and eosin, reconstituted following in vivo transplantation. Denuded rat tracheas were seeded with 10^4 $\alpha_6^{bri}CD71^{dim}$ KSCs and 5×10^5 lethally irradiated (15 Gy) p0 HFK support cells and harvested after 6 **(a)** and 10 weeks **(b)**. 10^6 HFKs were transplanted and harvested at 4 weeks; intact epidermis was recovered from tracheas after overnight dispase treatment **(c)**. 5×10^4 cells recovered from transplant **(c)** of freshly isolated HFKs were serially transplanted with 5×10^5 lethally irradiated (15 Gy) p0 HFK support cells and harvested after 6 weeks **(d)**. (**a–d**) n=2. Scale bars: main panel = 100 μm, inset = 25 μm.

keratinocytes have also been transplanted successfully following fractionation into subsets using Hoechst 33342 (7). More recently, we have used this transplant model to reconstitute both murine and human oesophageal epithelium (8, 12).

3.6. Conclusions

We have described a convenient, technically simple and versatile long-term in vivo model for the investigation of biological properties of skin regeneration from limiting numbers of freshly isolated keratinocytes. This procedure can be easily extended to the study of the regenerative capacities of FACS-purified epidermal stem cells and their committed progeny, from both murine (7) and human (11, 13) origin, thus providing a technique for assaying sustained stem cell activity and consolidating their biological characterization.

The rat trachea in vivo long-term epithelialization model can help to bring biologically relevant insights into the following questions: (i) What is the proliferative potential and the self-renewal ability of the epithelial stem cells and their progeny in a long-term in vivo system? (ii) What other epithelial or dermal cell populations regulate long-term epithelialization?

This model is also amenable to other studies investigating the role of specific factors or genes that may have been modulated in either epidermal or dermal cells (e.g. from genetically altered strains of mice or human cell lines) in regulating epithelial proliferation, differentiation, carcinogenesis, polarity, tissue architecture, etc. It can also be used to assay the effects of therapeutic drugs, cytokines or inhibitors for cytotoxicity.

4. Notes

1. If the implanted keratinocytes are obtained from a mouse, the culture media and the transplant media must be adapted accordingly. Refer to (7, 8, 11).
2. RTs filled with support cells alone are used as negative controls.
3. The tracheas are permeable to liquid. The medium used to fill the RT will drain from the RT within a couple of hours.

Acknowledgements

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Chapter 27

Identification of Epithelial Stem Cells In Vivo and In Vitro Using Keratin 19 and BrdU

Danielle Larouche, Amélie Lavoie, Claudie Paquet, Carolyne Simard-Bisson, and Lucie Germain

Abstract

Progress in the identification of skin stem cells and the improvement of culture methods open the possibility to use stem cells in regenerative medicine. Based on their quiescent nature, the development of label retention assays allowed the localization of skin stem cells in the bulge region of the pilosebaceous units and in the bottom of rete ridges in glabrous skin. The development of markers such as keratin 19 also permits their study in human tissues. In this chapter, protocols to identify skin stem cells based on their slow-cycling property and their expression of keratin 19 will be described in detail. The methods include the labeling of skin stem cells within mouse or rat tissues in vivo, the labeling of proliferative human cells in vitro using 5-bromo-2-deoxyuridine (BrdU), and the detection of keratin 19 and BrdU by immunofluorescence or immunoperoxidase staining.

Key words: Cell culture, Epidermis, Hair follicle, Human, Keratinocytes, Keratin 19, Mouse, Proliferation, Rat, Regenerative medicine, Stem cells, Vibrissa.

1. Introduction

Given their application in regenerative medicine, the identification and culture of skin epithelial stem cells are of great interest. These cells are relatively undifferentiated, retain a high capacity for self-renewal throughout their lifetime, have a large proliferative potential, and are normally slow cycling in situ. Furthermore, epithelial cells presenting stem cell properties express keratin 19 (K19) in human and rodent (mouse) tissues, as well as in their derivate cultured cells (1, 2). In animals, synthetic analogue of DNA bases such as ^{3}H-thymidine (1, 3, 4) or 5-bromo-2-deoxyuridine (BrdU) (5) are useful to study the cycling dynamics of the cells.

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BrdU can be incorporated into the newly synthesized DNA of replicating cells during the S phase of their cycle. A single pulse of BrdU followed by a short chase period allows the identification of dividing cells (phase S) at the moment of the pulse. To identify stem cells, the BrdU-labeling period must be long enough to allow stem cells to complete at least one cellular cycle. Next, a prolonged chase period results in dilution of the BrdU in DNA of the cells that actively proliferate. Then, slow-cycling cells, also called label-retaining cells (LRC), conserve a greater amount of BrdU over this chase period and can be identified with anti-BrdU antibodies by immunohistochemical methods.

In this chapter, the BrdU-labeling procedure which allows the localization of stem cells or actively cycling cells in mouse skin in vivo (2) will be described in detail. The utilization of BrdU to study the cycling dynamics of human keratinocytes in vitro is also detailed. The methods include immunolabeling procedures allowing the detection of BrdU and/or K19. The identification of stem cells in culture as well as in cutaneous substitutes is a crucial step in the development of tissue substitutes with long-term regeneration potential.

2. Materials

2.1. In Vivo BrdU Labeling of Slow-Cycling Cells (Label-Retaining Cell Method)

1. 1 day-old mice (Charles River, St-Constant, QC) (*see* **Note 1** and **Note 2**).
2. 5-bromo-2-deoxyuridine (BrdU 1.5 mg/mL) (cat. no. B5002, Sigma-Aldrich, St-Louis, MO). Wear mask and gloves. Dissolve 15 mg in 10 mL of saline solution (0.9% NaCl (cat. no. S271, Fisher Scientific, Nepean, ON) diluted in distilled water (dH_2O)). Sterilize by filtration through a Millex-GF 0.22 μm filter unit (cat. no. SLGV033RS, Millipore, Bedford, MA), distribute in single use aliquots, and store at –80°C.
3. 1 mL BD Yale™ tuberculin glass syringe with BD luer-lok™ tip (cat. no. 512027, Becton Dickinson, Franklin Lakes, NJ).
4. Needles 30 G × 1/2 in. (cat. no. 305106, Becton Dickinson).

2.2. In Vivo BrdU Labeling of Actively Dividing Cells

1. Adult mice (Charles River) (*see* **Note 2**).
2. 5-bromo-2-deoxyuridine (BrdU 10 mg/mL) (cat. no. B5002, Sigma-Aldrich). Wear mask and gloves. Dissolve 100 mg in 10 mL of saline solution (0.9% NaCl (cat. no. S271, Fisher Scientific) diluted in dH_2O). Sterilize by filtration through a Millex-GV 0.22 μm filter unit (cat. no. SLGV033RS, Millipore), distribute in single use aliquots, and store at –80°C.

3. 1 mL syringe (cat. no. SS-01T, Terumo, Somerset, NJ).
4. Needles 27 G × 1/2 in. (cat. no. NN-2713R, Terumo Medical Corporation, Elkton, MD).

2.3. Tissue Preservation and Sectioning

1. OCT compound (Miles Inc., Elkhart, IN).
2. Small container with liquid nitrogen.
3. Dissecting jeweler microforceps (cat. no. 08-953F, Fisher Scientific).
4. Dissecting curved scissors (cat. no. 08-935, Fisher Scientific).
5. Superfrost glass slides (cat. no. 12-550-15, Fisher Scientific).
6. Slide warmer (cat. no. 12-594, Fisher Scientific).

2.4. Immunoperoxidase Staining of BrdU on Tissue Cryosection

2.4.1. Materials

1. Diamond pencil (cat. no. 08-675, Fisher Scientific).
2. Formaldehyde 0.37%. Make a 3.7% buffered formaldehyde solution: dissolve 8 g $NaH_2PO_4 \cdot H_2O$ (cat. no. S369, Fisher Scientific) and 13 g Na_2HPO_4 (cat. no. S374, Fisher Scientific) in 500 mL of dH_2O, then add 100 mL of formaldehyde 37% (cat. no. F-5940, ACP, Montreal, QC). Complete to 1 L with dH_2O. Dilute the 3.7% buffered formaldehyde solution in dH_2O at a proportion of 1:9.
3. Cold methanol (cat. no. A412, Fisher Scientific). Cool at –20°C.
4. 0.07 N NaOH. Dilute 10 N NaOH solution (cat. no. 5674-02, JT Baker, Phillipsburg, NJ) in dH_2O at a proportion of 1:142.
5. Phosphate-buffered saline-calcium (PBS-Ca): 137 mM NaCl (cat. no. S271, Fisher Scientific), 2.7 mM KCl (cat. no. P285, Fisher Scientific), 6.5 mM Na_2HPO_4(cat. no. S374, Fisher Scientific), 1.5 mM KH_2PO_4(cat. no. P217, Fisher Scientific), 0.5 mM $MgCl_2.6H_2O$ (cat. no. M8266, Sigma-Aldrich), 0.9 mM $CaCl_2.2H_2O$ (cat. no. C7902, Sigma-Aldrich). To make 1 L, dissolve 8 g NaCl, 0.2 g KCl, 0.92 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.17 mL $MgCl_2.6H_2O$ of a 2.8 M solution kept at –20°C and 0.131 g $CaCl_2 \cdot 2H_2O$ in dH_2O. Complete to 1L with dH_2O. Verify that pH is 7.4 (*see* **Note 3**).
6. PBS-Ca-BSA. PBS-Ca containing 1% BSA. Dissolve 1 g bovine serum albumin (BSA) (cat. no. A2153, Sigma-Aldrich) in 100 mL PBS-Ca.
7. Hydrogen peroxide 0.3%. Dilute 100 µL hydrogen peroxide 30% (v/v) (cat. no. H1009, Sigma-Aldrich) in 9.9 mL dH_2O.
8. ID Labs IDetect™ super stain system (HRP) (cat. no. IDST1007, ID Labs Biotechnology Inc., London, ON). Store at 4°C.

a. ID Labs™ anti-polyvalent biotinylated antibody (yellow solution). Biotinylated anti-immunoglobulin serum (anti-mouse, rat, rabbit, and guinea pig). Ready to use.

b. ID Labs™ staining reagent (red solution). Horseradish peroxidase (HRP)-labeled ultra streptavidin. Ready to use.

9. Sigma fast™ 3,3-diaminobenzidine (DAB) (cat. no. D4293, Sigma-Aldrich). Dissolve one Sigma fast™ urea H_2O_2 tablet with one Sigma fast™ DAB tablet in 5 mL dH_2O. Pass through a 45 μM-pore-filter Millex-HV (cat. no. SLHV025LS, Millipore). Protect from light (*see* **Note 4**).

10. Vector® M.O.M.™ Immunodetection Kit Basic (cat. no. BMK-2202, Vector Laboratories, Inc., Burlingame, CA).
    a. M.O.M.™ blocking reagent. Use at a dilution of 1:28 in PBS-Ca.
    b. M.O.M.™ diluent. Use at a dilution of 1:12.5 in PBS-Ca.

11. Harris hematoxylin (cat. no. 30473, Intermedico, Markham, ON). Pass through a grade 4 filter paper (cat. no. 1004185, Whatman®) before each use.

12. Microscope slide mailer (cat. no. 4456, Somagen Diagnostics, Edmonton, AL).

13. Microscope slide rack (cat. no. 4465A, Somagen Diagnostics).

14. Mounting media. 137 mM NaCl (cat. no. S271, Fisher Scientific), 2.7 mM KCl (cat. no. P285, Fisher Scientific), 6.5 mM Na_2HPO_4(cat. no. S374, Fisher Scientific), 1.5 mM KH_2PO_4 (cat. no. P217, Fisher Scientific), 22 mM NaN_3(cat. no. S-202, Sigma-Aldrich), 1.5% gelatin (cat. no. G8-500, Fisher Scientific). To make 62 mL, dissolve 0.8 g gelatin in hot water. Add 0.224 g NaCl, 0.0056 g KCl, 0.0258 g Na_2HPO_4, 0.0056 g KH_2PO_4, and 0.04 g NaN_3. Add 25 mL of dH_2O. Adjust pH to 7.6 using pH paper (cat. no. 9583, EMD Chemicals Inc., Gibbstown, NJ). Complete to 40 mL with dH_2O. Add 12 mL glycerol (cat. no. 800687, MP Biomedicals, Solon, OH).

15. Coverslips 24 × 50 mm, #1 (cat. no. 12545F, Fisher Scientific).

2.4.2. Primary antibody

a. Mouse anti-BrdU. Mouse IgG_1 anti-BrdU, clone B44, 50 μg/mL (cat. no. 347580, Becton Dickinson). Dilute in M.O.M.™ diluent at a final concentration of 10 μg/mL (*see* **Note 5**).

b. Unrelated anti-mouse IgG_1 (control antibody). Mouse IgG_1, clone X40 (cat. no. 349040, Becton Dickinson). Dilute in M.O.M.™ diluent at the same concentration than the mouse anti-BrdU.

2.5. Simple or Double Immunofluorescence Staining of BrdU and K19 on Tissue Cryosection

2.5.1. Materials

1. Acetone (cat. no. AXO116-1, EMD Chemicals Inc.). Cool at –20°C.
2. Microscope slide mailer (cat. no. 4456, Somagen Diagnostics).
3. Microscope slide rack (cat. no. 4465A, Somagen Diagnostics).
4. Diamond pencil (cat. no. 08-675, Fisher Scientific).
5. Hoechst solution. Dissolve 25 mg of Hoechst 33258 (cat. no. B2883, Sigma-Aldrich) in 50 µL of dH_2O to make 100,000 × solution. To dilute 100,000 × solution to 1 ×, add dH_2O.
6. Mounting media (*see* **Section 2.4.1**; 14).
7. Coverslips 25 × 50 mm, #1 (cat. no. 12545F, Fisher Scientific).
8. Microscope (e.g., Nikon Eclipse E600) equipped with epifluorescence.
9. PBS-Ca-BSA (*see* **Section 2.4.1**; 6).

2.5.2. Primary Antibodies

a. Guinea pig anti-mouse keratin 19. A site-directed polyclonal antibody was raised in guinea pig against the specific amino acid sequence 391-404 (NH2-Glu-Ala-His-Tyr-Asn-Asn-Leu-Pro-Thr-Pro-Lys-Ala-Ile-OH) of mouse keratin 19 (6). Sera were tested and used at dilutions of 1:500 to 1:2000 in PBS-Ca-BSA.

b. Mouse anti-BrdU-FITC. Fluorescein isothiocyanate (FITC)-conjugated mouse IgG_1 anti-BrdU, clone B44, 50 µg/mL (cat. no. 347583, Becton Dickinson). Dilute in PBS-Ca-BSA at a final concentration of 10 µg/mL (*see* **Note 5**).

c. Unrelated mouse IgG_1-FITC (control antibody for mouse anti-BrdU-FITC). FITC-conjugated mouse IgG_1, clone X40 (cat. no. 349041, Becton Dickinson). Dilute in PBS-Ca-BSA at the same concentration than the mouse anti-BrdU-FITC.

2.5.3. Secondary Antibodies

d. Goat anti-guinea pig-DTAF. Fluorescein dichlorotriazine (DTAF)-conjugated affinipure goat anti-guinea pig IgG (H+L) (cat. no. JPG015003, Accurate Chemical & Scientific Corporation, Westbury, NY). Use at a dilution of 1:100 in PBS-Ca-BSA (*see* **Note 5**).

e. Goat anti-guinea pig-Texas red. Texas red-conjugated affinipure goat anti-Guinea Pig IgG (H+L) (cat. no. 106-075-003, Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). Use at a dilution of 1:200 in PBS-Ca-BSA (*see* **Note 5**).

2.6. Monolayer Culture of Human Keratinocytes with an Irradiated S3T3 Feeder Layer on Coverslips

2.6.1. Keratinocyte Culture Medium Preparation

1. Preparation of keratinocyte culture medium (for final concentration *see* **Table 27.1**):
   a. DME-Ham. Dulbecco's modified Eagle's medium (DME) (cat. no. 12800, Invitrogen, Oakville, ON):Ham's F12 medium (cat. no. 21700, Invitrogen), 3:1, 3.07 g/L $NaHCO_3$ (36.54 mM) (cat. no. S233, Fisher Scientific), 24.3 mg/L adenine (0.18 mM) (cat. no. A2786, Sigma-Aldrich), 312.5 µL/L 2 N HCl (cat. no. SA431-500, Fisher Scientific). Dissolve in apyrogenic ultrapure water. Adjust pH to 7.1. Sterilize by filtration through a 0.22 µm low-binding disposable filter. Aliquot and store at 4°C.

