Chapter 10

Analysis of Gene Expression in Skin Using Laser Capture Microdissection

Briana Lee, Mikhail Geyfman, Bogi Andersen, and Xing Dai

Abstract

Gene expression analysis is a useful tool to study the molecular mechanisms underlying skin development and homeostasis. Here we describe a method that utilizes laser capture microdissection (LCM) to isolate RNAs from localized areas of skin, allowing the characterization of gene expression by RT-PCR and microarray technologies.

Key words Laser capture microdissection, Epidermis, Hair follicle, Stem/progenitor cells, mRNA, Reverse transcription, Gene expression

1 Introduction

Mammalian skin is a complex organ with a multitude of epithelial and stromal cell types and harbors various appendages such as hair follicles which themselves are "miniorgans." As the skin epithelial compartment develops, it acquires multiple cell types with specific cellular functions. The interfollicular epidermis continuously self-renews throughout life, owing to the activity of epidermal stem/progenitor cells (1, 2). In contrast, hair follicles undergo periodic cycles of proliferation and apoptosis, a process referred to as the hair cycle in which regeneration is fueled by stem cells located in distinct anatomic locales (e.g., bulge, secondary hair germ, and isthmus) (3). A thorough understanding of the molecular mechanisms governing epidermal/hair follicle development and regeneration will benefit from a systematic analysis of gene expression in the constituent cell types at different developmental and regeneration stages.

Physical separation, especially when used in combination with enzyme digestion, provides a useful way for isolating distinct skin cell populations. A time-tested method is to separate epidermis from its underlying dermis using trypsin/dispase followed by

Kursad Turksen (ed.), *Skin Stem Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 989,
DOI 10.1007/978-1-62703-330-5_10,

Table 1
Summary of markers used to identify skin epithelial stem/progenitor cell populations

Marker	Location	Reference
α6 Integrin	Basal epidermis	(10)
Sca1	Basal epidermal cells	(11)
CD34	Bulge	(12)
Lgr5	Lower bulge, hair germ	(13)
Lgr6	Isthmus	(14)
Lrig1	Infundibulum	(15)
MTS24	Infundibulum	(16)
Blimp1	Infundibulum	(17)
Gli1	Upper bulge	(18)
Ephrin β1	Transient amplifying cells of the hair matrix	(19)

physical peeling. However, this method does not allow the separation of distinct epidermal cell types. Moreover, epidermis of earlier developmental stages (e.g., E13.5) is extremely thin and difficult to peel off. The culture of primary keratinocytes from skin offers a valid substitute for analysis; however, it is unavoidably accompanied by a deviation from the true in vivo gene expression program due to altered growth conditions and lack of normal epithelial–mesenchymal interactions.

In recent years, fluorescence activated cell sorting (FACS) based on various surface markers has emerged as a powerful strategy to isolate distinct populations of skin epithelial stem cells, leading to the accumulation of tremendous amount of knowledge about adult stem cell behavior (4) (Table 1). However, the utility of this methodology is limited by the availability of markers. FACS has also been applied to cases where the desired cell types are labeled with green fluorescent protein (GFP) using cell type-specific promoters or based on characteristic cell behaviors (e.g., slow-cycling of bulge stem cells) (5, 6). However, these approaches typically involve complex breeding of transgenic mouse lines and sometimes drug administration to achieve precisely controlled GFP expression in a spatiotemporal manner.

For reasons discussed above, laser capture microdissection (LCM), which allows the collection of cells from histological sections, offers a useful alternative approach to compare gene expression in various skin cell types during development as well as in adulthood. Unlike FACS, this method enables one to directly identify and recover cell populations without reliance on known cell-surface markers or undertaking of complicated and expensive transgenic experiments.

LCM offers the added advantage of permitting the isolation of cells in their normal in vivo settings. This is particularly relevant for study of stem cells, which are known to reside in niches which include, in addition to stem cells themselves, nearby signaling cells and extracellular matrix (7). LCM allows one to collect an intact niche without disrupting its components as is required by FACS. Additionally, it is possible to use immunofluorescence to label a subset of cells within small regions, thus guiding the capture of specific cells (8).

