Isolation and Long-Term Culture of Human Hair-Follicle Melanocytes

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We report a method to establish long-term cultures of melanocytes derived from human hair follicles. Normal human scalp was transected 1 mm below the epidermis, and hair follicles in the remaining dermis were isolated by collagenase treatment. Hair-follicle cell suspensions were prepared by trypsin/ethylenediamine tetraacetic acid treatment and cultured in a mixture of Eagle's minimum essential medium (supplemented with 12-O-tetradecanoyl-phorbol-13-acetate and cholera toxin) and keratinocyte serum-free medium. After contaminating fibroblasts and keratinocytes were removed, cells with two distinct morphologies remained. These included large, dendritic and deeply pigmented cells, which did not proliferate and which disappeared by the third passage, and small bipolar cells, which initially were unpigmented, proliferated very rapidly, and became pigmented after the addition of 3-isobutyl-1-methylxanthine to the culture medium. Both cell types were melanocytes as confirmed by electron microscopy and by staining with antibodies to S-100, G$_{D3}$, and melanosomal antigens. The availability of cultured hair-follicle melanocytes will facilitate investigations of the role of these cells in normal and abnormal hair biology. *Key words: amelanotic melanocyte/outer root sheath/electron microscopy. J Invest Dermatol 104:86–89, 1995*

Much of the recent progress in hair research has resulted from advances in culture methodology for intact hair follicles [1,2] and their cellular components [3–6]. Human hair-follicle cells grown successfully in culture include follicular papilla fibroblasts [3], outer root sheath (ORS) keratinocytes [4], and germinative epidermal cells from the hair matrix [6]. By contrast, hair-follicle melanocytes have not been cultured, perhaps because of their relatively low numbers in the hair follicle [7].

Hair-follicle melanocytes consist of two morphologically and functionally different types: pigmented melanocytes (dendritic and dopa-positive), present in the infundibulum and bulb, and amelanotic melanocytes (nondendritic and dopa-negative), present in the ORS of the middle and lower follicle [8]. Little is known of the fate of either melanocyte population after anagen; i.e., whether they are lost with resorption of the lower follicle during catagen/telogen or whether they dedifferentiate and become nondendritic and amelanotic [9].

Availability of reliable methods of growing hair-follicle melanocytes in culture will lead to a better understanding of the role of these cells in hair growth and disease. Here, we describe a method to establish hair-follicle melanocytes in long-term culture.

MATERIALS AND METHODS

Isolation of Hair Follicles Normal human scalp (approximately 15–20 cm^2) was obtained from four individuals (two men and two women; mean age 40 years) during routine plastic surgery and placed in Eagle's minimum essential medium (Mediatech, Washington, DC) containing antibiotics. The epidermis and upper 1 mm of dermis were removed with a scalpel. Hair follicles were isolated by cutting the tissue into 1-cm^2 pieces and incubating these in the above culture medium supplemented with 0.50% collagenase Type V (Sigma, St. Louis, MO) and 5% fetal bovine serum (Intergen, Purchase, NY) for 1–2 h at 37°C.

Preparation of Hair-Follicle Single-Cell Suspensions Hair follicles were freed of contaminating dermal tissue by exhaustive washing in phosphate-buffered saline (Gibco, Grand Island, NY). Washing was continued until populations of hair follicles appeared pure by microscopic examination **(Fig 1)**. Single-cell suspensions were obtained by treating the isolated hair follicles with 0.05% trypsin and 0.53 mM ethylenediamine tetraacetic acid (EDTA) for 5–10 min at 37°C. The cells were plated onto 35-mm plastic tissue culture dishes with Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Intergen), 0.2 μg/ml cholera toxin (List Biological Laboratories, Campbell, CA), 50 nM 12-O-tetradecanoylphorbol-13-acetate (Sigma), 0.05 mg/ml gentamicin, 2.5 μg/ml fungizone, and keratinocyte serum-free medium (Gibco). When present, contaminating fibroblasts were removed with geneticin treatment (G418 sulfate, Gibco) [10], and keratinocytes were removed by differential trypsinization.

Induction of Melanogenesis in Hair-Follicle Melanocytes Amelanotic hair-follicle melanocytes (AM-melanocytes) were cultured in the above medium supplemented with 10^{-4} M 3-isobutyl-1-methylxanthine (IBMX).