Table 27.1
Keratinocyte culture medium

Component	Quantity (mL)	Final concentration
DME-Ham	950	95% (v/v)
Fetal clone II	50	5% (v/v)
Insulin 1000 ×	1	5 µg/mL
Hydrocortisone 500 ×	2	0.4 µg/mL
Cholera toxin 1000 ×	1	10^{-10}M
Epidermal growth factor 1000 ×	1	10 ng/mL
Penicillin G-gentamicin 500 ×	2	Penicillin G 100 IU/mL Gentamicin 25 µg/mL

   b. Fetal clone II serum (cat. no. SH30066, HyClone, Logan, UT). Thaw in cold water. Inactivate in hot water (56°C) for 30 minutes. Distribute in single-use aliquots and store at –20°C.
   c. Insulin 1000 × (cat. no. I1882, Sigma-Aldrich). Dissolve 250 mg in 50 mL 0.005 N HCl (125 µL 2 N HCl/50 mL apyrogenic ultrapure water) to make a 1000 × stock solution (0.87 mM). Sterilize by filtration through a 0.22 µm low-binding disposable filter, distribute in single-use aliquots, and store at –80°C.
   d. Hydrocortisone 500 × (cat. no. 386698, Calbiochem, San Diego, CA). Dissolve 25 mg in 5 mL of 95% ethanol (4.8 mL 99% ethanol (Les Alcools de Commerce Inc., Brampton, ON)/0.2 mL apyrogenic ultrapure water. Complete to 125 mL with DME-Ham to make a

500 × stock solution (0.53 mM). Sterilize by filtration through a 0.22 μm low-binding disposable filter, distribute in single-use aliquots, and store at –80°C.

e. Cholera toxin 1000 × (cat. no. C8052, Sigma-Aldrich). Dissolve 1 mg in 1 mL of apyrogenic ultrapure water. Complete to 118.18 mL with DME-Ham supplemented with 10% (v/v) Fetal clone II to make a 1000 × stock solution (10^{-7} M). Sterilize by filtration through a 0.22 μm low-binding disposable filter, distribute in single-use aliquots, and store at –80°C.

f. Epidermal growth factor 1000 × (cat. no. GF-010-8, Austral Biologicals, San Ramon, CA). Dissolve 500 μg in 2.5 mL of 10 mM HCl. Complete to 50 mL with DME-Ham supplemented with 10% (v/v) Fetal clone II to make a 1000 × stock solution. Sterilize by filtration through a 0.22 μm low-binding disposable filter, distribute in single-use aliquots, and store at –80°C.

g. Penicillin G and gentamicin 500 × (cat. no. P3032 and G1264, respectively, Sigma-Aldrich). Dissolve 50,000 IU/mL of penicillin G and 12.5 mg/mL of gentamicin sulfate in apyrogenic ultrapure water to make a 500 × stock solution. Sterilize by filtration through a 0.22 μm low-binding disposable filter, distribute in single-use aliquots, and store at –80°C.

2. Thaw all components at 4°C. To make 1 L, refer to **Table 27.1**. Store the culture medium at 4°C.

2.6.2. Irradiated S3T3 Culture Medium Preparation

1. Preparation of irradiated S3T3 (iS3T3) culture medium components (for final concentration, *see* **Table 27.2**).
   a. DME. Dulbecco's modified Eagle's medium (DME) (cat. no. 12800, Invitrogen), 3.7 g/L (44 mM) $NaHCO_3$ (cat. no. S233, Fisher Scientific). Dissolve in apyrogenic ultrapure water. Adjust pH to 7.1. Sterilize by filtration through a 0.22 μm low-binding disposable filter, aliquot, and store at 4°C.

Table 27.2
iS3T3 culture medium

Component	Quantity (mL)	Final concentration
DME	900	90% (v/v)
Fetal calf serum	100	10% (v/v)
Penicillin G-gentamicin 500 ×	2	Penicillin G 100 IU/mL Gentamicin 25 μg/mL

b. Fetal calf serum (cat. no. SH30396, HyClone). Thaw in cold water. Inactivate in hot water (56°C) for 30 minutes. Distribute in single-use aliquots and store at –20°C.

c. Penicillin G-gentamicin 500 ×.

2. Thaw all components at 4°C. To make 1 L, refer to **Table 27.2**.

3. The iS3T3 culture medium can be stored at 4°C for 10 days.

2.6.3. Materials

1. 75 cm^2 culture flask of keratinocytes cultured on iS3T3 feeder layer at 80% confluence as described (7).

2. Keratinocyte culture medium (*see* **Section 2.6.1**).

3. Irradiated Swiss 3T3 (iS3T3) (#CCL-92, ATCC, Manassas, VA). To obtain about 8–10 × 10^6 cells, seed 1 × 10^6 cells in a 75 cm^2 culture flask (tissue culture flask; cat. no. 35310, BD Falcon, Franklin Lakes, NJ) with 20 mL of DME. Incubate for 4 days in 8% CO_2, 100% humidity atmosphere at 37°C. Irradiate at 6000 rads with a Gammacell irradiator (^{60}Co source) (*see* **Note 6).**

4. Culture-phosphate-buffered saline-penicillin G/gentamicin (culture-PBS-P/G): 137 mM NaCl (cat. no. S271, Fisher Scientific), 2.7 mM KCl (cat. no. P285, Fisher Scientific), 6.5 mM Na_2HPO_4 (cat. no. S374, Fisher Scientific), 1.5 mM KH_2PO_4 (cat. no. P217, Fisher Scientific). Dissolve in apyrogenic ultrapure water to make a 10 × stock solution. Store at room temperature. To dilute 10 × PBS to 1 ×, add apyrogenic ultrapure water. Verify pH is 7.4. Sterilize by filtration through a 0.22 μm low-binding disposable filter. Store at room temperature. Before use, add penicillin G-gentamicin 500 × stock solution by diluting these additives to 1 ×. Store at 4°C.

5. Trypsin/EDTA. 2.8 mM D-glucose (cat. no. DX0145, EMD Chemicals Inc.), 0.05% (w/v) trypsin 1-500 (cat. no. 7003, Intergen, Toronto, ON), 0.00075% (v/v) phenol red (Phenol red solution 0.5%, sterile-filtered, cat. no. P0290, Sigma-Aldrich), 100 000 IU/L penicillin G (cat. no. P3032, Sigma-Aldrich), 25 mg/L gentamicin (cat. no. G1264, Sigma-Aldrich), 0.01% (w/v) EDTA (EDTA, disodium salt, cat. no. 8993, J.T. Baker, Phillipsburg, NJ). Dissolve in 1 × culture-PBS-P/G. Adjust pH to 7.45. Sterilize by filtration through a 0.22 μm low-binding disposable filter, distribute in single-use aliquots, and store at –20°C.

6. 50 mL tube (cat. no. 352070, BD Falcon).

7. Trypan blue (cat. no. T8154, Sigma-Aldrich).

8. Hemacytometer (cat. no. 1475, Hausser Scientific, Buffalo, NY).

9. 6-well culture plate (cat. no. 35046, Becton Dickinson).

10. Coverslips 22 × 22 mm, #1 (cat. no. 12-542B, Fisher Scientific).

11. 5-bromo-2-deoxyuridine 1 mM (BrdU 1 mM) (cat. no. B5002, Sigma-Aldrich). Wear mask and gloves. Dissolve 31 mg BrdU in 10 mL of culture-PBS-P/G. Sterilize by filtration through a 0.22 μm low-binding disposable filter, distribute in single-use aliquots, and store at –80°C.
12. Methanol (cat. no. L-6778, Fisher Scientific). Cool at –20°C.
13. Formaldehyde 0.37% (*see* **Section 2.4.1**; 2).
14. Parafilm (cat. no. PM-992, Pechiney, Menasha, WI).

2.7. Double Immunofluorescence Staining of BrdU and K19 in Monolayer Culture of Human Keratinocytes

2.7.1. Materials

1. Microscope slide double frosted 25 × 75 × 1 mm (cat. no. 12-552-5, Fisher Scientific).
2. NaOH 0.07 N (*see* **Section 2.4.1**; 4).
3. PBS-Ca (*see* **Section 2.4.1**; 5).
4. PBS-Ca-BSA (*see* **Section 2.4.1**; 6).
5. Hoechst (*see* **Section 2.5.1**; 5).
6. Mounting media (*see* **Section 2.4.1**; 14).

2.7.2. Primary Antibodies

a. Mouse anti-BrdU. *See* **Section 2.4.2**; a.

b. Unrelated anti-mouse IgG_1 (control antibody). *See* **Section 2.4.2**; b.

c. Mouse anti-human K19-Alexa Fluor® 488. Mouse monoclonal IgG_{2a} anti-cytokeratin peptide 19, clone CCD-27 (cat. no. CRL-1475, ATCC). The mouse anti-human K19 antibody was conjugated to Alexa Fluor® 488 using the Alexa Fluor® 488 protein labeling kit (cat. no. A10235, Invitrogen) according to the specifications of Invitrogen. Dilute in PBS-Ca-BSA at a final concentration of 5 μg/mL (*see* **Note 5**).

d. Unrelated anti-mouse IgG_{2a}-Alexa Fluor® 488 (cat. no. 557703, BD Pharmingen™, Mississauga, ON). Dilute in PBS-Ca-BSA at the same concentration than the mouse anti-human K19-Alexa Fluor® 488.

2.7.3. Secondary Antibody

a. Goat anti-mouse-rhodamine. Rhodamine-conjugated goat anti-mouse IgG_1 (cat. no. AP-130R, Chemicon, Temecula, CA) (*see* **Note 5**).

3. Methods

3.1. In Vivo BrdU Labeling of Slow-Cycling Cells (Label-Retaining Cell Method)

All further manipulations were approved by the institution's animal care and use committee (Comité de protection des animaux de laboratoire de l'Université Laval).

1. Thaw BrdU 1.5 mg/mL aliquots at room temperature.

2. Manipulate neonate mice with gloves (*see* **Note 7**).
3. Weigh neonate mice (the average weight of a neonate mouse is 1–1.5 g). To inject mice with 50 mg BrdU/kg of body weight, multiply mouse weight by 0.033 to obtain the volume of BrdU 1.5 mg/mL solution to inject.
4. Inject each mouse intraperitoneally with 50 mg BrdU/kg of body weight.
5. Repeat steps 1 to 4 twice daily, every 12 hours, for the first 5 days of post-natal life.
6. Sacrifice the mice 28 days after birth, excise pieces of skin, and process for tissue preservation immediately (*see* **Section 3.3**).

3.2. In Vivo BrdU Labeling of Actively Dividing Cells

All further manipulations were approved by the institution's animal care and use committee (Comité de protection des animaux de laboratoire de l'Université Laval).

1. Thaw BrdU 10 mg/mL aliquots at room temperature.
2. Weigh mice (the average weight of a mouse is 10–30 g). To inject mice with 50 mg BrdU/kg of body weight, multiply mouse weigh by 0.005 to obtain the volume of BrdU 10 mg/mL solution to inject.
3. Inject each mouse with syringe and needle intraperitoneally with 50 mg BrdU/kg of body weight twice daily, every 12 hours.
4. Sacrifice the mice 1 day after the BrdU injections, excise pieces of skin, and process for tissue preservation immediately (*see* **Section 3.3**).

3.3. Tissue Preservation and Sectioning

1. Cut external hairs with curved scissors.
2. Cut the mouse biopsies from different anatomical sites into small pieces (2–5 mm wide rectangles).
3. Sponge the tissue fluid excess with absorbent paper.
4. Coat completely the tissue with OCT. Try to eliminate bubbles.
5. Put liquid OCT on a tissue holder and use forceps to immerse it for 2–3 seconds in liquid nitrogen, remove it before the OCT turns completely white.
6. Take the OCT-coated piece of tissue using forceps and place it on the liquid OCT on the tissue holder.
7. Add OCT on top of the tissue sample to coat completely the tissue to prevent if from freeze-drying.
8. Take the tissue holder with forceps and immerse it in liquid nitrogen for approximately 15 seconds. The OCT should be white but should not crack.
9. Store OCT blocks at –80°C until use.
10. Cut the tissue in a cryostat (4–6 μm thick sections) and place the tissue sections on superfrost glass slides.

11. Dry the sections on a warm plate at 37°C for 30 minutes.
12. Process slides for immunohistochemistry or store the slides at −20°C (they can be kept for few weeks).

3.4. Immunoperoxidase Staining of BrdU on Tissue Cryosection

1. Take the tissue sections from the freezer and leave them at room temperature for 5 minutes to remove excess of humidity. If the tissue sections are small, they can be encircled by etching with a diamond pencil.
2. Formaldehyde fixation. Cover each tissue section with 25–50 µL of formaldehyde 0.37% solution. Incubate 10 minutes at room temperature.
3. PBS-Ca washes. Put the slides in a microscope slide rack and immerse it in a bath of PBS-Ca for 5 minutes. Repeat twice.
4. Methanol fixation. Fix the tissue sections by immersion in a bath of cold methanol for 10 minutes at –20°C.
5. Do three PBS-Ca washes (*see* step 3).
6. DNA dehybridization. Cover each tissue section with 25 µL of NaOH 0.07 N. Incubate 15 seconds at room temperature.
7. Do three PBS-Ca washes (*see* step 3).
8. Blocking of endogenous peroxidase activity. Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 25–50 µL of warm (37°C) hydrogen peroxide 0.3%. Incubate 5 minutes at room temperature.
9. Do three PBS-Ca washes (*see* step 3).
10. Blocking step. This step helps to reduce the non-specific binding of the secondary antibodies to mouse endogenous immunoglobulins. Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 25–50 µL of M.O.M.™ blocking reagent. Incubate 45 minutes at room temperature.
11. Do three PBS-Ca washes (*see* step 3).
12. Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 25–50 µL of M.O.M.™ diluent. Incubate 5 minutes at room temperature.
13. Primary antibody incubation. Remove the excess of M.O.M.™ diluent and cover each tissue section with 25–50 µL of primary antibody diluted in M.O.M.™ diluent. Incubate 45 minutes at room temperature.
14. Do three PBS-Ca washes (*see* step 3).
15. Biotinylated secondary antibody incubation. Aspirate the liquid surrounding the tissue sections. Process rapidly to

avoid drying the tissue. Cover each tissue section with 25–50 µL of the ID Labs™ linking reagent (yellow solution). Incubate 20 minutes at room temperature.

16. Do three PBS-Ca washes (*see* step 3).
17. Streptavidin-HRP incubation. Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 25–50 µL of the I Labs™ staining reagent (red solution). Incubate 20 minutes at room temperature.
18. Do three PBS-Ca washes (*see* step 3).
19. DAB revelation. Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 100 µL of DAB. Incubate 2–5 minutes (*see* **Note 8**). The peroxidase product is brownish (*see* **Fig. 27.1Aa,b,g,h**). It is recommended to determine the appropriate revelation time for each experiment. Then reveal each tissue section and its appropriate control for the exact same time. Stop the reaction by immersion in a dH_2O bath.
20. Wash in dH_2O for 1 minute, repeat twice.
21. Harris's hematoxylin counterstain. Harris's hematoxylin counterstain is performed to visualize cell nuclei (*see* **Fig. 27.1Aa,b,g,h**).
    a. Immerse sections in the filtered Harris hematoxylin for 1 minute.
    b. Rinse in running tap water until the water is clear.
22. Wash in dH_2O for 1 minute, repeat three times.
23. Mounting slides:
    a. Warm the solid mounting media in warm water or in the microwave to liquefy it. Be careful to avoid a too high brought up temperature.
    b. For tissues on slides: place 1 drop of mounting media on each tissue section.
    c. Put a coverslip gently on the top, avoiding bubbles.
    d. Remove the excess of mounting media by draining on absorbent paper.
    e. Store at 4°C in the dark.
24. Examine the tissue sections under a light microscope.

3.5. Simple or Double Immunofluorescence Staining of BrdU and K19 on Tissue Cryosection

1. Take the tissue sections from the freezer and leave them at room temperature for 5 minutes to remove excess humidity. If the tissue sections are small, they can be encircled by etching with a diamond pencil.
2. Acetone fixation. Fix the tissue sections by immersion in cold acetone for 10 minutes at –20°C.