Currently methods are available for using the small amount of material obtained from LCM to perform molecular/biochemical assays such as RT-PCR, microarray gene expression studies, and even proteomic analyses (9). The shortcomings of LCM include a relatively high initial cost of the LCM microscope and contamination from adjacent tissue. However, experienced users report minimal contamination, and multiple tissue-specific genes can be used to accurately evaluate the relative amount of contaminating material in laser captured tissues.

2 Materials

For RNA isolation and cDNA synthesis, modified protocols and reagents from Qiagen RNeasy and Sensiscript kits, respectively, were utilized.

2.1 Preparing Skin Cryoblocks

1. Petri dishes (Bacterial grade).
2. Tissue-TekCryomold (15 mm × 15 mm × 5 mm), Electron Microscopy Sciences, Cat. No. 62352–15.
3. Tissue TekCryo-OCT, Fisher, Cat. No. NC9695545.
4. Dry ice.
5. Ice-cold PBS.
6. Fine scissors and forceps, RNAse Away-treated.

2.2 Sectioning Skin Cryoblocks

1. PEN-membrane slides, Leica, Cat. No. 11505158.
2. UV crosslinker (e.g., Stratagene UV Stratalinker 2400).
3. Tissue TekCryo-OCT.
4. Cryostat (e.g., Leica CM1850).
5. RNase Away, Fisher, Cat. No. 7003.
6. Ethanol Gold Shield, 200 proof.

2.3 Fixation and H&E Staining

1. 70% and 100% Ethanol.
2. Xylene, Fisher, Cat. No. X3S-4.
3. Acetone, Fisher, Cat. No. A929-4.
4. DEPC-treated water, Sigma, Cat. No. D5758 or home-made.

5. Meyer's hemotoxylin solution, Sigma, Cat. No. MHS1.
6. Slide mailers, Fisher, Cat. No. HS15986.

2.4 LCM

1. Flat cap 0.5 ml PCR tubes, Fisher, Cat. No. 14-222-265.
2. RNase away.
3. Buffer RLT, Qiagen, Cat. No. 79216.
4. Beta-mercaptoethanol (BME), Sigma, Cat. No. M3148.
5. Laser microdissection microscope (e.g., Leica LS-AMD).

2.5 RNA Isolation

1. RNeasyMicro kit, Qiagen, Cat. No. 74004.
   - Buffer RLT and RW1.
2. 20 ng/μl proteinase K, Fisher, Cat. No. BP1700-100.
3. 100% ethanol.
4. Microcentrifuge.

2.6 RNA Quality Control

1. Spectrophotometer.
2. Agilent 2100 Bioanalyzer.

2.7 Reverse Transcription

1. Sensiscript RT kit, Qiagen, Cat. No. 205221.
2. Random hexamer primers, Applied Biosystems, Cat. No. N8080127.
3. 37°C water bath.
4. Eppendorf tubes.

3 Methods

3.1 Preparing Skin Cryoblocks

1. Always use skin samples that have been harvested immediately before placing in cryoblock and keep the tissues on ice until freezing in OCT.
2. Excise a piece of back skin using fine scissors and forceps (pre-treated with RNase Away) and briefly wash with ice-cold PBS (see Note 1).
3. Place a cryoblock on a piece of dry ice and cover the bottom with a few drops of OCT medium. Allow it to freeze.
4. Place skin sample with epidermis facing up into the cryoblock and completely cover the tissue with OCT medium, being careful to avoid bubbles (see Note 2).
5. Wait until the OCT is frozen thoroughly and store the samples at −80°C or proceed immediately to sectioning step.

3.2 Sectioning

1. Clean the inside of the cryostat with ethanol and RNase away.
2. Incubate slides in UV crosslink chamber on maximum power for 30 min.

3. Adjust cryostat chamber temperature to –25°C and place OCT tissue samples into the chamber for 20 min to allow the tissue to equilibrate to chamber temperature.
4. Cut 8 μm sections and place no more than four slices onto one membrane slide (see Note 3).
5. Keep slides at –80°C for up to 1 week in a tightly closed slide box until they are ready to be fixed and stained.

3.3 Fixation and H&E Staining

All materials, including slide containers and forceps should be treated with RNase away prior to use.