Immunofluorescence Staining for Melanocyte Markers Cells were identified as melanocytes in part by expression of S-100 protein, G$_{D3}$, and melanosome-related antigens. Cells were grown on LabTek glass chamber slides (Miles Laboratories, Naperville, IL), fixed in 100% methanol, and stained conventionally with the following antibodies: anti–S-100 polyclonal IgG antibody (Sigma), MEL-1 monoclonal antibody against the ganglioside G$_{D3}$ (Signet Laboratories, Dedham, MA), NKI/beteb monoclonal antibody against premelanosomal antigens (Monosan, Caltag, San Francisco, CA), and TA99 monoclonal antibody against tyrosinase-related protein-1
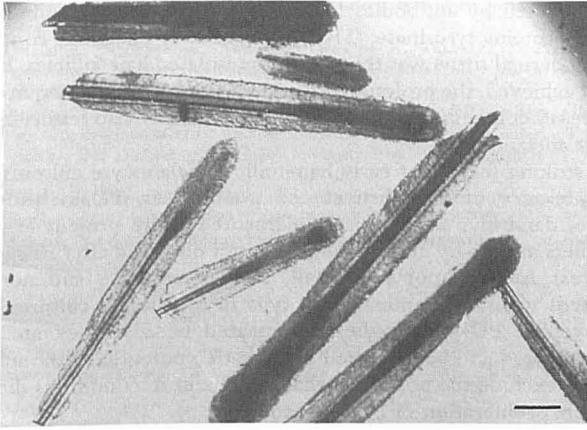
Figure 1. **Pure population of hair follicles**. Follicles were isolated from scalp tissue after 0.50% collagenase treatment and removal of contaminating dermal tissue. Bright-field illumination. *Bar*, 220 μm.

Figure 2. **Pigmented (PD) and unpigmented (AM) cells migrating from a hair-follicle fragment after 7 d in primary culture**. Most unpigmented cells are small and bipolar. Melanosomes are clearly visible in PD-melanocytes. Bright-field illumination. *Bar*, 18 μm.

(TRP-1; a gift from Dr. Alan Houghton, Memorial Sloan Kettering, NY). Neonatal fibroblasts were stained similarly as negative controls.

Dopa and Combined Dopa-Premelanin Reaction Melanocytes were stained for dopa and the dopa-premelanin reaction, as described previously [11]. Similarly treated neonatal foreskin fibroblasts were used as controls.

Transmission Electron Microscopy Cells were grown to confluence on 35-mm dishes, fixed in 2.5% glutaraldehyde in 0.1 M/l cacodylate buffer (pH 7.4), and processed conventionally.

RESULTS

Isolation of Hair-Follicle Cells Hair follicles were isolated from normal scalp dermis, which was separated from the upper dermis with a scalpel. Absence of epidermis was confirmed microscopically in all cases. Collagenase treatment of 1-cm² chunks of dermis permitted isolation of hair follicles in all stages of growth **(Fig 1)**. Individual cells were released from the isolated hair follicle by trypsin/EDTA treatment and plated onto tissue culture dishes with medium.

Less than 0.1% of plated hair-follicle cells consisted of cells assumed to be melanocytes based on their intense pigmentation and dendricity (hereafter called PD-melanocytes for pigmented and dendritic melanocytes). Most of the remainder consisted of round unpigmented cells.

Establishment of Hair-Follicle Melanocytes in Culture Twelve hours after plating, some cells attached to the substratum. These cells included PD-melanocytes, small nonpigmented melanocyte-like cells (based on their characteristic smooth, bipolar, i.e., "neuronal" shape), occasional keratinocytes (identified by their cobblestone morphology), and fibroblasts (distinguished from AM-melanocytes on the basis of their size and their broad and flattened shape). Keratinocyte growth was retarded in this culture medium supplemented by 12-O-tetradecanoylphorbol-13-acetate, and fibroblast growth was controlled by geneticin treatment.

AM-melanocytes were smaller and bipolar, whereas PD-melanocytes commonly formed clusters of up to 10 cells. Intimate contacts occurred between the two melanocyte populations, with AM-melanocytes extending cytoplasmic processes to PD-melanocytes, and vice versa. In some cases, PD- and AM-melanocytes could be seen to emerge from small fragments of poorly dissociated hair-follicle tissue that fortuitously attached to the substratum **(Fig 2)**. This observation suggests that PD- and AM-melanocytes can coexist in the same hair-follicle anatomic sites. The presence of PD-melanocytes in these fragments suggests that the fragments originated from the hair bulb, as pigmented melanocytes do not exist elsewhere in the hair follicle.