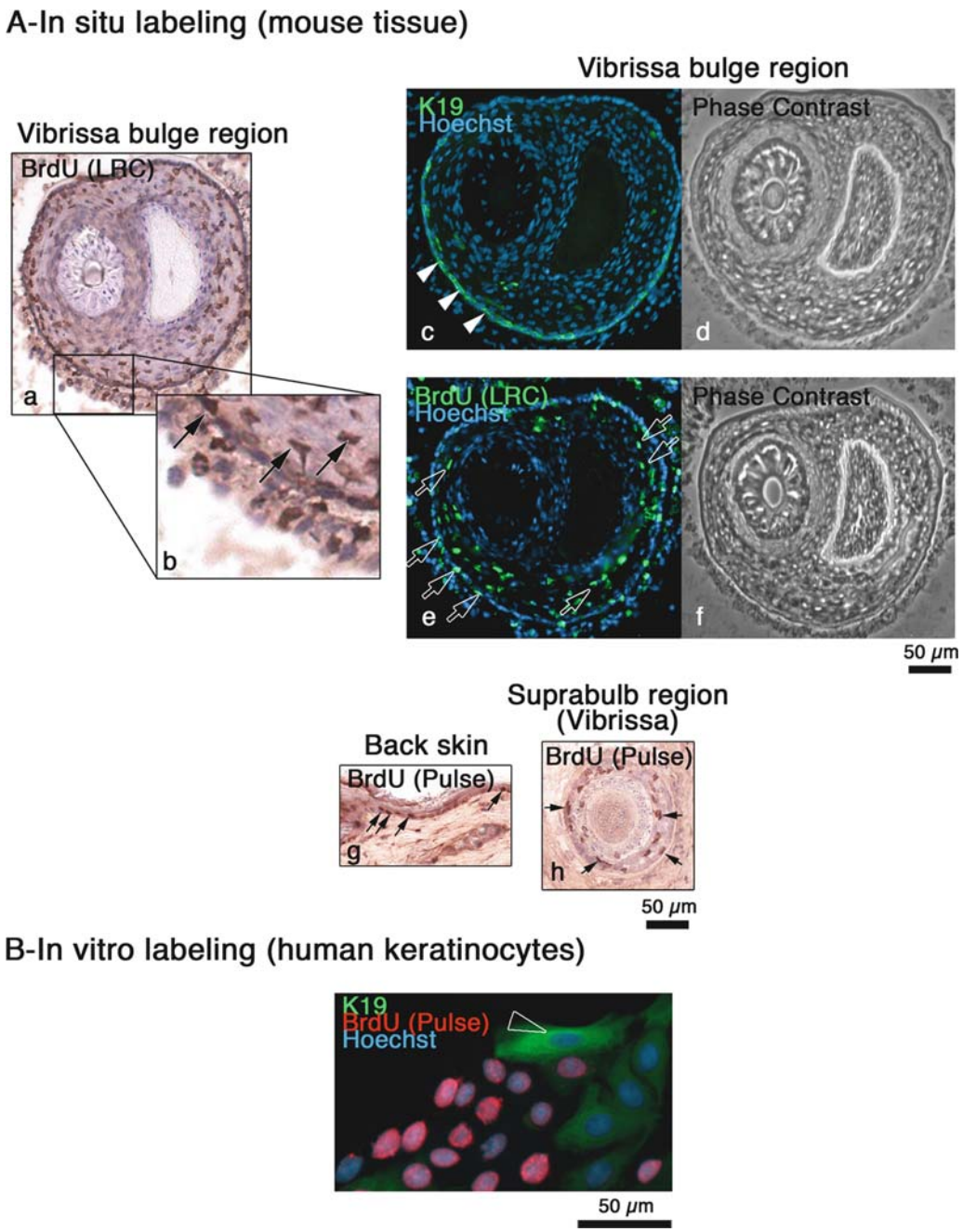

Figure 27.1. (**A)** BrdU labeling of mouse tissue. (a–f) Consecutive vibrissa bulge sections from adult FVB mouse that have received repeated BrdU injections between day 1 and 5 after birth, as described in **Section 3.1**. (d, f) Phase contrast micrographs corresponding to (c) and (e), respectively. (b) High magnification of boxed area in (a). (g, h) Vibrissa bulge sections from adult FVB mouse that have received two BrdU injections 1 day before the biopsy was taken, as described in **Section 3.2**. (a, b, g, h) Label-retaining cells (LRC) identified by immunoperoxidase labeling of BrdU as described in **Section 3.4**. Note the brownish coloration of BrdU-labeled nuclei (arrows in a, b, g, h). (c, e) Immunofluorescence staining of K19 (green staining, arrowheads in c) and BrdU (green staining, open arrows in e) on consecutive sections, as described in **Section 3.5**. **(B)** BrdU labeling of human keratinocytes cultured in monolayer. Double immunofluorescence labeling of K19 (green, open arrowhead) and BrdU (red nuclei) as described in **Section 3.7**.

3. PBS-Ca washes. Put the slides in a rack and immerse it in a bath of PBS-Ca for 5 minutes. Repeat twice.
4. For a single labeling of BrdU, go to step 10.
5. First primary antibody incubation. Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 25–50 μL of the primary antibody. Put PBS-Ca-BSA or the unrelated antibody on the control slides. Incubate 45 minutes at room temperature. For BrdU-K19 double immunofluorescence staining, use the guinea pig anti-mouse keratin 19 as the primary antibody.
6. Do three PBS-Ca washes (*see* step 3).
7. Secondary antibody incubation. Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 25–50 μL of the secondary antibody (anti-guinea pig-Texas red). Incubate 30 minutes at room temperature. Protect from light. For double immunofluorescence staining of K19 and BrdU, use the goat anti-guinea pig-Texas red as the secondary antibody. For single immunofluorescence staining of K19, goat anti-guinea pig-DTAF secondary antibody can also be used, then go to step 10.
8. Do three PBS-Ca washes (*see* step 3).
9. Second primary antibody incubation. Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 25–50 μL of the primary antibody (mouse anti-BrdU-FITC). Use the unrelated mouse IgG_1-FITC as control. Incubate 45 minutes at room temperature. Protect from light.
10. Do three PBS-Ca washes (*see* step 3).
11. The tissue sections or cells can be counterstained with Hoechst (nuclei staining) as follows:
    a. Distilled H_2O washes. Put the slides in a rack, and immerse it in two successive baths of dH_2O, 2 minutes by wash. Be careful to avoid detachment of the tissue sections from the slides. Repeat twice.
    b. Cover with 25–50 μL of the 1 × Hoechst solution.
    c. Incubate 10 minutes in the dark.
    d. Do three dH_2O washes (*see* step a).
12. Mounting slides. *See* **Section 3.4**; 23.
13. Store at 4°C in the dark to avoid any bleaching of the fluorescence.
14. Tissue sections are examined under a microscope equipped with epifluorescence. For an example of the single immunofluorescence staining of K19 and BrdU on consecutive sections of vibrissa follicles, see **Fig. 27.1A**; c and e, respectively.

3.6. Monolayer Culture of Human Keratinocytes

We describe here the monolayer culture of human keratinocytes on glass coverslips, from the trypsinization of keratinocytes already in culture. For a complete description of the extraction, culture, subculture, cryopreservation, and thawing of human keratinocytes, see Larouche et al. (7).

3.6.1. Culture of Human Keratinocytes

All further manipulations are performed under a sterile laminar flow hood cabinet.

From a 75 cm^2 culture flask of keratinocytes cultured on iS3T3 feeder layer at 80% confluence:

1. Wash the culture flask with 2 mL of warm (37°C) trypsin/EDTA and remove it.
2. Add 8 mL of trypsin/EDTA. Incubate at 37°C until the cells are detached from the flask.
3. Add 8 mL of keratinocyte culture medium (37°C). Collect the cell suspension. Put into a 50 mL tube. Wash the flask with 2 mL of keratinocyte culture medium, collect the cell suspension, and add it to the 50 mL tube (total 18 mL).
4. Count the cells and measure the viability by trypan blue staining. The cell viability should be superior to 80%.
5. Centrifuge cell suspension at 300 × *g* for 10 minutes at room temperature.
6. In the 6-well culture plate, put one 22 mm × 22 mm glass coverslip by well.
7. Resuspend cell pellet in a given volume of complete keratinocyte culture medium (*see* step 8).
8. Seed 3125 keratinocytes and 20,833 iS3T3 by cm^2: prepare a cellular suspension containing 15,000 keratinocytes and 100,000 iS3T3 by mL of keratinocyte culture medium. With a 45° angle, drip in drops with a plastic pipet 2 mL of the cellular suspension in the center of each coverslip. Be careful with the 6-well plate manipulation to limit the liquid vortex effect leading to heterogeneous distribution of the cells.
9. Incubate in 8% CO_2, 100% humidity atmosphere at 37°C. Change culture medium three times a week.
10. To mark proliferative cells, add 10 µL of BrdU 1 mM by mL of keratinocyte culture medium, for a final BrdU concentration of 10 µM 1 day before the end of the experiment.
11. Cell fixation:
    a. Remove the keratinocyte culture medium.
    b. Culture-PBS-P/G washes. Put 2 mL of cold (4°C) culture-PBS-P/G by well, wait 2 minutes, and remove it. Repeat twice.
    c. Put 2 mL of cold methanol 100% by well. Incubate 10 minutes at –20°C.

d. Do three culture-PBS-P/G washes (*see* step b).

e. Seal the 6-well culture plate cover with parafilm to avoid that liquid dries up.

f. Keep at 4°C until labeling.

3.7. Simple or Double Immunofluorescence Staining of BrdU and K19 in Monolayer Culture of Human Keratinocytes

Further manipulations are performed on human keratinocytes cultured on glass coverslips as described in **Section 3.6**.

1. Aspirate completely the culture-PBS-P/G surrounding the coverslips. Process rapidly to avoid drying the fixed cells.
2. DNA dehybridization. Cover each coverslip with 300 µL of NaOH 0.07 N for 15 seconds.
3. PBS-Ca washes. Put 2 mL of PBS-Ca per well, wait 5 minutes, and remove it. Repeat twice.
4. First primary antibody incubation. Aspirate the liquid surrounding the coverslips. Process rapidly to avoid drying the fixed cells. Cover each coverslip with 150 µL of the primary antibody. Put PBS-Ca-BSA or the unrelated antibody on the control samples. For double immunofluorescence staining of BrdU and K19, put the mouse anti-BrdU as the primary antibody. Incubate 45 minutes at room temperature.
5. Do three PBS-Ca washes (*see* step 3).
6. Secondary antibody incubation. Aspirate the liquid surrounding the coverlips. Process rapidly to avoid drying the fixed cells. Cover each coverslip with 150 µL of the secondary antibody (anti-mouse-rhodamine) diluted in PBS-Ca-BSA. Incubate 30 minutes at room temperature. Protect from light.
7. Do three PBS-Ca washes (*see* step 3).
8. Second primary antibody incubation. Aspirate the liquid surrounding the coverslips. Process rapidly to avoid drying the fixed cells. Cover each coverslip with 150 µL of the primary antibody (mouse anti-K19-Alexa Fluor® 488). Use the unrelated mouse IgG_{2a}-Alexa Fluor® 488 as control. Protect from light.
9. Do three PBS-Ca washes (*see* step 3).
   a. Cells can be counterstained with Hoechst (nuclei staining). *See* **Section 3.5**; 11.
10. Mounting slides. For fixed cells on coverslips:
    a. Place 1 drop of mounting media on a microscope slide. Gently place the coverslip with the cells facing down on the mounting media, avoiding bubbles.
    b. Remove the excess of mounting media by draining on absorbent paper.
11. Store at 4°C in the dark to avoid any bleaching of the fluorescence.

12. Cells fixed on coverslips are examined under a microscope, equipped with epifluorescence. For an example of the double immunofluorescence staining of K19 and BrdU in cultured human keratinocytes, see **Fig. 27.1B**.

4. Notes

1. It is recommended to have pregnant females and to wait for the newborn delivery.
2. The label-retaining cells method can be adapted for rats.
3. Five times concentrated PBS solution can be prepared and kept at 4°C, but the $CaCl_2$ should be omitted, and added only after dilution of PBS to 1 ×. Ten times concentrated solution can also be prepared and kept at 4°C.
4. DAB solution must be used within 6 hours.
5. The working dilution can differ according to the product lot. It is recommended that user determines its optimal working dilution by titration assay.
6. Irradiated S3T3 can be kept 1 week in 8% CO_2, 100% humidity atmosphere at 37°C. However, the cell yield may fall by approximately 10–15% per day.
7. Manipulation of neonate mice with gloves avoids their rejection by the mother (because of the human smell).
8. The revelation time should be adjusted in function of the tissue. Follow the reaction under a phase microscope to evaluate the optimal revelation time.

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Chapter 28

Isolation and Culture of Hair Follicle Pluripotent Stem (hfPS) Cells and Their Use for Nerve and Spinal Cord Regeneration

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Abstract

The hair follicle is dynamic, cycling between growth (anagen), regression (catagen), and resting (telogen) phases throughout life. We have demonstrated that nestin-expressing hair follicle stem cells give rise to follicle structures during early anagen or growth phase of the hair follicle. Nestin-expressing hair follicle stem cells appear in the hair follicular stem cell area, the permanent upper hair follicle immediately below the sebaceous glands and above the bulge area. The nestin-expressing hair follicle stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro. Furthermore, the hair follicle stem cells promote the recovery of peripheral nerve and spinal cord injury. We have termed these cells hair follicle pluripotent stem (hfPS) cells. These results suggest that hfPS cells provide an important accessible, autologous source of adult stem cells with potential for use in regenerative medicine.

Key words: Hair follicle, Nestin, Stem cell, Hair follicular stem cell area, Differentiation, Neuron, Glial cell, Melanocyte.

1. Introduction

The hair follicle bulge area has an abundance of, easily accessible actively-growing, pluripotent adult stem cells. Nestin, a protein marker for neural stem cells, also is expressed in follicle stem cells and their immediate, differentiated progeny (1). The fluorescent protein GFP, whose expression is driven by the nestin regulatory element in transgenic mice, can be used to image the fate of the hair follicle stem cells. The pluripotent nestin-driven GFP stem cells are positive for the stem cell marker CD34 but negative for keratinocyte marker keratin 15, suggesting their relatively undifferentiated state. These cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells,

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and melanocytes in vitro (2). In vivo studies show the nestin-driven GFP hair follicle stem cells can differentiate into blood vessels and neural tissue after transplantation to the subcutis of nude mice (3).

Equivalent hair follicle stem cells, derived from transgenic mice with beta-actin-driven GFP, implanted into the gap region of a severed sciatic nerve greatly enhance the rate of nerve regeneration and the restoration of nerve function. The follicle cells transdifferentiate largely into Schwann cells, which are known to support neuron regrowth. Function of the rejoined sciatic nerve was measured by contraction of the gastrocnemius muscle upon electrical stimulation. After severing the tibial nerve and subsequent transplantation of hair follicle stem cells, walking print length and intermediate toe spread significantly recovered, indicating that the transplanted mice recovered the ability to walk normally (4).

Hair follicle stem cells were also transplanted to the severed spinal cord in mice. Most of the transplanted cells also differentiated into Schwann cells that apparently facilitated repair of the severed spinal cord. The rejoined spinal cord re-established extensive hind-limb locomotor performance. These results suggest that hair follicle stem cells can promote the recovery of spinal cord injury. Thus, hair follicle stem cells provide an effective accessible, autologous source of stem cells for the promising treatment of peripheral nerve and spinal cord injury (5).

In the mouse, nestin-expressing stem cells are located in the upper hair follicle immediately below the sebaceous glands just above the hair follicle bulge area. The nestin-expressing stem cells are K15-negative. Nestin-negative, K15-positive cells, on the other hand, are located in the bulge area of the mouse hair follicle and can differentiate only to keratinocytes (6).

Analogous to the mouse, in the intact human hair follicle dissected from the scalp, the cells immediately below the sebaceous glands just above the bulge area are nestin-positive and K15-negative. In contrast, the hair follicle stem cells in the bulge are nestin-negative, K15-positive (6).

Thus, the hair follicles of mice and men appear to have two populations of stem cells: a pluripotent type and an apparent unipotent type. These stem cells have potential for regenerative medicine and hair growth, respectively. Our results suggest that, the hair follicle stem cells have clinical potential as adult stem cells due to easy access and capability for differentiation (6). We have termed these stem cells hair follicle pluripotent stem (hfPS) cells (12).

