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Cultivation of Normal Human Epidermal Melanocytes in the Absence of Phorbol Esters

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1. Introduction

An important approach in studies of normal, diseased, and malignant cells is their growth in culture. The isolation and subsequent culture of human epidermal melanocytes has been attempted since 1957 ***(1–5)***, but only since 1982 have pure normal human melanocyte cultures been reproducibly established to yield cells in sufficient quantity for biological, biochemical, and molecular analyses ***(6)***. Selective growth of melanocytes, which comprise only 3–7% of epidermal cells in normal human skin, was initially achieved by suppressing the growth of keratinocytes and fibroblasts in epidermal cell suspensions with the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) and the intracellular cyclic adenosine 3′, 5′ monophosphate (cAMP) enhancer cholera toxin, respectively, which both also act as melanocyte growth promoters. However, phorbol ester is metabolically stable and has prolonged effects on multiple cellular responses ***(6)***. Recent progress in basic cell-culture technology, along with an improved understanding of culture requirements, has led to an effective and standardized isolation method, and special TPA-free culture media for selective growth and long-term maintenance of human melanocytes. The detailed description of this new method is aimed at encouraging its use in basic and applied biological research.

2. Materials

1. Normal skin-transporting medium: The medium for collecting normal skin is composed of Hanks balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (HBSS; Gibco-BRL Grand Island, NY, cat. no. 21250-089) supplemented with penicillin (100 U/mL; USB, Cleveland, OH, cat. no. 199B5), streptomycin (100 µg/mL;

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USB, cat. no. 21B65), gentamicin (100 µg/mL; BioWittaker, Walkersville, MD, cat. no. 17-518Z), and fungizone (0.25 µg/mL; JRH Biosciences, Lenexa, KS, cat. no. 59-604-076). After sterilization through a 0.2-µm filter, the skin-transporting medium is transferred into sterile containers in 20-mL aliquots and stored at 4°C for up to 1 mo.

2. Epidermal isolation solution: Dissolve 0.48 g of dispase (grade II, 0.5 U/mg; Boehringer Mannheim, Indianapolis, IN, cat. no. 165859) in 100 mL of phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Cellgro® by Mediatech, Herndon, VA, cat. no. MT21-031-CM) containing 0.1% bovine serum albumin (BSA) (fraction V; Sigma, St. Louis, MO, cat. no. A9418) to yield a final dispase activity of 2.4 U/mL. Sterilize the enzyme solution through a 0.2-µm filter, aliquot into 5-mL tube, and store at –20°C for up to 3 mo.
3. Cell-dispersal solution: The cell-dispersal solution contains 0.25% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA) and is purchased from Cellgro by Mediatech, cat. no. 25-053-CI. Store at 4°C for up to 1 mo.
4. TPA-free melanocyte growth medium (TPA-free MGM): The following stock solutions and reagents are required:
   a. MCDB153 (Sigma, cat. no. M7403): Dissolve MCDB153 powder in ~approx 700 mL ddH_2O, add 1.18 g sodium bicarbonate (Sigma, cat. no. S5761), adjust pH to 7.4 ± 0.02, bring the total volume to 1 L with ddH_2O, sterilize through a 0.2-µm filter, and store light-protected at 4°C for up to 3 wk. Use 87 mL per 100 mL complete MGM.
   b. Heat-inactivated fetal bovine serum (FBS; Cansera, Etobicoke, ON, Canada, cat. no. CS-C08-100): Heat FBS in manufacturer's bottle at 56°C water bath for 20 min and store at 4°C for up to 3 wk. Use 2 mL per 100 mL complete MGM.
   c. Chelated FBS: Add 15 g Chelax 100 (Sigma, cat. no. C7901) to 500 mL FBS, stir to mix at 4°C for 1.5 h, filter-sterilize with a 0.2-µm filter, prepare 10-mL single-use aliquots, and store at –20°C for up to 3 mo.
   d. L-Glutamine, 200 m*M* stock (Cellgro by Mediatech, cat. no. MT25-005-C1): Prepare 1-mL single-use aliquots and store at –20°C for up to 6 mo.
   e. Cholera toxin (Sigma, cat. no. C3021), 40 n*M* (3.33 µg/mL) stock: Dissolve 500 µg of cholera toxin in 150 mL ddH_2O, sterilize through a 0.2-µm low protein-binding filter (Millipore, Marlborough, MA, cat. no. SLGV025LS), divide into 250-µL aliquots, and store at 4°C for up to 1 yr. Use 50 µL/100 mL MGM to give a final cholera toxin concentration of 20 p*M*.
   f. Recombinant human basic fibroblast growth factor (rh-bFGF; Research Diagnostics, Flanders, NJ, cat. no. RDI-118bx), 0.57 µg/mL stock: Dissolve 4 µg of rh-bFGF in 7 mL of 0.1% BSA; Sigma, cat. no. A9647) in Ca^{2+}- and Mg^{2+}-free PBS. Pre-wet a 0.2-µm low protein-binding filter with Ca^{2+}- and Mg^{2+}-free PBS containing 0.1% BSA before filter-sterilizing rh-bFGF stock solution to avoid loss of the recombinant protein due to nonspecific binding to the filter. Prepare 500-µL aliquots and store at –20°C for up to 3 mo. Add 200 µL of rh-bFGF stock per 100 mL MGM to yield a final concentration of rh-bFGF of 1.14 ng/mL.