1. Prepare the following slide holders containing: ice-cold acetone, ice-cold 70% ethanol, DEPC water (x2), Meyer's hematoxylin, 70% ethanol, 100% ethanol (x2), and xylene (x2).
2. Incubate slides in the following order:

   Fixation

   - Cold acetone for 5 min.
   - Cold 70% ethanol for 1 min.
   - Wash with DEPC water (RNase-free) for 10 s.
   - H&E staining
   - Meyer's hematoxylin for 30 s.
   - Wash with DEPC water for 10 s.

   Dehydration

   - 70% ethanol for 10 s.
   - 100% ethanol for 30 s.
   - 100% ethanol for another 30 s.
   - Xylene for 30 s.
   - Xylene for 5 min (see Note 4).
3. Proceed immediately to LCM.

3.4 LCM (Refer to Leica LMD Manual for Details and Illustrations of Cap Loading and Basic Operation)

1. Add 30 μl BME per 1 ml RLT buffer.
2. Insert a flat cap PCR tube into the tube holder on the sample collection tray.
3. Load the cap of PCR tubes with 75 μl BME/RLT buffer.
4. Insert a LCM membrane slide facing down.
5. Align the sample tray according to operational instructions.
6. Cut the desired area by tracing a line around the outside region of the area so that the laser is cutting the cells directly adjacent to your desired cells (see Note 5).
7. Collect at least 2,000 epidermal keratinocytes (see Fig. 1a) or hair follicle keratinocytes or dermal papillae cells in one tube (see Fig. 2a). Make sure that a total dissection time does not

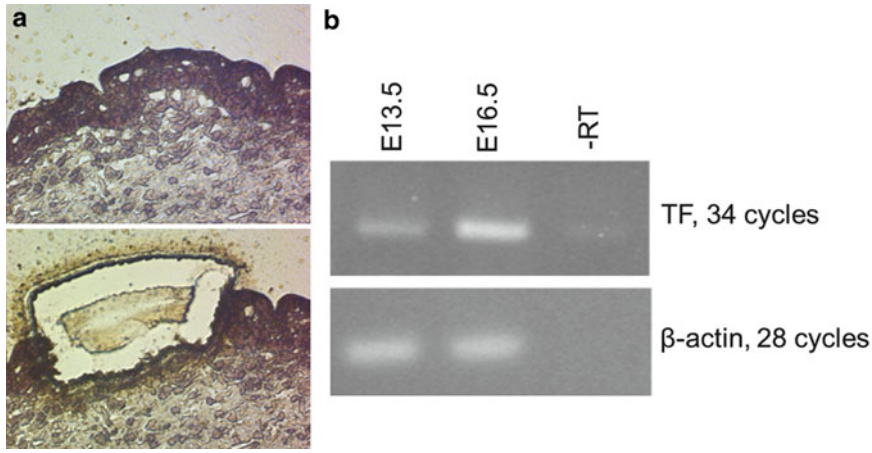

Fig. 1 LCM of embryonic epidermal cells. (**a**) E16.5 skin sections before and after LCM. Cutting was performed with attempt to minimize burning of desired epidermal cells while avoiding the capture of dermal cells. (**b**) Semi-quantitative RT-PCR was performed on RNAs prepared from E13.5 and E16.5 laser captured epidermal cells as shown in (**a**), using primers against a transcription factor (TF) and housekeeping gene β-actin