2. Materials

2.1. Reagents

1. Transgenic mice with nestin-regulatory-element-driven green fluorescent protein (ND-GFP mice) (7)

2. GFP-expressing transgenic mice (GFP mice) (Jackson Laboratories) (8); C57BL/6 mice and C57BL *nu/nu* mice (e.g., Charles River Labs, Wilmington, MA, Taconic Labs Germantown, NY or Harlan Teklad, Los Angeles, CA)
3. DMEM-F12 (GIBCO-BRL, Grand Island, NY) containing B-27 (GIBCO-BRL) and 1% penicillin–streptomycin (GIBCO-BRL) with 1% methylcellulose (Sigma-Aldrich St. Louis, MO). The culture was supplemented every 2 days with basic FGF at 20 ng/ml (Chemicon, Temecula, CA)
4. RPMI medium 1640 (Cellgro, Herndon, VA) containing 10% FBS
5. 96-well uncoated tissue-culture dishes (BD Biosciences, San Jose, CA)
6. SonicSeal four-well chamber slides (Nunc)
7. Anti-βIII-tubulin mAb (1:500, Tuj1 clone; Covance Research Products, Berkeley, CA)
8. Anti-neurofilament 200 polyclonal Ab (1:80; Sigma-Aldrich)
9. Anti-GABA polyclonal Ab (1:200; Chemicon)
10. Anti-neuronal-specific enolase mAb (1:800; Lab Vision, Fremont, CA)
11. Anti-tyrosine hydroxylase polyclonal Ab (1:100; Chemicon)
12. Anti-glial fibrillary acidic protein (GFAP) mAb (1:100; Molecular Probes, Invitrogen, Carlsbad, CA)
13. Anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) mAb (1:50; Lab Vision)
14. Anti-keratin 5/8 (K5/8) mAb (1:250; Chemicon)
15. Anti-keratin 15 (K15) mAb (1:100; Lab Vision)
16. Anti-smooth muscle actin mAb (1:200; Lab Vision)
17. Anti-BrdUrd mAb (1:10; BD PharMingen, San Jose, CA)
18. Anti-CD31 mAb (1:50; Chemicon)
19. Anti-CD34 mAb (1:10; BD PharMingen)
20. The following are secondary Abs: Alexa Fluor® 568-conjugated goat anti-mouse (1:200; Molecular Probes); Alexa Fluor® 568-conjugated goat anti-rabbit (1:200; Molecular Probes); and Alexa Fluor® 647-conjugated chicken anti-rat (1:200; Molecular Probes)
21. Mouse-on-Mouse (MOM) immunodetection kit (Vector Laboratories, Burlingame, CA)
22. Ig horseradish peroxidase detection kit (BD PharMingen)

2.2. Equipment

1. OV-100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan)

2. IMT-2 inverted microscope equipped with a mercury lamp power supply (Olympus)
3. Hamamatsu C5810 three-chip cooled color CCD camera (Hamamatsu Photonics Systems, Hamamatsu-city, Japan)
4. FluorVivo Imaging System (Indec Systems, Santa Clara, CA) iBox Imaging System (UVP LLC, Upland, CA)
5. Sony VCR model SLV-R1000 (Sony)
6. Image Pro Plus 3.1 software (Media Cybernetics, Bethesda, MA)
7. 1 ml 27G2 latex-free syringe (BD Bioscience)
8. 25-ml Hamilton syringe (Fisher Scientific, Santa Ana, CA)
9. D470/40 excitation filter (Chroma Technology, Rockingham, VT)
10. GG475 emission filter (Chroma Technology)
11. Cloning cylinders (Bel-Art Products, Pequannock, NJ)
12. Hemocytometer (Reichert Scientific Instruments)
13. Blunt-end hook (Fine Science Tools, Foster City, CA)
14. 33-gauge needle (Fine Science Tools)

2.3. Equipment Setup

2.3.1. Whole-Body Imaging Equipment

The Olympus OV-100 small animal imaging system (Olympus), containing an MT-20 light source and a DP70 CCD camera, can be used for whole-body and skin-flap imaging in live mice at variable magnifications (9). The optics of the OV100 fluorescence imaging system have been specially developed for macro-imaging as well as micro-imaging with high light gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Four individually optimized objective lenses, parcentered and parfocal, provide a 10^5-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has lenses mounted on an automated turret with a high magnification range of $\times 1.6$ to $\times 16$ and a field of view ranging from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images are captured directly on a PC (Fujitsu Siemens). Images are processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 (Corel) and Cell (Olympus Biosystems).

Many other fluorescence imaging systems can also be used. For example, a Leica fluorescence stereo microscope (model MZ16) equipped with a mercury 50 W lamp power supply can be used. Selective excitation of GFP is produced through a D425/60 band pass filter and 470 DCXR dichroic mirror. Emitted fluorescence is collected through a longpass filter (GG475; Chroma Technology). Under anesthesia, the experimental animals can be examined with the microscope and the images can be

acquired with a Hamamatsu C5810 three-chip cooled color charge-coupled-device camera (Hamamatsu Photonics Systems). Images can also be processed for contrast and brightness and analyzed with the use of Image-Pro Plus software (Media Cybernetics). High-resolution images of 1,024 × 724 pixels can be captured directly on a PC or continuously through video output on a high-resolution VCR (e.g., Sony model SLV-R1000).

Alternatively, the FluorVivo Imaging System (Indec Systems) can be used with any type of CCD camera. The FluorVivo has its own measurement software. FluorVivo uses extremely bright, solid state, LED illuminators and a full color CCD camera to provide high-speed, multi-color imaging of up to three animals with single exposures. The instrument's high speed acquisition permits *in vivo* monitoring of both static and dynamic processes, as well as real-time recordings of fluorescence-guided surgeries. FluorVivo's fully integrated software provides complete control of the instrument, ease of use, and powerful analytical tools for extracting quantitative data from acquired images.

The UVP iBox Small Animal Imaging System (UVP LLC) is capable of fluorescent protein imaging with a range of cameras that use front and back illuminated CCDs with sizes up to a 43 mm diagonal, greatly expanding the applications for high resolution, large-field-of-view and increased-throughput imaging. The iBox imaging system can be configured with both monochrome and color CCDs, with CCD resolution currently up to 8.3 megapixels and sensitive to a wide range of spectrum (CFP to near infrared). The range of fast lenses includes several interchangeable, fully automated optics: a 50 mm f1.2, 28 mm f 1.8, and a 24 -70 mm f2.8 zoom lens. These lenses give maximum imaging flexibility, with the field of view ranging from one to several animals. At f1.2, the typical exposures are less than 50 msec, minimizing the effect of animal movement. The camera, optics, sample platform position, and excitation and emission filters are under full software control, permitting reproducible and rapid imaging with software presets and macros.

Please refer to **Note 1** when using any of the imaging systems.

3. Methods

3.1. Isolation of Mouse Nestin-Expressing Pluripotent Hair Follicle Stem Cells from the Hair Follicle Stem Cell Area

1. Isolate ND-GFP cells from the hair follicle stem cell area. To isolate the vibrissa follicles, cut the upper lip containing the vibrissa pad of ND-GFP mice and expose its inner surface. Dissect the vibrissa follicles under a binocular microscope. Pluck the vibrissa from the pad by pulling them gently by the neck with fine forceps. Wash the isolated vibrissae in DMEM-F12 (GIBCO-BRL) containing B-27 (GIBCO-BRL) and 1%

penicillin–streptomycin (GIBCO-BRL). Do all surgical procedures under a sterile environment. Please refer to **Notes 2–4** when performing the surgical procedures. The hair follicle stem cell area contains ND-GFP-expressing cells. Isolate the cells under fluorescence microscopy. Suspend the isolated cells in 1 ml DMEM-F12 containing B-27 with 1% methylcellulose (Sigma-Aldrich), and 20 ng ml1 basic FGF (bFGF) (Chemicon) (3, 10). Culture the cells in 24-well tissue-culture dishes (Corning) at 37°C in a 5% CO_2 95% air tissue-culture incubator. After 4 weeks, the ND-GFP-expressing hair follicle stem cells form colonies. Please refer to **Notes 5–8**.

2. For differentiation, centrifuge the colonies to remove growth factor-containing DMEM-F12 medium. Resuspend the cells into fresh RPMI medium 1640 (Cellgro, Herndon, VA) containing 10% FBS. Culture colonies in SonicSeal four-well chamber slides (Nunc). Please refer to **Notes 9 and 10**.
3. Clone cells from ND-GFP cell colonies that had been cultured for 2 months by trypsinizating and serially diluting cells into DMEM-F12 containing B-27 in 96-well uncoated tissue-culture dishes (BD Biosciences). Assess each chamber microscopically for the presence of a single cell. Supplement the medium with 1% methylcellulose and 20 ng/ml bFGF. Change the medium every 2 days. After 4 weeks of clonal expansion, switch the ND-GFP colonies to RPMI medium 1640 containing 10% FBS in SonicSeal four-well chamber slides for differentiation. Label ND-GFP cells with BrdUrd for 7 days. Immunostain the cells for anti-βIII-tubulin and anti-BrdUrd mAbs.
4. Isolate ND-GFP-expressing hair follicles from telogen dorsal skin pelage hair follicles under fluorescence microscopy. Wash the follicles in DMEM-F12 containing B-27 and 1% penicillin–streptomycin. Suspend the ND-GFP-expressing hair follicle stem cells in 1 ml DMEM-F12 containing B-27 and 20 ng/ml bFGF. Culture the cells in 24-well tissue-culture dishes at 37°C in 5% CO_2 95% air. The cells form ND-GFP colonies by 4 weeks. For differentiation, transfer the ND-GFP colonies to SonicSeal four-well chamber slides, where the colonies are resuspended in fresh RPMI medium 1640 containing 10% FBS. Transfer ND-GFP colonies to SonicSeal four-well chamber slides in the presence of DMEM-F12 containing B-27 and 20 ng ml bFGF. Please refer to **Notes 9 and 10**.
5. For immunohistochemistry use the following primary Abs: anti-βIII-tubulin mAb (1:500, Tuj1 clone; Covance Research Products, Berkeley, CA); anti-neurofilament 200 polyclonal Ab (1:80; Sigma-Aldrich); anti-GABA polyclonal Ab (1:200; Chemicon); anti-neuronal-specific enolase mAb (1:800; Lab Vision, Fremont, CA); anti-tyrosine hydroxylase polyclonal Ab (1:100; Chemicon); anti-GFAP mAb (1:100; Molecular

Probes); anti-CNPase mAb (1:50; Lab Vision); anti-keratin 5/8 (K5/8) mAb (1:250; Chemicon); anti-keratin 15 (K15) mAb (1:100; Lab Vision); anti-smooth muscle actin mAb (1:200; Lab Vision); anti-BrdUrd mAb (1:10; BD PharMingen); anti-CD31 mAb (1:50; Chemicon); and anti-CD34 mAb (1:10; BD PharMingen). The following secondary Abs were used: Alexa Fluor® 568-conjugated goat anti-mouse (1:200; Molecular Probes); Alexa Fluor® 568-conjugated goat anti-rabbit (1:200; Molecular Probes); and Alexa Fluor® 647-conjugated chicken anti-rat (1:200; Molecular Probes). For BrdUrd immunocytochemistry, treat cells as described above. For quantification of the percentage of cells producing a given marker protein, photograph at least three fields in any given experiment and determine the number of positive cells relative to the total number of cells. Immunocytochemical staining of βIII-tubulin and K15 in the ND-GFP cells is detected with the Mouse-on-Mouse (MOM) immunodetection kit (Vector Laboratories). CD31 and CD34 are detected with the Ig horseradish peroxidase detection kit (BD PharMingen).

3.2. Sciatic Nerve Regeneration Using Hair Follicle Stem Cells

1. Prepare GFP-expressing hair follicle stem cells as described above, but in this case, using C57BL/6 GFP mice.
2. Differentiate hair follicle stem cells as described above.
3. Transplant differentiated GFP-expressing hair follicle stem cell colonies between severed sciatic or tibial nerve fragments in immunocompetent C57BL6 mice under tribromoethanol anesthesia. The skin incision is closed with nylon sutures (6–0). After 2 months, directly observe the sciatic nerve of the transplanted mouse by fluorescence microscopy under anesthesia.
4. Embed the sciatic nerve samples in tissue-freezing embedding medium and keep at –80°C overnight. Cut frozen sections 5 μm thick with a Leica CM1850 cryostat and air-dry. Observe the sections under fluorescence microscopy.
5. Use the frozen sections for the immunofluorescence staining of βIII-tubulin, glial fibrillary acidic protein, K15, and smooth muscle actin as described above. For quantification of the percentage of cells producing a given marker protein in any given experiment, photograph at least three fields and determine the number of positive cells relative to the total number of cells.
6. Observe the sciatic nerve in the live mouse and the excised sciatic nerve directly under an Olympus IMT-2 inverted microscope or equivalent equipped with a mercury lamp power supply. The microscope needs a GFP filter set (Chroma Technology, Brattleboro, VT).

7. Stimulate the sciatic nerve with an electric stimulator. Use an electric stimulator (FGK-1S, Medical Access, Tokyo) that can deliver repetitious electric pulses of 0.05 mA at 10 Hz with pulse widths of 0.5 msec to stimulate control mice, mice with severed sciatic nerves and mice that had GFP hair follicle stem cells injected to join the severed nerve.
8. Measure the gastrocnemius muscle lengths and calculate the difference of the gastrocnemius muscle lengths (from lateral epicondyle of femur to heel) before and after contraction by the electric stimulator. Each experimental group consists of 10 mice.
9. Obtain walking tracks by using a corridor open at one end to a darkened compartment. The animal's feet are soaked in Higgins7 black waterproof ink (Sanford, Bellwood, IL), and the animal is allowed to walk multiple times to obtain measurable prints. Evaluate the tracks for print length and intermediate toe spread.
10. Perform multiple linear regression analysis with values derived from each of the parameters. Values are derived for each parameter by subtracting the normal value (normal left side) from the hair follicle stem cell-transplanted value (experimental right side) and dividing by the normal value. Each experimental group consists of seven mice. Groups include control mice, mice with a severed tibial nerve only, and mice with the tibial nerve enjoined by injected hair follicle stem cells. PL is the print length, NPL is the normal left print length, EPL is the experimental right print length, IT is the intermediate toe spread, NIT is the normal left intermediate toe spread, and EIT is the experimental right intermediate toe spread.
11. Express the experimental data as the mean ± SD. Perform statistical analysis by using a two-tailed Student's *t*-test.

Regarding repair of peripheral nerve injury by hair follicle stem cells, refer to **Note 11**.

3.3. Spinal Cord Regeneration Using Hair Follicle Stem Cells

1. Transplant differentiated GFP-expressing stem cell colonies to the thoracic region of the spinal cord in C57BL/6 immunocompetent mice. Perform a laminectomy at the 10th thoracic spinal vertebrae, followed by transversal cut using a binocular microscope. Transplant the GFP-expressing stem cells between the severed thoracic region (spinal level T10) of the spinal cord in C57BL/6 immunocompetent mice.
   a. After 2 months, directly observe the spinal cord of the transplanted mice by fluorescence microscopy under anesthesia. Excise spinal cord samples of the transplanted mice under anesthesia. Embed the spinal cord samples in tissue freezing embedding medium and freeze at –80°C overnight.
   b. Cut frozen sections 5 μm thick with a Leica CM1850 cryostat and air-dry. Directly observe the sections by fluorescence microscopy that are then used for immunofluoresence

stain with βIII-tubulin, GFAP, CNPase, K15, and SMA. Use the following primary antibodies: anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; Covance Research Products, Inc., Berkeley, CA), anti-glial fibrillary acidic protein (GFAP) monoclonal (1:200; Lab Vision, Fremont, CA), anti-2′-3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) monoclonal (1:50; Lab Vision), anti-K15 monoclonal (1:100; Lab Vision), and anti-smooth muscle actin (SMA) monoclonal (1:200; Lab Vision). Use the following secondary antibodies: Alexa Fluor® 568 goat anti-mouse (1:200; Molecular Probes, Eugene, OR) or Alexa Fluor® 568-conjugated goat anti-rabbit (1:200; Molecular Probes). For quantification of the percentage of cells producing a given marker protein, photograph at least three fields and determine the number of positive cells relative to the total number of GFP-expressing cells.

2. Transplant differentiated GFP-expressing stem cell colonies to the lumbar region of the spinal cord in C57BL/6 immunocompetent mice. Using a binocular microscope, perform a laminectomy at the second lumbar spinal vertebrae, followed by a transversal cut. Transplant the GFP-expressing stem cell colonies between the severed lumbar region (spinal level L2) of the spinal cord in C57BL/6 immunocompetent mice.
3. Evaluate the regeneration efficacy of GFP-expressing stem cells transplanted in the severed thoracic and lumbar regions of the spinal cord. Conduct performance analyses for 12 weeks using the Basso–Beattie–Bresnahan (BBB) locomotor rating scale (11). Each group consists of five to seven mice.
4. Observe the spinal cord in the live mouse with transplanted GFP-expressing hair follicle stem cells using an Olympus IMT-2 inverted microscope equipped with a mercury lamp power supply. The microscope has a GFP filter set.
5. Express the experimental data as the mean ± SD. Perform statistical analysis using the two-tailed Student's *t*-test (5).