g. Recombinant human, rat, pig, or rabbit endothelin-3 (rET-3; American Peptide Company, Sunnyvale, CA, cat. no. 88-5-10), 100 µ*M* (264 µg/mL) stock: Dissolve 500 µg of rET-3 in 1.89 mL of 0.1% BSA in Ca^{2+}- and Mg^{2+}-free PBS. Filter-sterilize rET-3 stock solution by passage through a 0.2-µm low protein-binding filter pre-wet with PBS containing 0.1% BSA. Make 200-µL single-use aliquots and store at –20°C for up to 3 mo.
h. Recombinant human stem cell factor (rhSCF; R&D systems, Minneapolis, MN, cat. no. 255-SC-050), 10 µg/mL stock: Add 50 µg rhSCF to 5 mL of 0.1% BSA in Ca^{2+}- and Mg^{2+}-free PBS, filter-sterilize through a 0.2-µm low protein-binding membrane pre-wet with PBS containing 0.1% BSA, prepare 100-µL single-use aliquots, and store at –20°C for up to 3 mo.
i. Heparin (Sigma, cat. no. H3149), 1 µg/mL stock: Prepare heparin storage stock at 1 mg/mL by dissolving 1 mg of heparin sodium salt in 1 mL of Ca^{2+}- and Mg^{2+}-free PBS, filter-sterilizing through a 0.2-µm filter, dividing into 10-µL aliquots, and storing at 4°C for up to 6 mo. Before making up the complete medium, 1 µg/mL heparin stock solution is prepared fresh by diluting 1 µL of 1 mg/mL storage stock with 1 mL of PBS. Use 100 µL of 1 µg/mL heparin stock for 100 mL complete medium to yield a final heparin concentration of 1 ng/mL.

TPA-free MGM is prepared as follows: Mix 87 mL of MCDB153 with 2 mL heat-inactivated FBS, 10 mL chelated FBS, 1 mL L-glutamine (200 m*M* stock), 50 µL cholera toxin (40 n*M* stock), 200 µL bFGF (0.57 µg/mL stock), 200 µL ET-3 (100 µ*M* stock), 100 µL SCF (10 µg/mL stock), and 100 µL heparin (1 µg/mL stock) to give final concentrations of 12% FBS, 2 m*M* L-glutamine, 20 p*M* cholera toxin, 1.14 ng/mL bFGF, 100 n*M* ET-3, 10 ng/mL SCF, and 1 ng/mL heparin. Store TPA-free MGM at 4°C for up to 8 d.

5. Trypsin–versene solution: Make a 5X stock by mixing 0.5 mL of 2.5% trypsin solution (BioWittaker, cat. no. 17-160E) with 100 mL of versene composed of 0.1% EDTA (Fisher, Pittsburgh, PA, cat. no. 02793-500) in Ca^{2+}- and Mg^{2+}-free PBS (pH 7.4). To prepare trypsin–versene solution, dilute 5X stock with Ca^{2+}- and Mg^{2+}-free HBSS to give a final concentration of 0.0025% trypsin and 0.02% EDTA.
6. Cell-preservative medium: Prepare 5% (v/v) dimethyl sulfoxide (DMSO; Sigma, cat. no. D2650) in 95% heat-inactivated FCS as needed.