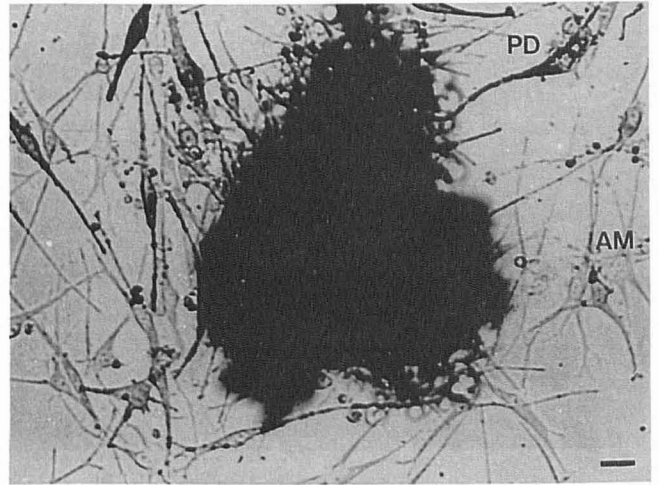Marked proliferation of AM-melanocytes, but not of PD-mela-nocytes, was observed after 12–16 d in culture **(Fig 3)**. Primary cultures reached confluence after approximately 2–3 weeks. At this time, the predominant cell type was AM-melanocytes **(Fig 3)**. The melanocyte cultures were split 1:2 and re-treated with geneticin, if necessary, to remove any residual fibroblasts. Remaining keratinocytes were removed by differential trypsinization. PD-melanocytes, keratinocytes, and fibroblasts were absent from cultures after the third passage. AM-melanocyte cultures could be maintained for at least 30 passages (approximately 60 doublings over 9 months).

Cell Characterization To confirm that AM-cells were indeed melanocytes, we immunostained the cells with melanocyte-specific antibodies. Greater than 99% of the cells (after passage 3) expressed S-100 protein and the ganglioside G_{D3} (data not shown). All pigmented cells also expressed premelanosomal antigens, recognized by NKI/beteb antibody **(Fig 4)**, and TRP-1, recognized by TA99 antibody (data not shown). These findings confirm that AM-cells were indeed melanocytes.

Most AM-melanocytes were positive for the dopa-premelanin reaction when grown in the absence of IBMX, but became dopa-positive in IBMX-supplemented culture medium **(Fig 5)**. This further confirms their identity as melanocytes. Control exper-
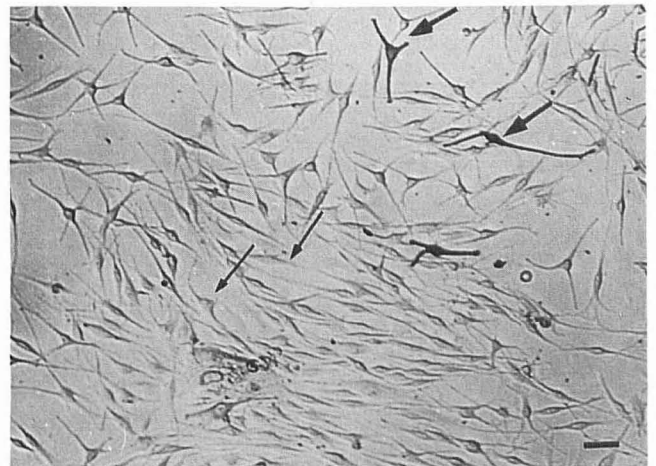

Figure 3. **Hair-follicle melanocytes after 18 d in culture (passage 1)**. Note the greater number of AM-melanocytes (*thin arrows*) compared to PD-melanocytes (*thick arrows*). Bright-field illumination. *Bar*, 60 μm.
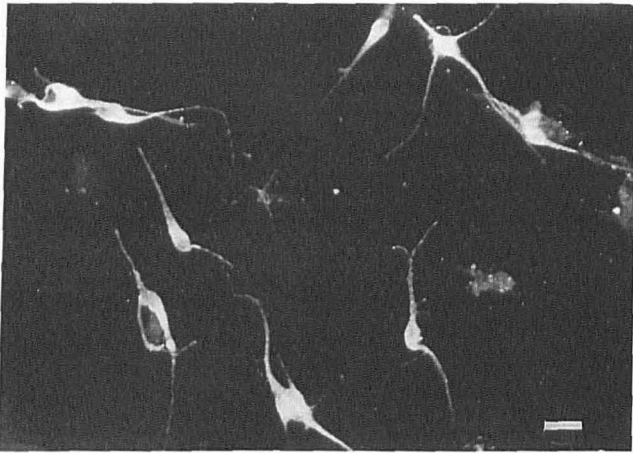
Figure 4. Immunofluorescence staining of hair-follicle melanocytes for premelanosomal antigens (100 and 7 kD). *Bar,* 20 μm.

iments using neonatal foreskin fibroblasts were negative in these staining procedures.