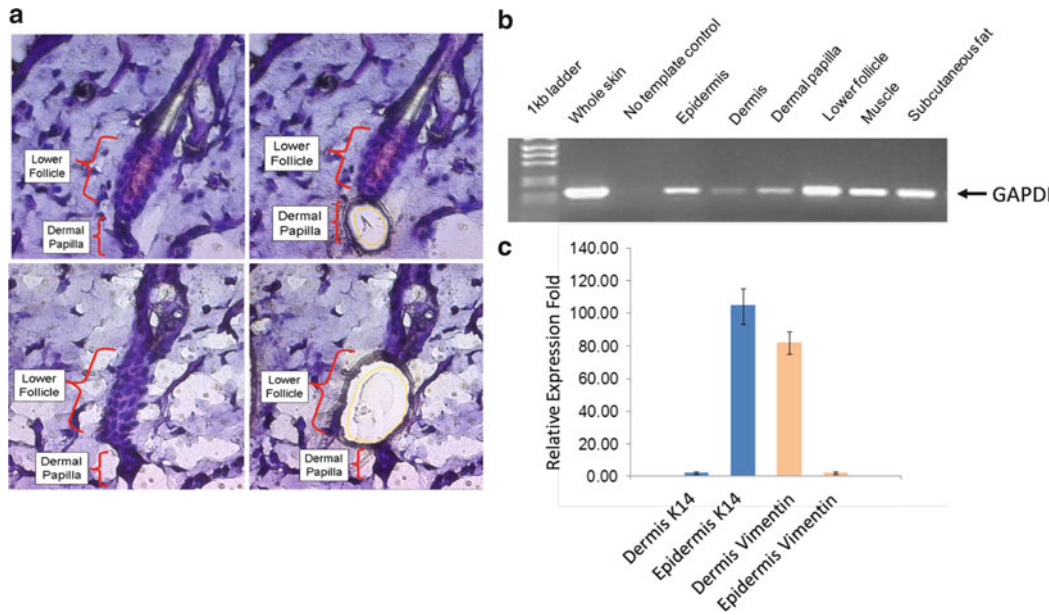

Fig. 2 LCM of adult hair follicle cells. (**a**) Adult skin sections before and after LCM. *Top*, collection of dermal papilla cells. *Bottom*, collection of the lower part of the follicle containing the bulge area. Cutting was performed with attempt to minimize contamination while avoiding UV laser induced thermal damage of RNA. (**b**) RT-PCR analysis of RNAs prepared from laser captured cells of epidermis, dermis, dermal papilla, lower follicle, muscle, and subcutaneous layer. A 1/10 of cDNA generated was used to PCR amplify a ~100-bp segment of GAPDH (35 cycles). (**c**) qRT-PCR analysis of the expression of Keratin 14 (basal epidermis-specific gene) and Vimentin (dermis-specific gene) in laser captured dermis and epidermis

exceed 1 h. Therefore we recommend processing no more than two slides at a time.

8. Vortex tissue in buffer for 2 min at full speed.
9. Process samples immediately or store at –80°C for up to a week (see Note 6).

3.5 RNA Isolation

1. If possible, carry out all pipetting steps using filter tips in a sterile laminar flow hood and clean equipment with RNase away.
2. RNA extraction using RNeasy Micro Kit.
   (a) Vortex for a few minutes at full speed if the samples have been stored at –80°C.
   (b) Adjust volume of tissue harvests to 150 µl (you can combine two 75 µl tubes or just add another 75 µl RLT buffer to one tube).
   (c) Vortex at full speed for 30 s.
   (d) Add 295 µl RNase-free water.
   (e) Add 5 µl proteinase K (20 ng/µl). Mix by pipetting.
      - Incubate at 55°C for 10–15 min.
   (f) Microfuge for 3 min at 12,000 rpm (10,000 × *g*).
   (g) Pipette supernatant into a new tube provided with the kit.
   (h) Add 225 µl of 100% RNase-free ethanol.
      - Mix by pipetting.
   (i) Transfer to RNeasy MiniElute spin column with 2 ml collection tube.
   (j) Close the lid of the microfuge gently and microfuge for 15 s at 10,000 rpm (8,000 × *g*).
      - Optional: run the flow-through on the column again. This may increase the amount of RNA that binds to the column; however, you also run the risk of increasing salt contamination.
      - Discard the flow-through.
   (k) Add 700 µl Buffer RW1 to the spin column.
      - Microfuge 15 s at 10,000 rpm (8,000 × *g*).
      - Discard the flow-through and collection tube.
   (l) Place spin column in a new 2 ml collection tube provided with the kit.
      - Add 500 µl Buffer RPE (ethanol added).
      - Microfuge 15 s at 10,000 rpm (8,000 × *g*).
      - Discard the flow-through.

(m) Add 500 μl fresh 80% ethanol (use RNase-free water) to the spin column.
- Microfuge 15 s at 10,000 rpm (8,000 × *g*).
- Discard the flow-through and the collection tube. Do not let spin column contact the flow-through when removing the column.

(n) Place spin column in a new 2 ml collection tube provided with the kit.
- Microfuge at full speed (14,000 × *g*) for 5 min. Leave the spin column lid open (evaporates ethanol).
- Discard the flow-through and the collection tube.