3. Methods

3.1. Day 1

1. Prepare the following in a laminar flow hood: one pair each of sterile forceps, curved scissors, and surgical scalpel blade; 5 mL of epidermal isolation solution (*see* **Subheading 2.2.**) in a sterile centrifuge tube; 10 mL of Ca^{2+}- and Mg^{2+}-free HBSS in a sterile nontissue-culture Petri dish; and 10 mL of 70% ethanol in a separate sterile Petri dish.
2. Soak the skin specimens in 70% ethanol for 1 min. Transfer skin to the Petri dish containing HBSS to rinse off ethanol (*see* **Notes 1** and **2**).

3. Cut skin-ring open, and trim off fat and sc tissue with scissors (*see* **Note 3**).
4. Cut skin into pieces (approx 5 × 5 mm^2) using the surgical scalpel blade with one-motion cuts (*see* **Note 4**).
5. Transfer the skin pieces into the tube containing epidermal isolation solution. Cap, invert, and incubate the tube in the refrigerator at 4°C for 18–24 h (*see* **Note 5**).

3.2. Day 2

1. Remove the tube containing the sample from the refrigerator and incubate at 37°C for 5 min.
2. Prepare the following in a laminar flow hood: two pairs of sterile forceps and a surgical scalpel blade; two empty sterile nontissue-culture Petri dishes; 5 mL of cell-dispersal solution; and 10 mL of Ca^{2+}- and Mg^{2+}-free HBSS in a 15-mL centrifuge tube.
3. Pour tissue in epidermal isolation solution into one of the empty Petri dishes. Separate the epidermis (thin, brownish, translucent layer) from the dermis (thick, white, opaque layer) using the forceps. Hold the dermal part of the skin piece with one pair of forceps, and the epidermal side another. Gently tease them apart. Discard the dermis immediately (*see* **Note 5**). Transfer the harvested epidermal sheets to an empty Petri dish, add a drop of Ca^{2+}- and Mg^{2+}-free HBSS to prevent tissue from drying. Repeat the above described procedure for each piece of tissue and then mince them into smaller pieces (approx 2 × 2 mm^2) with a surgical scalpel blade (*see* **Note 5**).
4. Transfer the collected epidermal sheets from the Petri dish to the centrifuge tube containing 5 mL of cell-dispersal solution. Incubate the tube at 37°C for 5 min. Vortex the tube vigorously or use repetitive pipet motions to release single cells from epidermal sheets. Wash the resulting single-cell suspension once with 10 mL of Ca^{2+}- and Mg^{2+}-free HBSS. Centrifuge for 5 min at 800*g* at room temperature. Carefully aspirate the supernatant, which may contain remaining stratum corneum. Resuspend the pellet with 5 mL TPA-free MGM (*see* **Note 6**).
5. Plate the resulting epidermal cell suspension in a T25 cell-culture vessel. Incubate at 37°C in 5% CO_2/5% air for 48–72 h without disturbance.

3.3. Subsequent Maintenance, Subcultivation, Cryopreservation, and Thawing

1. Wash culture with MGM on d 4 to remove nonadherent cells, which may include but are not limited to keratinocytes and fragments of stratum corneum. Medium change should be performed twice a week thereafter. Seventy percent confluent primary melanocyte cultures can be obtained in approx 1 wk (*see* **Note 7**).
2. Subcultivation: Primary cultures established from foreskins usually reach 70% confluence within 7–9 d after plating. At this point, cultures are treated with trypsin–versene solution (*see* **Subheading 2., step 5**) at room temperature for 2–3 min, harvested with Leibovittz's L-15 (Gibco-BRL, cat. no. 41300-070) containing 10% heat-inactivated FBS, centrifuged at 2000 rpm for 3 min, resuspended in

TPA-free MGM, reinoculated at approx 10^4 cells/cm^2, and serially passaged. Medium is changed twice each week.

3. Cryopreservation: Melanocyte suspensions harvested by trypsin–versene and Leibovitz's L-15 containing 10% heat-inactivated FBS are centrifuged at 800*g* for 5 min and resuspended in cell-preservative medium (*see* **Subheading 2., step 6**) containing 5% DMSO as a cryopreservative. Cells are normally suspended at a density of 10^6/mL and transferred to cryotubes. The tubes are then placed in a plastic sandwich box (Nalgene™ Cryo 1°C Freezing container; Nalge, Rochester, NY, cat. no. 5100-0001), which is immediately transferred to a –70°C freezer. The insulation of the freezing container ensures gradual cooling of the cryotubes and results in more than 80% viability of cells upon thawing. After overnight storage in the –70°C freezer, the cryotubes are placed in permanent storage in liquid nitrogen.
4. Thawing: The melanocyte suspension is thawed by incubating the cryotube in a 37°C water bath. When the cell-preservative medium is almost, but not totally, defrosted, the outside of the tube is wiped with 70% alcohol. The cell suspension is then withdrawn, quickly diluted in TPA-free MGM at room temperature, centrifuged, and resuspended in fresh TPA-free MGM. Cell viability is determined by Trypan blue exclusion. The resulting melanocytes are then seeded at a density of 10^4 cells/cm^2.