Transmission electron microscopy of AM-melanocytes grown in the absence of IBMX revealed amelanotic cells, whereas cells from IBMX-treated cultures contained melanosomes in all four stages of maturation (data not shown).

DISCUSSION

In this study, we describe a method of establishing hair-follicle melanocytes in long-term culture. Although melanocytes of epidermal origin and most other hair-follicle cell types have been cultured successfully [3–6], this is the first report of the successful long-term culture of normal human hair-follicle melanocytes. Greater than 99% of the cells expressed S-100 protein, ganglioside G_{D3}, (pre)melanosome antigens (100 and 7 kD), and TRP-1, indicating that they were melanocytes [12,13]. In addition, the cells became pigmented and dopa-positive when grown in IBMX-supplemented medium, further indicating that they were melanocytes.

Important features of this method of hair-follicle melanocyte culture include the following. First, melanocytes were isolated not only from hair bulbs, but also from the middle to lower ORS region. Hair-bulb melanocytes are relatively few in number. The majority of these cells are highly differentiated [9] and thus may have limited proliferative potential. By contrast, ORS melanocytes are poorly differentiated, lack pigment and dendrites, and are identifiable *in vivo* by antibodies to premelanosomes. They cannot

be identified by antibodies to mature melanosomes or the functional proteins tyrosinase, TRP-1, or TRP-2.* Second, contaminating dermal tissue was removed from isolated hair follicles. If this is not achieved, the prolonged treatment with geneticin, required to kill heavy contamination with fibroblasts, usually also results in loss of the melanocytes.

A striking feature of early hair-follicle melanocyte cultures was the presence of two distinct cell populations. PD-melanocytes (large, dendritic, and intensely pigmented) were present in small numbers in primary cultures and were not observed after passage 3, whereas AM-melanocytes (small, bipolar, smooth, and nonpigmented) were the dominant cell type in established cultures. We believe the PD-melanocytes disappeared because they are well differentiated and have limited proliferative potential, although we cannot exclude the possibility that these culture conditions did not support proliferation of PD-melanocytes.

Both PD- and AM-melanocytes were of hair-follicle origin, as these cells were derived from isolated hair follicles, the epidermis was removed from scalp specimens before hair-follicle collection, and this separation was confirmed microscopically. In addition, both PD- and AM-melanocytes could be seen to migrate from small fragments of hair follicle that had fortuitously remained attached during primary culture. We believe that PD-melanocytes originated from the hair bulb, as no similar cells have been shown to exist elsewhere in the hair follicle. We believe that AM-melanocytes are likely to originate, in large part, from the ORS, as these cells share important morphologic features with ORS melanocytes. These features were apparent in the AM-melanocyte population immediately after isolation, and therefore are unlikely to be induced by culture conditions.

The significant proliferative activity of AM-melanocytes supports the notion that ORS melanocytes may provide a reservoir of melanocytes for hair bulbs during early anagen [9], during hair regrowth in alopecia areata, and for repigmentation of the epidermis in vitiligo [14], burns, and dermabrasion [15].

In conclusion, we have successfully established hair-follicle melanocytes in long-term culture and shown the existence of two morphologically different populations of melanocytes in hair follicles: one undifferentiated (AM-melanocytes) and the other more differentiated (PD-melanocytes). Only the former was observed to proliferate. These cells will provide a useful tool to examine the role of follicular melanocytes in hair biology and disease.

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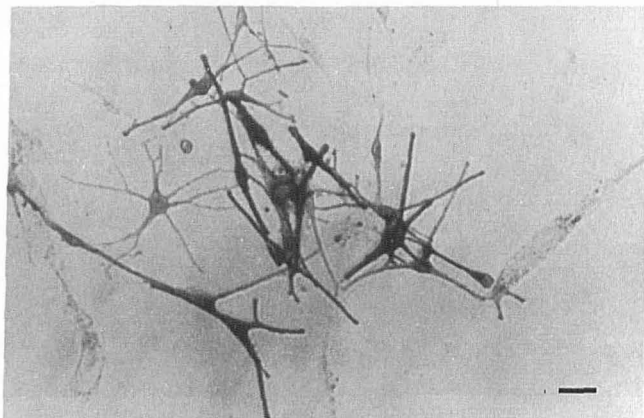

Figure 5. AM-melanocytes grown in IBMX-supplemented medium for 2 weeks and stained with L-dopa. *Bar,* 20 μm.

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