Regarding repair of spinal cord injury by hair follicle stem cells, refer to **Note 12**.

3.4. Isolation of Human Nestin-Expressing Pluripotent Hair Follicle Stem Cells from the Hair Follicle Stem Cell Area

3.4.1. Culture of Whole and Plucked Hair Follicles from Human Scalp

1. Obtain samples from surgical specimens of normal human scalp skin. To isolate whole hair follicles, cut the scalp skin containing the hair follicle pad and expose its inner surface. Dissect the scalp hair follicles under a binocular microscope and pluck from the pad by pulling them gently by the neck with fine forceps and wash in DMEM-F12 (GIBCO-BRL, Grand Island, NY) containing B-27 (GIBCO-BRL) and 1% penicillin–streptomycin (GIBCO-BRL). Perform all surgical procedures in a sterile environment. Isolate the hair follicle stem cell area from the hair follicles. Immediately locate the

hair follicle stem cell area below the sebaceous glands and above the hair follicle bulge area. The hair follicle stem cell area contains nestin-positive and K15-negative cells. Isolate the hair follicle stem cell area under a binocular microscope and suspend in 1 ml DMEM-F12 containing B-27 with 1% methylcellulose (Sigma-Aldrich) and add 20 ng ml^{-1} basic FGF (bFGF) (Chemicon, Temecula, CA) every 2 days. Culture cells in 24-well tissue-culture dishes (Corning, Aliso Viejo, CA) in a 37°C, 5% CO^2/95% air tissue-culture incubator. After 4 weeks, the nestin-positive human hair follicle cells should form a colony. For differentiation, centrifuge the hair follicle cells, remove the growth factor-containing supernatant, and resuspend the colony in fresh RPMI 1640 medium (Cellgro, Herndon, VA) containing 10% fetal bovine serum (FBS) in the SonicSeal 4-well chamber slides (Nunc Inc., Rochester, NY).

2. Obtain plucked hair follicles by removing the scalp hairs with a depilation forceps and wash in DMEM-F12 containing B-27 and 1% penicillin–streptomycin. Perform all surgical procedures under a sterile environment. Divide the hair follicle into three parts (upper, middle, and lower parts) and suspend in DMEM-F12 containing B-27 supplemented with 20 ng ml^{-1} bFGF added every 2 days. Culture cells in 24-well tissue-culture dishes in a 37 °C, 5% CO^2/95% air tissue-culture incubator. After 4 weeks, the middle parts of the hair follicle should form colonies. For differentiation, centrifuge the cell colonies, remove the growth factor-containing supernatant, and resuspend the colonies into fresh RPMI 1640 medium containing 10% FBS in SonicSeal 4-well chamber slides.

3.4.2. Immunofluorescence Staining and Quantification

Use immunofluorescence to stain the cells which differentiate in cell colonies formed from the nestin-positive and K15-negative cells in the intact hair follicle. Use the following primary antibodies: anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; Covance Research Products Inc., Berkeley, CA), anti-nestin polyclonal (1:200; Chemicon, Temecula, CA), anti-S100 polyclonal (1:200; Chemicon), anti-glial fibrillary acidic protein (GFAP) monoclonal (1:200; Lab Vision, Fremont, CA), anti-2′-3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) monoclonal (1:50; Lab Vision), anti-K15 monoclonal (1:100; Lab Vision), and anti-smooth muscle actin (SMA) monoclonal (1:200; Lab Vision). Secondary antibodies are fluorescein-conjugated sheep anti-mouse IgG (1:10; Chemicon) and tetramethylrhodamine-conjugated swine anti-rabbit IgG (1:80; Nordic Immunological laboratories, Tilburg, The Netherlands). DAPI (Molecular Probes) is used for nuclear counterstaining.

3.4.3. Nestin RT-PCR

Extract total RNA from human endothelium (HMvEC) using ISOGEN or equivalent (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. Reverse transcribe 1 μg of the total RNA into cDNA in a 20 μl reaction mixture containing 1 × RT buffer (GIBCO-BRL, Rockville, MD, USA), 0.5 mM of dNTPs (Takara Shuzo Co., Otsu, Japan), 0.5 μg of oligo-dT primer, 40 U of RNase inhibitor (Boehringer Mannheim Co., Mannheim, Germany), 200 U M-MLV reverse transcriptase (GIBCO-BRL), and 10 mM DTT (GIBCO-BRL). After incubation at 43°C for 1 hour and then at 95°C for 3 min, amplify the cDNA using 1 μl of the cDNA preparation for nestin in a 25 μl reaction mixture containing 10 mM of Tris-HCl, pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% (W/V) gelatin, 0.2 mM dNTPs, 25 pM 5′ and 3′ oligonucleotide primers, and 2.5 U Taq polymerase (Perkin-Elmer, Branchburg, NJ). Pre-incubate the reactions in a DNA thermocycler 480 (Perkin-Elmer Cetus, Norwalk, CT) for 94°C for 2 min and then 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds and extention at 72°C for 1 min), followed by a final 7 min extension at 72°C. Use the following primers for cDNA amplification: nestin (5′ GGCAGCGTTGGAACAGAGGTTGGA 3′, 5′ CTCTAAACT GGAGTGGTCAGGGCT 3′, 718 bp) (10), and β-actin (5′ primer: GTGGGGCGCCCCAGGCACCA; 3′ primer: CTCCTTAATGTCACGCACGATTTC). Electrophoresis the PCR product in 1.5% agarose gel which is visualized by staining with ethidium bromide.

3.4.4. In Situ Hybridization of Nestin mRNA

Use a human nestin cDNA PCR product containing a 718 bp fragment. Subclone the cDNA using a TA cloning kit. Generate sense and antisense nestin RNA probes using SP6 or T7 RNA polymerases. Label the probes with digoxigenin-11-UTP using in vitro transcription. Digoxigenin-label RNA probes at a concentration of 100 μg per ml. Mount serial cryostat sections of biopsy material on aminopropylsilane-coated slides, followed by fixation in freshly prepared 4% paraformaldehyde. Permeate with 1 μg/ml proteinase K for 15 min at 37°C. Acetylate the sections for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), and dehydrate in ethanol. Perform hybridization in a sealed, humid box for 16 hours at 50°C in hybridization solution (Boehringer Mannheim). Wash the slides in 50% formamide/2 × sodium citrate/sodium chloride buffer, treated with RNase A (20 μg per ml); further wash in 2 × sodium citrate/sodium chloride buffer for 20 min at 50°C, two changes of 0.2 × sodium citrate/sodium chloride buffer for 20 min at 50°C, and then block with blocking solution (Boehringer Mannheim) at room temperature for 1 hour. After blocking, wash the sections and incubate with anti-digoxigenin and Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim.) at room temperature for 30 min. Wash the

sections and then incubate with nitroblue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate at 37°C for 20 hours. Mount the slides and view by light microscopy.

3.5. Nestin, K15, and CD34 Expression in Plucked and Intact Hair Follicles of Human Scalp

Stain by immunofluorescence and immunohistochemistry for nestin, K15, and CD34 in plucked and intact human hair follicles. Detect with the secondary fluorescent antibodies described above using an anti-mouse immunoglobulin horseradish peroxidase detection kit (DAKO, Carpinteria, CA) following manufacturer's instructions. Use the following primary antibodies: anti-nestin polyclonal, anti-K15 monoclonal, and anti-CD34 monoclonal (1:10; Nichirei, Tokyo, Japan) (6).

4. Notes

1. Autofluorescence: It is important to minimize autofluorescence of the tissue and body fluids by using proper filters. Excitation filters should have a narrow band as close to 490 nm as possible to specifically excite GFP whose peak is distinct from that of the skin, tissues, and fluid of the animal. In addition, proper band-pass emission filters should be used with a cutoff of approximately 515 nm.
2. Bleeding : Bleeding should be avoided at the surgical site as hemoglobin will absorb the incident excitation light.
3. Dehydration : During biopsy procedures, it is essential to hydrate the animal by spraying normal saline on the open tissue.
4. Infection : When doing open biopsies or other invasive procedures, it is crucial to maintain a proper sterile operation field.
5. The procedures in these protocols describe the isolation and differentiation of hair follicle stem cells that can be used for the regeneration of injured peripheral nerves and the spinal cord. Moreover, we can locate the nestin-positive and K15-negative stem cells in mouse and human hair follicles.
6. **Figure 28.1** demonstrates that ND-GFP is highly expressed in the hair follicle stem cell area and newly formed outer-root sheath cells of the mouse (1).
7. **Figure 28.2** demonstrates that ND-GFP-expressing cells in the hair follicle stem cell area are CD34-positive and K15-negative. ND-GFP-expressing cells (keratinocyte progenitor cells) in the bulge area are K15-positive and CD34-negative (1).

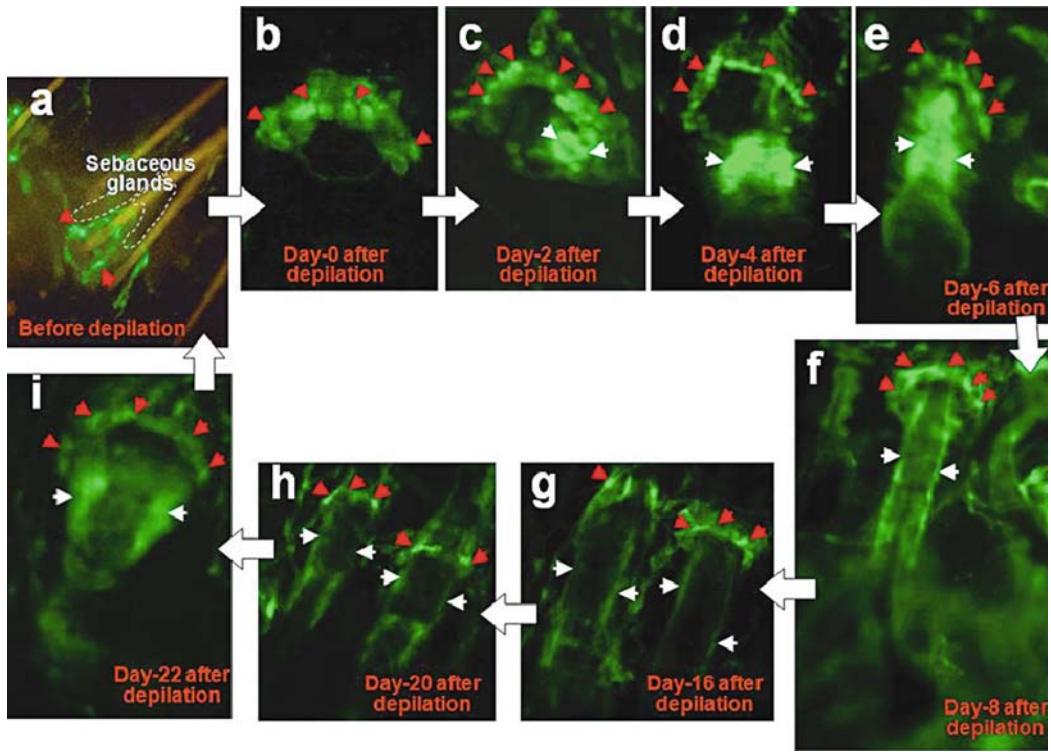

Fig. 28.1. Hair follicle stem cells in the hair growth cycle. (**a, b**) ND-GFP-expressing hair follicle stem cells (*red arrows*) located in the hair follicular stem cell area (the permanent upper hair follicle immediately below the sebaceous glands in the hair follicular stem cell area surrounding the bulge) in telogen phase. (**c, d**) Days 2 (**c**) and 4 (**d**) after anagen induction by depilation. Note the new hair follicle outer-root sheath cells (white arrow) formed directly from the nestin-GFP-expressing hair follicle stem cells. (**e, f**) Day 6 (**e**) and day 8 (**f**) after anagen induction by depilation. Note the nestin-GFP-expressing outer-root sheath cells (white arrows) in the upper two-thirds of the hair follicle. (**g–i**) Day 16 (**g**), day 20 (**h**), and day 22 (**i**) after depilation. Note in (**g**, **h,** and **i)** that the hair follicles are in the catagen phase and are undergoing regression and degeneration, including the ND-expressing cells in the outer-root sheath. The hair follicular stem cell area ND-GFP-expressing stem cells remain.

8. **Figure 28.3** demonstrates that ND-GFP-expressing cells in the hair follicle stem cell area are CD34-positive and K15-negative. ND-GFP-expressing cells in the hair follicle bulge area are K15-positive and CD34-negative (1).
9. **Figure 28.4** demonstrates that hair follicle cells labeled with ND-GFP behave as stem cells, differentiating to form much of the hair follicle in each hair growth cycle. Nestin also occurs in new perifollicular blood vessels, which are formed in response to follicular angiogenic signals during the anagen growth phase. This is seen most clearly by transplanting ND-GFP-labeled vibrissa (whisker) hair follicles to unlabeled nude mice. New ND-GFP-expressing vessels grow from the transplanted follicle, and these vessels increase when the local recipient skin is wounded (3).

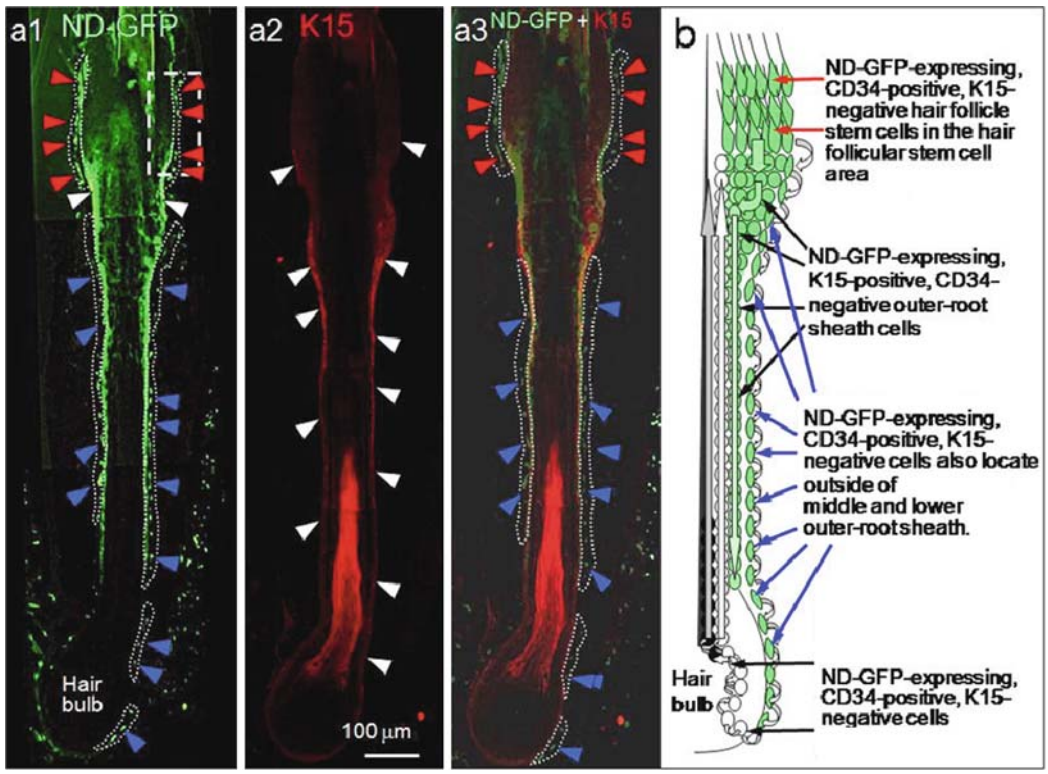

Fig. 28.2. ND-GFP-expressing, K15-negative, and CD34-positive cells locate not only in the hair follicular stem cell area but also outside of the middle and lower outer-root sheath. (**a1**) In frozen section of vibrissa follicle of ND-GFP transgenic mice, ND-GFP-expressing cells locate in hair follicular stem cell area (*red arrowheads*, white dashed areas), outside of the middle and lower outer-root sheath (*blue arrowheads, white dashed areas*), and outer-root sheath (*white arrowheads*). (**a2, a3**) Immunofluoresence staining of K15 showed that ND-GFP-expressing cells in hair follicular stem cell area (*red arrowheads, white dashed areas*) and ND-GFP-expressing cells around the middle and lower outer-root sheath (*blue arrowheads, white dashed areas*) were K15-negative. ND-GFP-expressing outer-root sheath cells in the bulge area were K15-positive and CD34-negative. ND-GFP-expressing cells in the outer-root sheath were K15-positive, (*white arrowheads*). (**b**) Schematic of vibrissa follicle of ND-GFP transgenic mice showing the position of ND-GFP-expressing cells in the section. ND-GFP-expressing cells in hair follicle stem cell area were CD34-positive and K15-negative (*red arrows*). ND-GFP-expressing outer-root sheath cells were K15-positive and CD34-negative (*black arrows*). ND-GFP-expressing cells outside of the outer-root sheath (*blue arrows*) were CD34-positive and K15-negative. ND-GFP-expressing, CD34-positive, K15-negative hair follicle stem cells in the growing outer-root sheath are directly derived from the follicle stem cell area (black arrows).