3.4. Results

3.4.1. Minimal Growth Requirements

Earlier studies of normal melanocytes ***(6–8)*** were done using media containing bovine pituitary extracts, which provides a host of poorly characterized growth-promoting activities. Deprivation of serum and brain tissue extracts from media has led to the delineation of four groups of chemically defined melanocyte mitogens.

1. Peptide growth factors, including bFGF ***(9–12)***, which is the main growth-promoting polypeptide in bovine hypothalamus and pituitary extracts, insulin/insulin-like growth factor-1 (IGF-1; ***13***), epidermal growth factor (EGF; ***14,15***), transforming growth factor-α (TGF-α; ***16***), endothelins (ET; ***17–21***), hepatocyte growth factor/scatter factor (HGF/SF; ***22–24***), and stem cell factor (SCF; ***10,25–28***).
2. Calcium, because reduction of Ca^{2+} concentrations in TPA-containing MGM from an optimal 2.0 to 0.03 m*M* reduces cell growth by approx 50% ***(20)***, and cation-binding proteins, such as tyrosinase at 10^{-11} *M*, and ceruloplasmin at 0.6 U/mL ***(29)***.
3. Enhancers of intracellular levels of cAMP, including α-melanocyte stimulating hormone (α-MSH) at 10 ng/mL ***(30)***; forskolin at 10^{-9} *M* ***(29)***, follicle stimulating hormone (FSH) at 10^{-7} *M* ***(31)***; and cholera toxin at 10^{-12} *M* ***(6,29,32,33)***.
4. Activators of protein kinase C (PKC), such as TPA ***(34)***, which is lipophilic and cannot be removed by simple washing, and 20-oxo-phorbol-12,13-dibutyrate

(PDBu; ***34***), which is a similar derivative, but more hydrophilic. Recent data suggest that the tigliane class phorbol compounds, such as 12 deoxyphorbol, 13 isobutyrate (DPIB), and 12 deoxyphorbol, 13 phenylacetate (DPPA), which possess diminished tumor-promoting activity, are also able to activate PKC as well as stimulate melanocyte proliferation ***(35)***.

3.4.2. Morphology

Human epidermal melanocytes grown in TPA-free MGM normally exhibit a dendritic morphology with varying degrees of pigmentation (*see* **Fig. 1**). By contrast, melanocytes maintained in the conventional TPA medium ***(36)*** are bi- or tri-polar.

3.4.3. Expression of Antigens

Extensive studies have been done to characterize the antigenic phenotype of malignant melanoma cells ***(37)***. On the other hand, very few attempts have been made to produce monoclonal antibodies (mAbs) to normal melanocytes ***(15,38)***. Cultured melanocytes share with melanoma cells the expression of a variety of cell-surface antigens (melanoma-associated antigens), including p97 melanotransferrin, integrin β_3 subunit of the vitronectin receptor, gangliosides GD_3 and 9-O-acetyl GD_3, chondroitin sulfate proteoglycan ***(15)***, and MelCAM/MUC18/CD146 ***(39,40)***. However, these antigens are not expressed by normal melanocytes *in situ* ***(41,42)***. **Table 1** summarizes the expression of antigens on melanocytes *in situ* and in culture. The observed divergent antigenic phenotype in culture and *in situ* suggested a role for the epidermal microenvironmental signals in controlling the melanocytic phenotype. Indeed, accumulating evidence indicates that undifferentiated keratinocytes can control proliferation, morphology, pigmentation, and antigen expression of melanocytes in coculture ***(43–47)***. Using coculture and three-dimensional (3D) skin reconstruct models, we have begun to characterize the molecular bases of the crosstalk between keratinocytes and melanocytes ***(48–50)***.