(o) Place spin column in a new 1.5 ml collection tube.
- Add 20 μl RNase-free water directly to the center of the spin column membrane.
- Microfuge for 1 min at full speed to elute the RNA.
- Run the eluate through the spin column again to increase the RNA yield.

3.6 Quality Control

1. Maintain RNase-free working conditions as described above.
2. Determine RNA concentration with a spectrophotometer measuring 260 nm absorption and Agilent Bioanalyzer to check RNA integrity (see Note 7).
3. Proceed to reverse transcription.

3.7 Reverse Transcription

1. Maintain RNase-free working conditions as described above.
2. Use the Sensiscript RT kit and random hexamer primers for reverse transcription.
3. Aqueous solutions of cDNA can be stored at –20°C.
4. Perform RT-PCR with housekeeping and tissue-specific genes in order to validate lack of contamination from unintended tissue and to estimate cDNA yield (see Figs. 1b and 2b, c).

4 Notes

1. For adult mouse skin, make sure to clip hair after sacrificing and prior to removal of mouse skin.
2. Since the specimen is no longer visible after OCT embedding, make a note of which way the epidermis is facing.
3. Be careful not to overlap the OCT sections, since that leads to loss of sections in subsequent staining steps.
4. Slides can be kept in xylene for longer than 5 min during transport to the LCM. We have not noted deleterious effects to the

tissue or purified material when slides had been kept in xylene for longer than 5 min.

5. If the line is drawn too close to the cells to be collected, the laser will burn them away. Draw a line just outside the area of interest (refer to Fig. 1a, 2a).
6. Once all samples are cut and captured, immediately process or store at −80°C. Do not leave samples out at room temperature for longer than 30 min.
7. For each 2,000-cell harvest, typical yield of RNA is 15–20 ng/μl for embryonic epidermis, 10–20 ng/μl for hair germ, 10–20 ng/μl for dermal papilla, and 20–30 ng/μl for the bulge.

References

1. Clayton E et al (2007) A single type of progenitor cell maintains normal epidermis. Nature 446(7132):185–189
2. Jensen KB et al (2009) Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis. Cell Stem Cell 4(5):427–439
3. Woo WM, Oro AE (2011) SnapShot: hair follicle stem cells. Cell 146(2):334–334e2
4. Fuchs E (2009) The tortoise and the hair: slow-cycling cells in the stem cell race. Cell 137(5):811–819
5. Rhee H, Polak L et al (2006) Lhx2 maintains stem cell character in hair follicles. Science 312(5782):1946–1949
6. Fuchs E, Horsley V (2011) Ferreting out stem cells from their niches. Nat Cell Biol 13(5):513–518
7. Spradling A, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414(6859):98–104
8. Murakami H, Liotta L et al (2000) IF-LCM: laser capture microdissection of immunofluorescently defined cells for mRNA analysis rapid communication. Kidney Int 58(3):1346–1353
9. Xu BJ et al (2009) Combining laser capture microdissection and proteomics: methodologies and clinical applications. Proteomics Clin Appl 4(2):116–123
10. Blanpain C et al (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 118(5):635–648
11. Albert MR, Foster RA, Vogel JC (2001) Murine epidermal label-retaining cells isolated by flow cytometry do not express the stem cell markers CD34, Sca-1, or Flk-1. J Invest Dermatol 117(4):943–948
12. Trempus CS et al (2003) Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J Invest Dermatol 120(4):501–511
13. Jaks V et al (2008) Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat Genet 40(11):1291–1299
14. Snippert HJ et al (2011) Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science 327(5971):1385–1389
15. Jensen KB, Collins CA et al (2009) Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis. Cell Stem Cell 4(5):427–439
16. Nijhof JG et al (2006) The cell-surface marker MTS24 identifies a novel population of follicular keratinocytes with characteristics of progenitor cells. Development 133(15):3027–3037
17. Horsley V et al (2006) Blimp1 defines a progenitor population that governs cellular input to the sebaceous gland. Cell 126(3):597–609
18. Brownell I et al (2011) Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. Cell Stem Cell 8(5):552–565
19. Lien WH et al (2011) Genome-wide maps of histone modifications unwind in vivo chromatin states of the hair follicle lineage. Cell Stem Cell 9(3):219–232