10. **Figure 28.5** demonstrates the isolation of multipotent nestin-positive, keratin-negative hair follicle stem cells. Hair follicle stem cells are isolated using the ND-GFP marker. These ND-GFP-expressing stem cells are primitive, because they express the stem cell marker CD34 but do not express the keratinocyte marker K15. The ND-GFP-expressing hair follicle stem cells in ND-GFP transgenic mice are isolated and suspended in DMEM-F12 containing B-27 supplemented with basic FGF every 2 days. After 4 weeks, ND-GFP-expressing

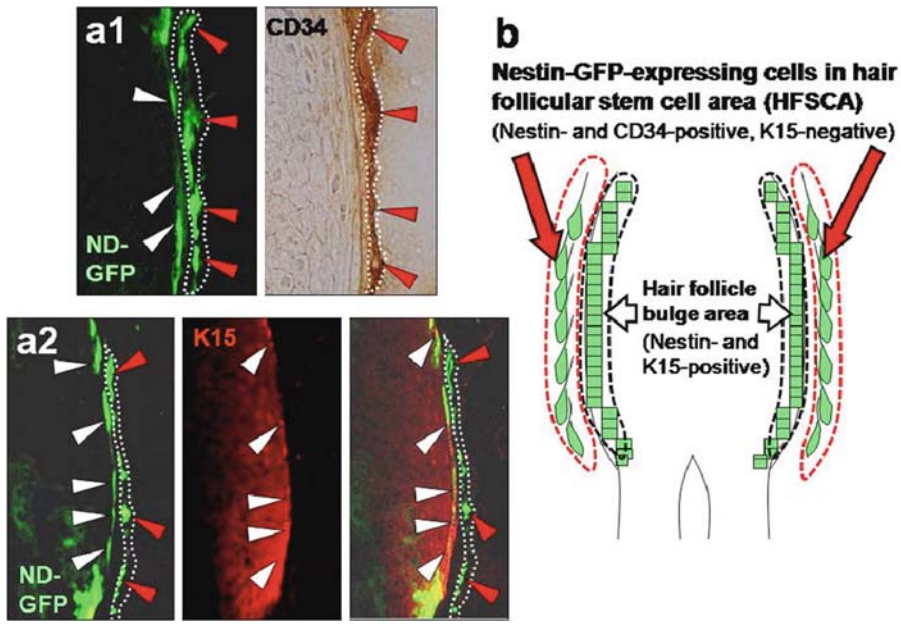

Fig. 28.3. (**a**) Higher magnification of white dashed box in **Fig. 28.2a**. ND-GFP-expressing cells in hair follicle stem cell area (*white dashed area, red arrowheads*) are CD34-positive and K15-negative. ND-GFP-expressing outer-root sheath cells in outer layer of the outer-root sheath (*white arrowheads*) are K15-positive and CD34-negative. (**b**) Schematic of hair follicular stem cell area and bulge area of ND-GFP transgenic mice. ND-GFP-expressing cells in hair follicle stem cell area (*red dashed area, red arrows*) are CD34-positive and K15-negative. ND-GFP-expressing outer-root sheath cells (keratinocyte progenitor cells) in outer layer of the outer-root sheath (hair follicle bulge area) (*black dashed area, white arrows*) are K15-positive and CD34-negative.

hair follicle stem cells formed the ND-GFP-expressing, CD34-positive, and K15-negative cell colonies. Upon transfer to RPMI-1640 medium containing 10% fetal bovine serum (FBS), the ND-GFP-expressing cells differentiated to neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro (2).

11. **Figure 28.6** demonstrates that hair follicle stem cells promote the recovery of peripheral nerve injury. We isolated the nestin- and CD34-positive and K15-negative stem cells from the hair follicle stem cell area in GFP transgenic mice (GFP mice). The nestin-expressing hair follicle stem cell area contains multipotent stem cells, which can differentiate into neurons, glial cells, keratinocytes, smooth muscle cells, and melanocytes. The isolated immature multipotent hair follicle stem cells differentiated to GFAP-positive glial cells and βIII-tubulin-positive neurons and could join the severed sciatic nerve. Most of the GFP-expressing hair follicle stem cells differentiated into GFAP-positive Schwann cells in the sciatic nerve of C57BL/6 immunocompetent mice. The regenerated sciatic nerve contracted the gastrocnemius muscle by

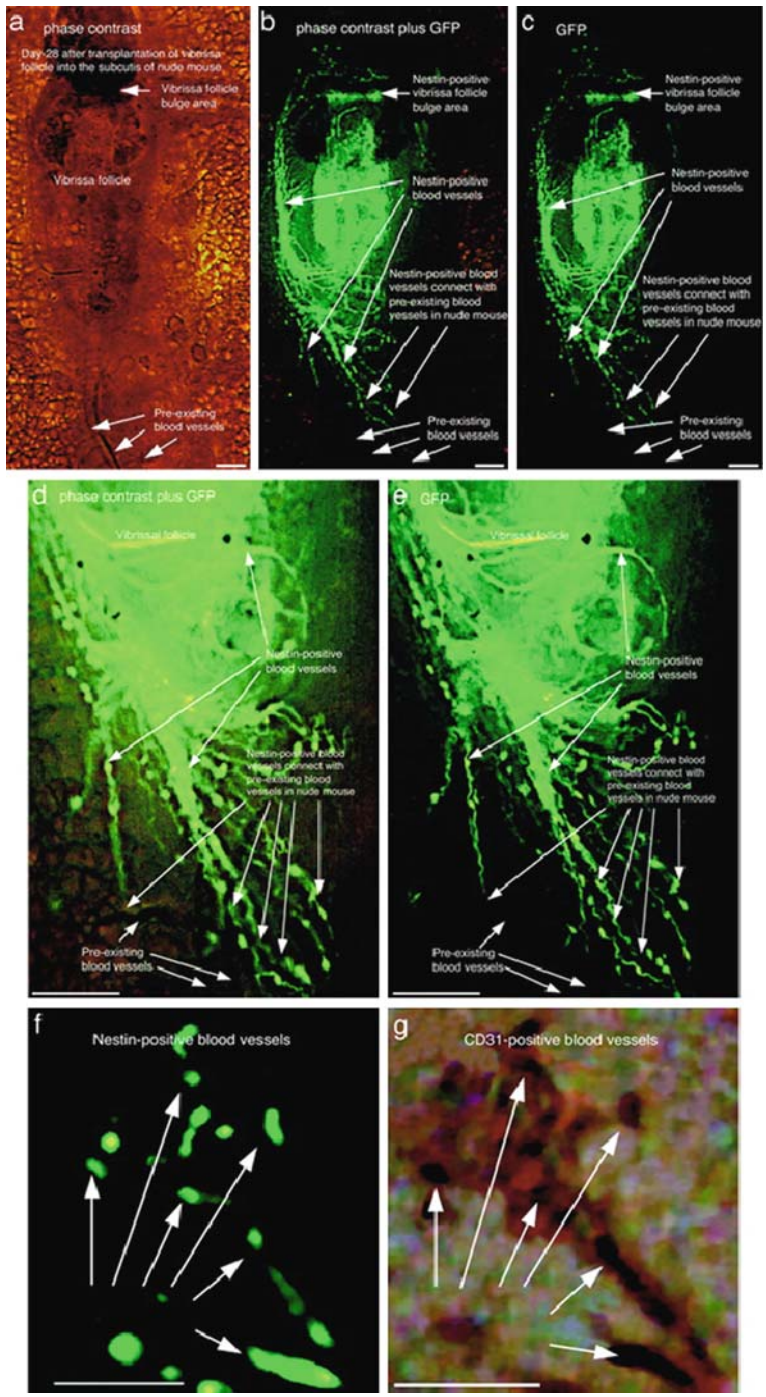

Fig. 28.4. Transplantation of an ND-GFP vibrissa follicle into the subcutis of a nude mouse. (**a**) Phase-contrast micrograph of follicle 28 days after transplantation. (**b**) Phase-contrast micrograph plus GFP fluorescence. (**c**) GFP fluorescence. In **(b and c)**, ND-GFP blood vessels (*arrows*) are seen growing from the transplanted ND-GFP hair follicle and associating with pre-existing blood vessels in the nude mouse skin. **(d and e)** Higher magnification of the ND-GFP vessels of (**b)** and (**c)**, respectively. (**f** and **g**) Colocalization of GFP and the endothelial cell marker CD31 (*arrows*). (**f)**is a fluorescent image, and (**g)** shows the same field air-dried and immunohistochemically stained with CD31. Scale bars, 100 μm (3).

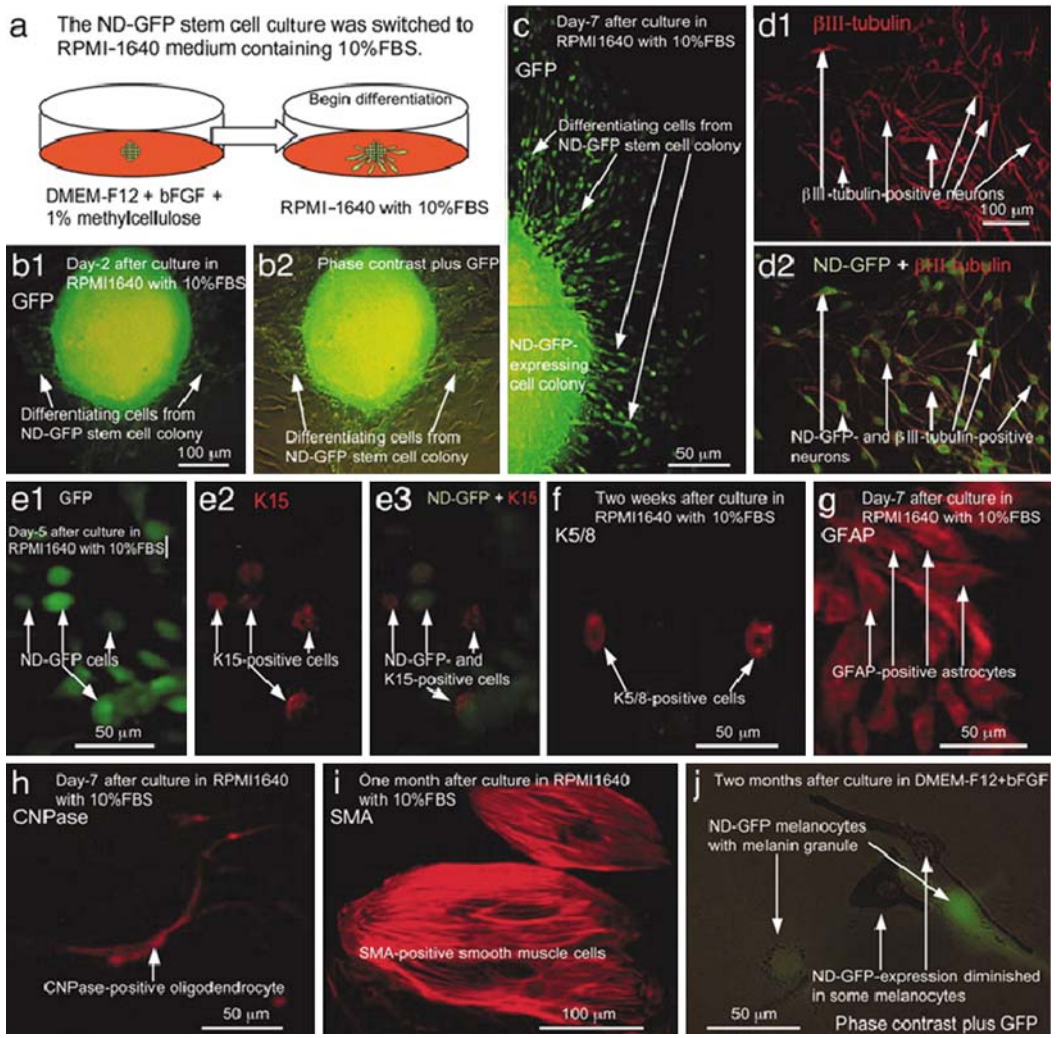

Fig. 28.5. Differentiation of ND-GFP hair follicle stem cells in vitro. (*a*) The ND-GFP-expressing cell culture was switched to RPMI-1640 medium containing 10% FBS from DMEM-F12 containing B-27 and 1% methylcellulose supplemented with bFGF added every 2 days. **(b1 and b2)** Two days after switching into RPMI-1640 medium containing 10% FBS, differentiating cells migrated out of the ND-GFP-expressing cell colony. **(c)** Seven days after switching to RPMI-1640 medium, many differentiating cells migrated out of the ND-GFP-expressing cell colony. **(d1 and d2)** ND-GFP-expressing cells differentiated to βIII -tubulin-positive neurons which maintain ND-GFP expression. **(e1–e3)** Five days after switching to RPMI-1640 medium, ND-GFP-expressing cells differentiated to K15-positive cells (red fluorescence, *arrows*) **(e2)**. The K15-positive cells still expressed ND-GFP. **(f)** ND-GFP-expressing cells differentiated to K5/8-positive cells 2 weeks after switching to RPMI-1640 medium. **(g)** Seven days after switching to RPMI-1640 medium, ND-GFP-expressing cells differentiated to GFAP-positive astrocytes. **(h)** Seven days after switching to RPMI-1640 medium, ND-GFP-expressing cells differentiated to 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)-positive oligodendrocytes. **(i)** At 1 month after culture in RPMI-1640 medium containing 10% FBS, ND-GFP-expressing cells differentiated to SMA-positive smooth muscle cells. **(j)** Two months after culture in DMEM-F12 containing B-27 and 1% methylcellulose supplemented with bFGF added every 2 days, ND-GFP-expressing cells differentiated to melanocytes containing melanin. Some melanocytes still expressed ND-GFP (2).