3.4.4. Growth Characteristics

Melanocytes from neonatal foreskin can be established with a success rate of 80% and have a maximum lifespan of 60 doublings, with a doubling time of

Fig. 1. *(see facing page)* Morphology of normal human epidermal melanocytes maintained in TPA-free medium supplemented with bFGF, ET-3, SCF, cholera toxin, and serum. A, post-plating d 1: Admixed in the background cellular and tissue debris, some cells (including melanocytes and occasional keratinocytes) though still rounded attach to the substratum. B, post-plating d 3: Attached melanocytes spread out on the substratum giving a bi- or tri-polar morphology. The occasional surviving keratinocytes

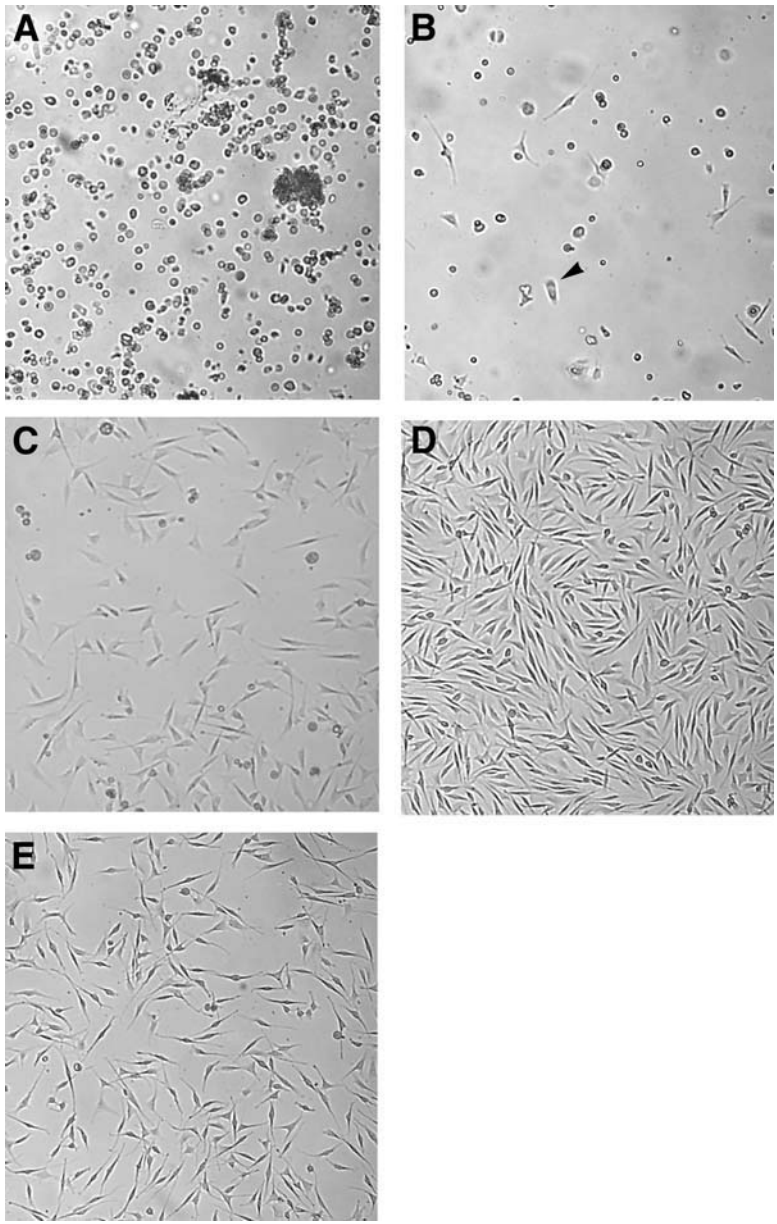

Fig. 1. *(continued)* are identified by their cobblestone/polygonal morphology (arrowhead). C, post-plating d 7: High serum concentration in the medium eliminates contaminated keratinocytes by induction of terminal differentiation. Pure melanocyte culture is usually established at this point. Melanocytes now appear more flattened and multidendritic. D, postplating d 9: Greater than 70% confluence is usually achieved by d 9, at which point the cells are ready to be subcultured. E, passage 1 melanocyte culture (1 d after splitting): Cells display the characteristic dendritic morphology.

Table 1
Expression of Antigens on Melanocytes *in situ* and in Culture[a]

Antigens	*in situ*	in culture
CD26	++++	++++
gp145	++	++
c-kit	++++	++++
TRP-1 (gp 75)	++++	++++
TRP-2 (DCT)	++++	++++
MITF	++++	++++
E-cadherin	++++	++++
α-catenin	++++	++++
β-catenin	++++	++++
integrin β3 subunit	–	++++
tenascin	–	++++
fibronectin	–	++++
chondroitin sulfate proteoglycan	±	++++
p97 melanotransferrin	–	++++
NGF-receptor (p75)	±	++++
9-O-acetyl GD3	±	+++
GD3	±	++++
HLA-DR	–	–
MelCAM/MUC18/CD146	–	++++

[a]–, lack of expression; ±, 0–20%; +, 20–40%, ++, 40–60%; +++, 60–80%; ++++, 80–100%.

1.5–4 d. Heavily pigmented cells isolated from black individuals have a shorter doubling time and tend to senesce after 20–30 doublings. By contrast, epidermal melanocytes from adult skin only grow in about 10% of cases and for no more than 10 doublings with a doubling time of 7–14 d. The cells do not grow beyond 70% confluence and exhibit signs of growth arrest by contact inhibition. Normal melanocytes do not proliferate anchorage independently in soft agar and are nontumorigenic in athymic nude mice ***(12,14)***.

4. Notes

1. Tissue source and collection: The source of tissue for melanocyte cultures are human neonatal foreskins obtained from routine circumcision and normal adult skin acquired from reduction mammoplasty. At the time of excision, the skin is placed into a sterile container with 20 mL of normal skin-transporting medium (*see* **Subheading 2.1.**) supplied in advance and kept near the surgical area at 4°C. Specimens are delivered immediately to the tissue-culture laboratory or stored at

4°C. Neonatal foreskins can be kept for up to 48 h, and normal adult skin, for up to 24 h. However, the fresher the specimens, the higher the yield of live cells on isolation.

2. Sterilization of skin specimens: Reduce contamination by a short treatment (1 min) of intact skin with 70% ethanol in a laminar flow hood. After sterilization, rinse samples thoroughly with Ca^{2+}- and Mg^{2+}-free HBSS.
3. Preliminary tissue preparation: Place tissue on a 100-mm nontissue-culture Petri dish, and remove most of the sc fat and membranous material with curved scissors.
4. Adjustment of tissue size for enzymatic digest: To improve reagent penetration, cut the skin samples into small pieces (approx 5×5 mm^2) rinsed in Ca^{2+}- and Mg^{2+}-free HBSS.
5. Dispase treatment: Because melanocytes are located just above the basement membrane in the epidermis, successful isolation requires effective separation of epidermis from dermis. Pieces of skin are incubated in epidermal isolation solution for up to 24 h at 4°C to allow detachment of epidermis from dermis. As originally described ***(51)***, dispase splits epidermis from dermis along the basement membrane. Each piece of skin is secured with two pairs of forceps; one holds the epidermis and the other the dermis. The epidermal sheet is then peeled apart from the dermis, transferred to a Petri dish, and minced with a scalpel blade to smaller fragments to expedite the subsequent cell dispersal. To prevent the epidermal sheets from drying, a drop of Ca^{2+}- and Mg^{2+}-free HBSS can be added to the Petri dish. To avoid potential sources of fibroblast contamination, dermal pieces should be discarded immediately once they are separated from the epidermis, and the forceps used to hold the dermis should never come in contact with the epidermal sheets and vise versa. Contaminated dermal fragments are easily recognized by their white opaque color in contrast to the yellowish-brown semitransluscent epidermis.
6. Cell dispersal techniques: A single-cell suspension is generated from clumps of epidermal tissue by enzymatic treatment with cell-dispersal solution containing trypsin at 37°C for 5 min followed by mechanical dissociation. After washing the cells once with Ca^{2+}- and Mg^{2+}-free HBSS to remove the enzyme, cells are then pelleted by centrifugation, resuspended and seeded in a T25 culture vessel. Extra caution should be taken to remove the supernatant when washing the cells, as the cells tend not to form a solid pellet because of the presence of remaining fragments of cornified materials. It is suggested that manual pipeting be used in place of suctioning.
7. Selective growth: Most methods for growing pure cultures of melanocytes from epidermal cell suspensions depend on optimal conditions that enable melanocytes, but not keratinocytes, to attach to a substrate and proliferate. These conditions include high oxygen tension ***(52)***, high seeding density ***(53)***, high Ca^{2+} concentration ***(54–56)***, and the presence of sodium citrate ***(57)***, 5-fluorouracil ***(58)***, and phorbol esters ***(6)***. The presence of phorbol esters not only suppresses the growth

of keratinocytes, but also promotes melanocyte growth. However, phorbol esters have been shown to reduce the numbers of melanosomes in human melanocytes in culture and to delay the onset of melanization ***(6)***. Thus, although these reagents support long-term culture of human melanocytes, they may have limited use in studies of melanocyte differentiation.