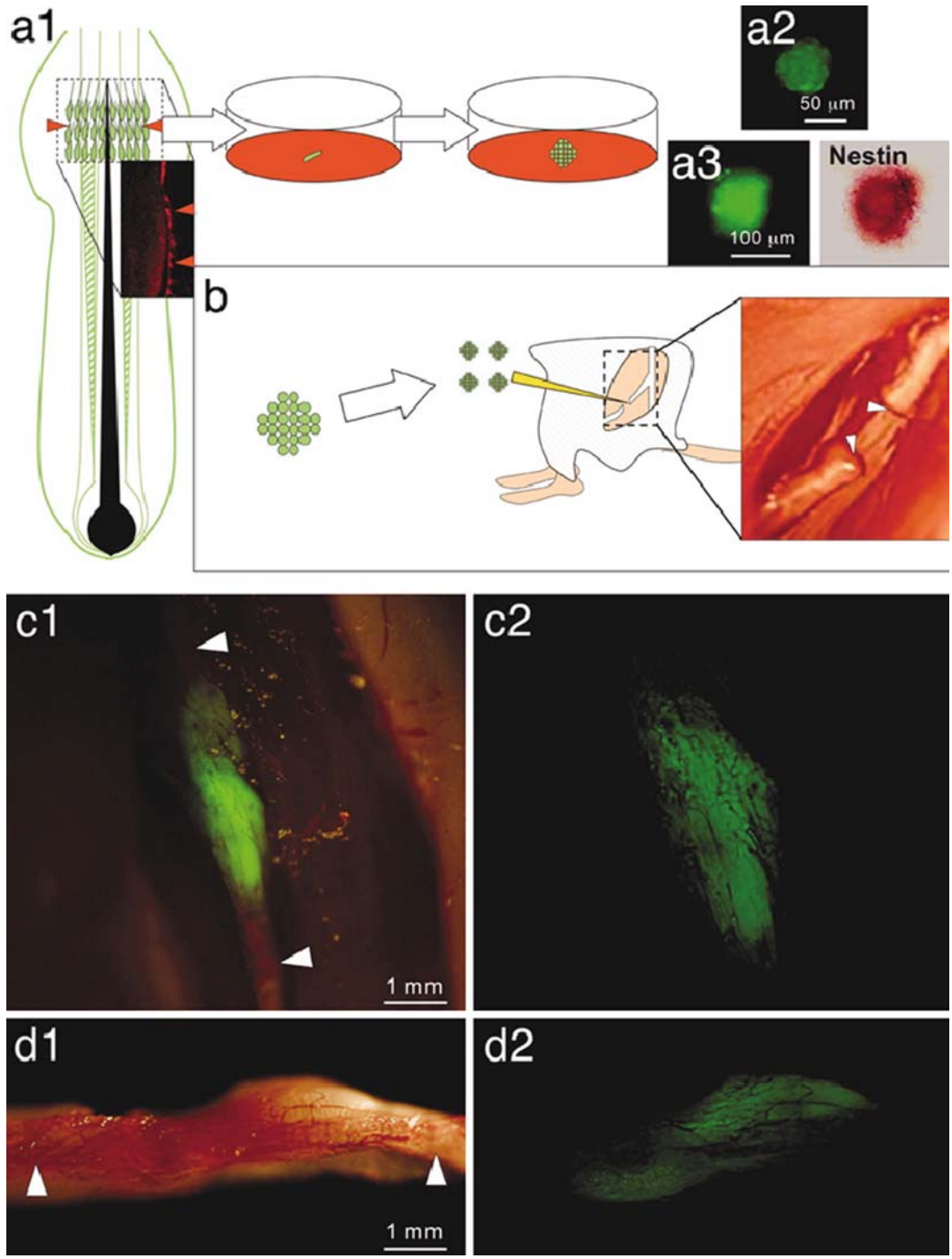

Fig. 28.6. Rejoining severed sciatic nerves with hair follicle stem cells. **(a1)** Schematic of vibrissa follicle of GFP transgenic mice showing the position of GFP- and nestin-expressing hair follicular stem cell area (*red arrowheads*). **(a2)** Colony formed from GFP-expressing hair follicle stem cells after 2 months in culture. **(a3)** GFP-expressing cells within the colony were nestin-positive. **(b)** GFP-expressing hair follicle stem cells grown for 2 months in DMEM-F12 medium containing B-27, 1% methylcellulose, and basic FGF were transplanted between the severed sciatic nerve fragments in C57BL/6 immunocompetent mice (*white arrowheads*). **(c1 and c2)** Fluorescence images from a live mouse. Two months after transplantation between the severed sciatic nerve, the GFP-expressing cells were visualized in the joined region of the severed sciatic nerve. *c2* shows higher magnification of *c1*. **(d1 and d2)** Brightfield (*d1*) and fluorescence (*d2*) images of an excised sciatic nerve. The pre-existing sciatic nerve is denoted by white arrowheads (4).

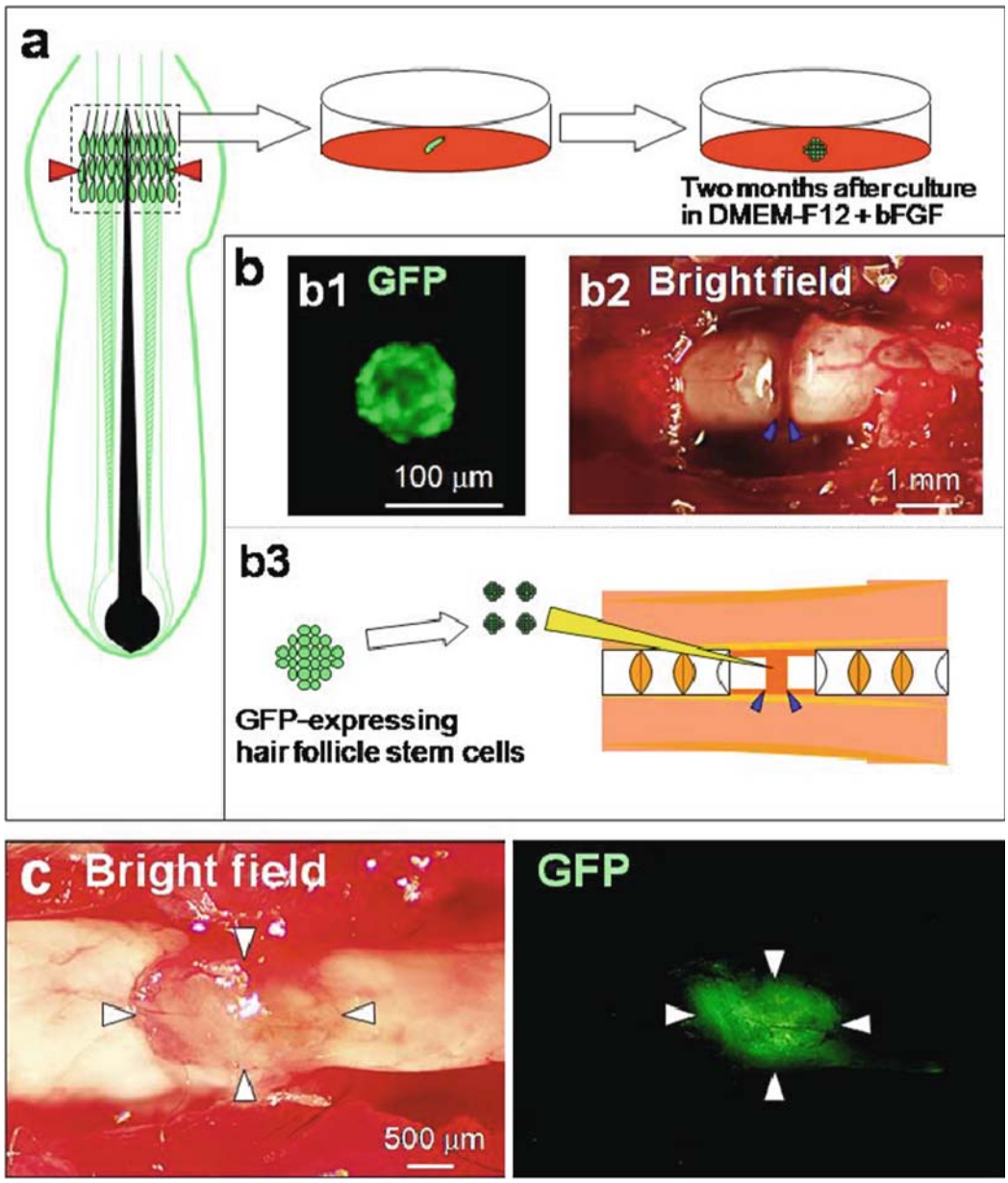

Fig. 28.7. Severed thoracic region of spinal cord joined by transplantation of hair follicle stem cells. **(a)** Schematic of vibrissa follicle of GFP transgenic mice shows the position of the GFP-expressing hair follicular stem cell area (*red arrowheads*). The GFP-expressing stem cells from hair follicular stem cell area were cultured for two months in DMEM-F12 medium containing B-27 supplemented with bFGF added every 2 days. After 2 months, GFP-expressing hair follicle stem cells from the hair follicle stem cell area formed the GFP-expressing hair follicle stem cell colonies. **(b, b1)** denotes a GFP-expressing hair follicle stem cell colony from the GFP-expressing hair follicular stem cell area. **(b2, b3)** The GFP-expressing stem cells were transplanted to the severed thoracic region of the spinal cord in C57BL/6 immunocompetent mice (*blue arrowheads*). **(c)** Two months after transplantation of GFP-expressing hair follicle stem cells between the severed thoracic region of the spinal cord, the GFP-expressing cells effected joining the severed thoracic region of the spinal cord in C57BL/6 immunocompetent mice (*white arrowheads*) (5).

electric stimulation. Walking print length and intermediate toe spread significantly recovered by transplantation of GFP-expressing hair follicle stem cells between the severed tibial nerve. These results suggest that hair follicle stem cells promote the recovery of peripheral nerve injury (4).

12. **Figure 28.7** demonstrates hair follicle stem cells promote the recovery of spinal cord injury. We isolated the nestin- and CD34-positive and K15-negative stem cells from the hair follicular stem cell area in GFP mice. We transplanted the GFP-expressing hair follicle stem cells between the severed thoracic regions of spinal cord. Most of the GFP-expressing hair follicle stem cells differentiated into GFAP- and CNPase-positive Schwann cells and joined the severed spinal cord of C57BL/6 immunocompetent mice. These results suggest that the hair follicle nestin-expressing stem cells differentiated into immature Schwann cells which promote axonal growth and functional recovery after spinal cord injury (5).

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Chapter 29

Limiting Dilution Analysis of Murine Epidermal Stem Cells Using an In Vivo Regeneration Assay

Lauren R. Strachan and Ruby Ghadially

Abstract

Epidermal stem cells are of major importance for tissue homeostasis, wound repair, tumor initiation, and gene therapy. Here we describe an in vivo regeneration assay to test for the ability of keratinocyte progenitors to maintain an epidermis over the long-term in vivo. Limiting dilution analysis of epidermal repopulating units in this in vivo regeneration assay at sequential time points allows the frequency of short-term (transit amplifying cell) and long-term (stem cell) repopulating cells to be quantified.

Keywords: Stem cell, Transit amplifying cell, Epidermis, Limiting dilution, Skin, Regeneration.

1. Introduction

In vivo assessment of epidermal stem cell function and frequency has been well recognized as an important goal (1–3). We have developed and used a long-term repopulating assay to test for sustained tissue regeneration and maintenance in vivo. This may be considered the most rigorous definition of an epidermal stem cell. For this assay dissociated keratinocytes regenerate a differentiated epidermis on top of dermal fibroblasts seeded on the subcutaneous fascia of immunodeficient mice. GFP-negative keratinocytes serve to ensure the production of an intact differentiated epidermis despite variations in the numbers of GFP-positive cells in the test population. For the test population, a range of dilutions of GFP-positive keratinocytes are used. Mixtures of test keratinocytes (GFP positive) are seeded into chambers along with a constant number of GFP-negative keratinocytes, and the

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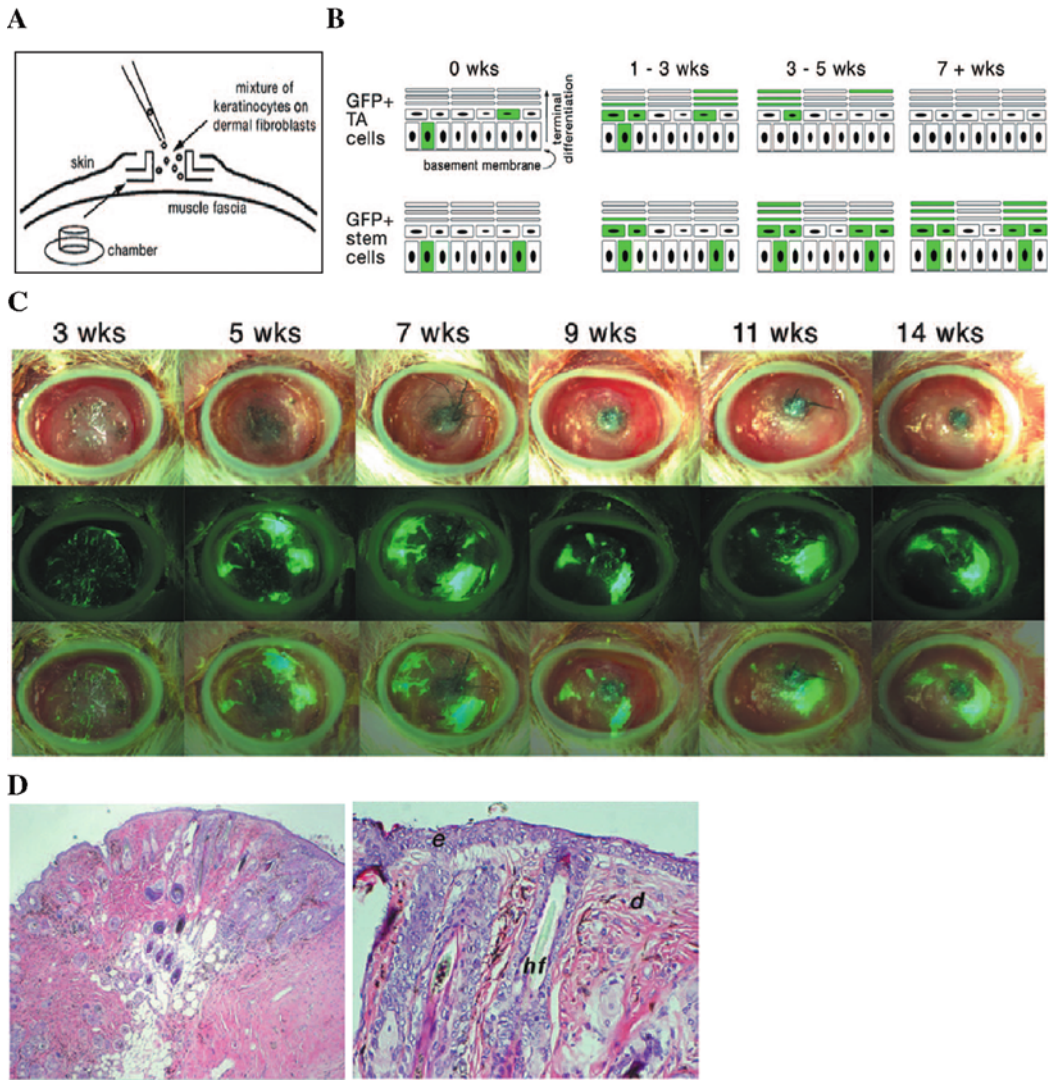

Fig. 29.1. Limiting dilution analysis for the determination of short-term (TA) and long-term (stem) repopulating cell frequency. **(A)** Diagram of the 6 mm diameter silicone chamber implanted onto the dorsal fascia of a NOD–SCID mouse into which the keratinocyte and fibroblast populations are seeded. **(B)** Schematic of epidermal layers where GFP-positive units derived from short-term repopulating cells (TA cells) disappear after 3–5 weeks (top row) or GFP-positive cells derived from long-term repopulating cells (stem cells) persist for 9–30 weeks. **(C)** Maintenance of GFP-positive repopulating units up to 14 weeks. Top panels show bright-field images of regenerated skin, middle panels show epifluorescence images taken at the same time, and bottom panels show an overlay of the GFP-positive repopulating units on the regenerated skin. **(D)** Hematoxylin and eosin staining of regenerated skin in cross section (10 ×, 20 ×). *d*: dermis, *e:* epidermis, *hf*: hair follicle.

presence of GFP-positive epidermal repopulating units is assessed 2–30 weeks after epidermal regeneration (**Fig. 29.1**). At each assessment the epidermis is scored as positive or negative, for the presence or absence of a cluster of GFP-positive cells. By seeding a range of doses of GFP-positive keratinocytes in this repopulating

assay and waiting until all transit amplifying cells and their progeny have differentiated and been lost from the epidermis, limiting dilution analysis allows the frequency of cells with long-term repopulating ability in a given population to be quantified (3).

Table 29.1 is presented as an example of how this type of assay can be used to compare the frequency of stem cells in various populations of keratinocytes. As seen in **Table 29.1**, keratinocytes that rapidly adhere to collagen were implanted at a range of doses. At the highest dose of 240,000 keratinocytes, all chambers were positive for GFP-positive repopulating units at all time points. At the lowest doses, of 1,900 and 7,500 keratinocytes, no chambers had GFP-positive repopulating units at any time point. At intermediate doses (e.g., 60,000 keratinocytes), it can be seen that, while at early time points (3 weeks) 9 of 10 chambers contained GFP-positive repopulating units, at later time points (e.g., 9 weeks) only 5 of the 10 chambers remained positive. At sequential time points the repopulating frequency decreases until only long-term repopulating cells remain and the repopulating unit frequency remains constant thereafter. Using Poisson distribution statistics the calculated frequency of repopulating units corresponds to the derived cell dose at which 37% of the tests yield a negative response (L-Calc software v1.1, www.stemcell.com). For the study in **Table 29.1**, we relied on previous work that found that the stem cell frequency of total unsorted cells at 9 weeks was 1 in 30,000 keratinocytes (3). As can be seen for the dose of 30,000 total unsorted keratinocytes at 9 weeks (**Table 29.1**), 4 out of 10 chambers are negative, consistent with Poisson statistic predictions. For each population of cells to be tested (for example, rapidly adherent cells, not rapidly adherent cells, unsorted cells) the doses to be used experimentally will depend on the expected long-term repopulating unit frequency.

Phenotypic analysis of hematopoietic stem cells in in vivo transplantation assays has allowed separation of long-term repopulating cells from cells detected in colony forming assays (4–11). These types of studies have defined a hierarchy of hematopoietic stem cell phenotypes (*see* (12), Figure 1). Primitive progenitors that represent the closest stem cell descendents which can be prospectively isolated from the true stem cell by flow cytometry are still multipotent, yet already have a decline in self-renewal capacity, underscoring long-term repopulating ability as the sine qua non of a stem cell (13, 14). These progenitors produce distinct highly proliferative colonies, which can differentiate into specific lineages, but unlike a true stem cell are unable to repopulate all hematopoietic lineages for the life of the animal. Thus in vivo transplantation assays have long been the gold standard for the study of hematopoietic stem cells and after almost 20 years remain so (15)].

Table 29.1
Frequency of repopulating units in rapidly adherent, not rapidly adherent, and unsorted murine keratinocytes over time.[1]

	Number of GFP+ cells[2]	3 weeks	5 weeks	7 weeks	9 weeks
Rapidly adherent cells	1,900	0/1	0/1	0/1	0/1
	7,500	0/7	0/7	0/7	0/7
	30,000	5/9	4/9	4/9	4/9
	60,000	9/10	8/10	5/10	5/10
	120,000	8/10	7/10	7/10	7/10
	240,000	5/5	5/5	5/5	5/5
Repopulating unit frequency		**1 in 47,501**	**1 in 63,657**	**1 in 81,635**	**1 in 81,635**
(±Standard Error)		(37,476–60,209)	(50,236–80,665)	(64,151–103,884)	(64,151–103,884)
(95% Confidence Interval)		(29,848–75,596)	(40,021–101,253)	(50,900–130,929)	(50,900–130,929)
Not rapidly adherent cells	7,500	0/3	0/3	0/3	0/3
	30,000	8/9	6/9	5/9	5/9
	60,000	9/10	9/10	8/10	8/10
	120,000	7/7	7/7	7/7	7/7
	240,000	5/5	5/5	5/5	5/5
Repopulating unit frequency		**1 in 21,094**	**1 in 27,630**	**1 in 36,196**	**1 in 36,196**
(±Standard Error)		(15,888–28,006)	(21,147–36,100)	(27,962–46,856)	(27,962–46,856)
(95% Confidence Interval)		(12,103–36,765)	(16,360–46,664)	(21,825–60,031)	(21,825–60,031)
Unsorted cells	30,000	8/10	7/10	6/10	6/10
Repopulating unit frequency		**1 in 18,640**	**1 in 24,918**	**1 in 32,741**	**1 in 32,741**
(±Standard Error)		(12,583–27,613)	(16,683–37,218)	(21,455–49,964)	(21,455–49,964)
(95% Confidence Interval)		(8,629–40,627)	(11,350–54,704)	(14,299–74,969)	(14,299–74,969)

1 Adapted from (17)
2 Added to chamber with 2 million GFP-negative cells.

One important question regarding functional assays for stem cells is what duration of repopulation distinguishes the true epidermal stem cell from a short-term repopulating cell. In the epidermis, the short-term repopulating cells are termed transit amplifying cells. Cell cycle duration has been estimated to be 4–5 days and transit amplifying cells go through approximately three divisions (16) before terminally differentiating. Our initial studies showed that there is no further decline in repopulating cell frequency after 7 weeks, indicating that at this point transit amplifying cells and their progeny have differentiated and been lost from the epidermis, and we are assaying the true long-term repopulating epidermal stem cell (3, 17). Thus in all subsequent epidermal stem cell studies we have selected an endpoint of 9 weeks or later to ensure the study of stem cells rather than short-term repopulating transit amplifying cells.

As noted in similar hematopoietic transplantation assays (18), estimates of stem cell frequency are most certainly underestimates since the detection efficiency of the assay procedure is not known, but is almost certain to be less than 1. In recognition of this we term the GFP-positive clusters of cells repopulation units rather than epidermal stem cell units. This does not undermine the value of comparing the relative frequency of progenitor cells in different cell populations. While this assay estimates that 1 in 10,000 basal cells is a truly primitive epidermal stem cell, similar to stem cell frequencies in other tissues (11, 18, 19), previous work on the epidermis showed that 1 in 10 basal cells was a colony forming stem cell (for review, see (20)). However, more recently it has been shown that colony forming cells do not all represent stem cells (1, 17, 20). These findings lead us to believe that stem cell frequency is significantly less than previously thought (20–23). Using the in vivo transplantation assay described here multiple studies have reported that the frequency of epidermal stem cells in young and in neonatal murine epidermis is approximately 1 in 10,000 basal cells (3, 17, 24), strengthening the argument that the frequency of epidermal stem cells is similar to that of other somatic stem cell populations.

In this chapter we describe a method to determine the frequency of short- and long-term repopulating epidermal progenitors in vivo using limiting dilution analysis. First, primary keratinocytes are isolated from GFP-positive and GFP-negative neonatal murine epidermis. Then fibroblasts are isolated from the GFP-negative skin. Next, a range of doses of GFP-positive test keratinocytes are prepared and left on ice, while silicone chambers are implanted onto the fascia of NOD–SCID mice. Fibroblasts and then keratinocytes are seeded into the chambers. The regenerated epidermis is imaged over time and analyzed for

presence or absence of GFP-positive repopulating units. Analysis of the positive and negative results is performed using Poisson statistics for limiting dilution analysis and allows a quantitative analysis of the repopulating unit frequency. The strengths of this assay are the long-term functional nature of the repopulation carried out in vivo, which allows for the distinction between true long-term repopulating stem cells and short-term repopulating (transit amplifying) cells and the ability to quantify the number of long-term repopulating epidermal stem cells.

2. Materials

2.1. Tissue and Reagents

1. C57BL/6-TgN(ACTbEGFP)1Osb mice (Jackson Laboratories)
2. NOD–SCID mice (Jackson Laboratories)
3. Ketamine (100 mg/mL)
4. Aceprozamine maleate (10 mg/mL; Henry Schein)
5. Sulfatrim (Actavis)
6. Forceps, fine-tip scissors, #15 disposable scalpels
7. Tegaderm and Coban (3M)
8. Forane (Baxter)
9. CNT-07 complete medium (Cell-N-Tech)
10. Hibiclens (Regent Medical)
11. HBSS-CMF (Invitrogen)
12. HBSS-CMF with 5× PSA (Penicillin, Streptomycin, and Amphotericin; Invitrogen)
13. 0.05% trypsin-EDTA (Invitrogen)
14. TNS (Trypsin Neutralizing Solution: HBSS-CMF + 5% chelexed FBS) (*see* **Section 2.2**)
15. Dispase (25 U/mL in HBSS-CMF; BD Biosciences)
16. 1% Collagenase Type 1A (Sigma; C9891) (*see* **Section 2.2**)
17. Hemacytometer (Fisher)
18. Trypan blue (Sigma)
19. UV lamp (Long-wave UV (365 nm) filter; Spectroline)
20. Silicone chambers (6-mm internal diameter; Renner GmbH, Germany; http://www.renner-gmbh.de)
21. Epifluorescence stereomicroscope (Stemi SV; Carl Zeiss, Inc.) with UV 488 nm filter
22. L-calc software (Stemsoft; www.stemsoft.com)

2.2. Reagent Preparation

1. Prepare chelexed FBS by mixing 100 g of Chelex 100 resin (200–400 mesh; Bio-Rad) with 500 mL FBS and stir at room temperature for 1 hour. Let settle and filter (0.2 μM).
2. Prepare a 1% (1 mg/mL) solution of Collagenase Type 1A (Sigma #C9891) in HBSS-CMF. Alilquot and store at –20°C. Use within 6 months.

3. Methods

3.1. Primary Isolation of Neonatal Murine Keratinocytes

1. Using the long-wave UV lamp to determine whether pups are GFP positive, collect 3–4-day-old GFP-positive and 3–4-day-old GFP-negative neonates (*see* **Note 1**).
2. Humanely euthanize neonates as per an approved IACUC protocol.
3. Using forceps, pinch skin and make a small incision with fine-tip scissors.
4. Remove truncal skin from neonates and place in CNT-07 medium at 4°C.
5. Scrape and remove the subcutaneous fat and wash once (1–2 minutes) in 10% Hibiclens and then twice in HBSS with 5 × PSA (1–2 minutes).
6. Place skin, epidermis up, in a 35 mm dish with 3 mL dispase and incubate 24 hours at 4°C, or 2.5 hours at 37°C.
7. Remove skin carefully from dispase and gently peel epidermis from dermis. To collect the dermal fibroblasts proceed to **Section 3.2**.
8. Place the epidermis in 2 mL of pre-warmed 0.05% trypsin-EDTA and incubate for 20 minutes at 37°C followed by gentle tapping and shaking to encourage keratinocyte separation from the stratum corneum.
9. Neutralize the trypsin with 6 mL TNS, then separate the keratinocytes from the stratum corneum by centrifugation at 500 rpm for 10 minutes. Remove the supernatant and resuspend the cell pellet in CNT-07 medium.
10. Using the hemacytometer and trypan blue, count the cells and determine the percentage of dead cells; approximately 3–4 million keratinocytes are usually recovered from each neonatal murine epidermis (*see* **Note 2**).
11. Centrifuge at 500 rpm for 5 minutes and resuspend the GFP-positive and GFP-negative keratinocyte populations in CNT-07 medium in the appropriate volume (*see* **Section 3.4**). Keep cells on ice while chambers are implanted.

3.2. Primary Isolation of Neonatal Murine Dermal Fibroblasts

1. Following dispase treatment and removal of the epidermis (**Section 3.1**), place the dermis in 2 mL of pre-warmed 0.05% trypsin and incubate for 25 minutes at 37° followed by 2 seconds of vortexing.
2. Neutralize trypsin with 6 mL TNS and place dermis in empty 100 mm dish.
3. Using 2 #15 scalpels, cut dermis into 2 mm^3 pieces; transfer pieces to 0.25%–0.5% collagenase (dilute from 1% stock in HBSS-CMF; add 0.3 mM $CaCl_2$). Incubate for 1 hour at 37°C; after slight vortexing a homogeneous cell slurry should be obtained.
4. Add 6 mL of HBSS-CMF and centrifuge at 1000 rpm for 20 minutes. Remove the supernatant and resuspend the cell pellet in CNT-07 medium.
5. After resuspension, strain cells through 100 μM cell strainer to remove debris and any non-dissociated tissue still remaining.
6. Using the hemacytometer and trypan blue, count the cells and determine the percentage of dead cells; approximately 5–7 million fibroblasts should be recovered from each pup dermis (*see* **Note 3**).
7. Centrifuge at 1000 rpm for 10 minutes and resuspend in the appropriate volume of CNT-07 medium (*see* **Section 3.4**). Keep cells on ice while chambers are implanted.

3.3. Chamber Implantation

1. Once the cells have been prepared at the appropriate dilutions and are stored on ice, the chambers can be implanted in preparation for cell transplantation (*see* **Note 5**).
2. Anesthetize host NOD–SCID mice by intramuscular injection of ketamine/aceprozamine (100 mg/kg ketamine/10 mg/kg aceprozamine, usually 0.03–0.05 mL of 1 mg/mL ketamine/0.1 mg/mL aceprozamine). The mouse should be fully sedated by 5–10 minutes as verified by testing the foot pad reflex. Sedation lasts 1–2 hours, providing ample time to implant the chamber and allow the cells to settle and adhere.
3. Using forceps pinch the dorsal skin in the midline just posterior to the shoulder blades and cut just below the forceps to excise a small ellipse of tissue approximately 5 mm in length.
4. Fold a sterilized chamber in half and clasp with forceps. Pinch up the skin on the anterior side of the excised site and insert folded chamber with forceps. Hold skin and inserted side of the chamber while using the forceps to bring the posterior side of the excised site around the rest of the chamber (*see* **Note 6**).

5. To prevent the chamber from moving around on the fascia, wrap the upper body of the mouse with Coban. Cut a small hole in the Coban first to allow the top of the chamber to be exposed.
6. Cells should be pipetted directly onto the muscle fascia in a total volume of 50–70 μL. Fibroblasts are added first, and then 5–10 minutes later the keratinocytes are added (*see* **Section 3.4**) Place Tegaderm over the chamber and adhere to the Coban.
7. Deliver Sulfatrim (200 mg sulfamethoxazole and 40 mg trimethoprim/200 mL water; protect from light) and carefully monitor the Coban and Tegaderm daily until the implantation has stabilized.
8. The Tegaderm should be replaced when necessary for 1–2 weeks after chamber implantation, after which time the chamber can be left open to the air.

3.4. Cell Transplantation

1. Resuspend the GFP-negative fibroblasts and keratinocytes at concentrations of 10^8 cells/mL. In order to ensure formation of a regenerated skin a minimum of 2×10^6 fibroblasts and 2×10^6 keratinocytes should be used, and these doses should be kept constant for each dilution of test keratinocytes.
2. The doses of test keratinocytes will depend on the expected stem cell frequency. For initial limiting dilution studies choose dilutions that range many orders of magnitude such as 10^6, 10^4, 10^3, and 10^2. For 10^6 test keratinocytes, a dilution which should yield all positive responses, resuspend the GFP-positive keratinocytes at a concentration of 50×10^6 cells/mL so that 20 μL equals 10^6 cells. Continue serially diluting the cells at concentrations that yield the desired test keratinocyte number in a 20 μL volume. Keep cells on ice while the chambers are being implanted.
3. Seed 20 μL of GFP-negative fibroblasts into the chamber first, wait 5–10 minutes for the cells to settle and adhere, then add 20 μL of GFP-negative keratinocytes and 20 μL of GFP-positive keratinocytes at the appropriate dilution.

3.5. Epifluorescence Imaging and Limiting Dilution Analysis

1. Regenerated epidermis can be imaged 1–2 weeks after chamber implantation and as desired after that (*see* **Note 7**).
2. For imaging, anesthetize mice (*see* **Section 3.3**) and place 100–200 μL of sterile PBS on top of the regenerated skin and scab.
3. After 20 minutes, gently dab away the remaining PBS and soaked scab with sterile kimwipes being careful not to disrupt the regenerated epidermis.

4. Place the mouse under the microscope and capture both bright-field and fluorescence images. It is important to place each mouse in the same orientation and to use the same exposure times for sequential observations (*see* **Fig. 29.1C**).
5. For limiting dilution analysis the regenerated epidermis is scored as positive if at least one GFP-positive epidermal cell cluster is detected.
6. Use the L-Calc software to perform statistical analysis as per the manufacturer's instructions and determine the repopulating unit frequency (*see* **Table 29.1**).
7. The regenerated skin can be excised for immunohistochemical analysis at the end of the imaging period (*see* **Fig. 29.1D**).

4. Notes

1. Pups that are 3–4 days old are optimal for the isolation of both follicular and interfollicular neonatal keratinocytes because separation of the epidermis from the dermis is easier and more complete than at later time points.
2. About 3.5–4 million keratinocytes can routinely be recovered from one day 4 neonatal skin, and less than 10% of the cells should be dead. Less recovery could be due to the following factors: subcutaneous fat incompletely removed resulting in only a partial epidermal–dermal separation; trypsin not adequately pre-warmed; keratinocytes not adequately separated from stratum corneum; keratinocytes not completely resuspended (still clumped) and lost during filtration
3. About 5–7 million fibroblasts can routinely be recovered from one day 4 neonatal skin, and less than 10% of the cells should be dead. Less recovery could be due to the following factors: dermis not adequately digested by collagenase (*see* **Note 4**); fibroblasts not completely resuspended (still clumped) and lost during filtration.
4. The collagenase treatment of the dermis is complete when no more tissue pieces are visible and a homogeneous cell slurry is obtained . Collagenase that is not properly prepared or has expired may not work as well.
5. The prepared cells are viable on ice for several hours, and the number of conditions per experiment will be determined by the number of chambers that can be implanted during that time.

6. The optimal fit of the chambers depends on the initial size of the ellipse of skin that is excised. If the excision is too large the chamber will have to be sutured in place (use 5.0 absorbable sutures). However, if the initial excision site is too small and/or chamber insertion takes many attempts, the host skin will be irritated and the mice tend to disrupt the chambers. The Coban should be tight but not tight enough to restrict breathing.
7. Scab removal should not be attempted prior to 2 weeks post-chamber implantation. During the initial 2 week regeneration period the epidermis is very fragile and should not be disturbed.

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SUBJECT INDEX

Note: The letters 'f', 't' and 'n' following locators refer to figures, tables and notes respectively.

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