In our original report dated back in 1987, when melanocytes were established in medium without TPA, they grew at doubling times of 4–7 d for the first 2–3 passages and senesced by passage 5. Initially, the cells assumed a spindle morphology, which changed by passage 3–5 to a flat, polygonal morphology ***(59)***. The flat, polygonal cells were unpigmented and proliferated slowly. Concomitant with the morphological and proliferative changes, there was a decrease in expression of the nerve growth factor (NGF) receptor and an increase in expression of gp145 (*see* **Table 2**). Recently, with the advance in melanocyte biology, we have devised a growth medium for human melanocytes (TPA-free MGM) based on the use of more physiologic mitogens that substituted for routinely used artificial and undefined agents. Important features of this method include the following. First, long-term culture of melanocytes in the absence of phorbol esters is achieved. Second, contamination by dermal fibroblasts, a common problem in establishing melanocyte culture, is dramatically reduced (from 15 to 20% to less than 5%) by minimal tissue manipulation during the isolation process. Third, melanocytes maintained in TPA-free MGM exhibit a more physiologic morphology (dendritic vs bi- or tri-polar) and a shorter population doubling time (1.5–4 vs 2–6 d) comparing to their counter parts grown in the conventional TPA medium (*see* **Table 2**). Abdel-Malek and co-workers also reported successful long-term proliferation sustained by TPA-free medium supplemented with 0.6 ng/mL bFGF, 10 n*M* ET-1, and 10 n*M* α-MSH ***(60)***.

There are other alternative media for melanocyte culture. TIP medium, a TPA-containing medium, consists of 85 n*M* TPA, 0.1 m*M* isobutylmethyl xanthine (IBMX), and 10–20 µg protein/mL placental extract in Ham's F-10 medium supplemented with 10% newborn calf serum ***(61)***. TPA-free medium ***(62)***, composed of Ca^{2+}-free M199 medium supplemented with 5–10% chelated FCS, 10 µg/mL insulin, 10 g/mL EGF, 10^{-9} *M* triiodothyronine, 10 µg/mL transferring, 1.4×10^{-6} *M* hydrocortisone, 10^{-9} *M* cholera toxin, and 10 ng/mL bFGF ***(62)***, can also support short-term culture of melanocytes.

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Table 2
Phenotype of Neonatal Foreskin Melanocytes in Culture[a]

	Growth Passage			Morphology[b] Passage			Pigmentation Passage			Antigen expression: NGFR Passage			Antigen expression: gp145 Passage		
Culture conditions	1	5	8	1	5	8	1	5	8	1	5	8	1	5	8
TPA-MGM without TPA[c]	+++	+	–	S	F	F	+	–	–	++++	–	–	–	+++	nt
TPA-MGM[d]	+++	+++	+++	B	B	B	+++	+++	+++	++	+++	+++	–	–	+
TPA-free MGM[e]	++++	++++	++++	D	D	D	++	++	++	nt	nt	nt	nt	nt	nt

[a]+ to ++++, Degree of growth, pigmentation, or antigen expression; –, no growth or >14 d doubling time, no pigmentation, and no expression of antigen; nt, not tested.

[b]S, spindle; F, flat, polygonal; B, bi- or tri-polar; D, dendritic.

[c]TPA-MGM without TPA ***(36)*** consists of four parts of MCDB153 supplemented with 2 m*M* $CaCl_2$, one part of Leibovitz's L-15, 2% heat-inactivated FCS, 5 µg/mL of insulin, 5 ng/mL EGF, and 40 µg protein/mL bovine pituitary extract.

[d]TPA-MGM ***(36)*** is composed of four parts of MCDB153 supplemented with 2 m*M* $CaCl_2$, one part of Leibovitz's L-15, 2% heat-inactivated FCS, 5 µg/mL of insulin, 5 ng/mL EGF, 40 µg protein/mL bovine pituitary extract, and 10 ng/mL TPA.

[e]TPA-free MGM, as described in **Subheading 2., step 4**.

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