The Fate of Hair Follicle Melanocytes During the Hair Growth Cycle

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The fate of the follicular pigmentary unit during the hair growth cycle has long been one of the great enigmas of both hair follicle and pigment cell biology. Although melanocytes are distributed in several different compartments of the anagen hair follicle, melanogenically active cells are located only in the hair bulb, where they are directly involved in hair shaft pigmentation. These pigment cells are readily detectable only when they become melanogenically active during anagen III of the hair growth cycle. Thus, their status during hair follicle regression (catagen), when melanogenesis is switched off, until they re-appear again as pigment-producing cells in the anagen III hair follicle, has remained poorly defined. Historically, it has been proposed that hair bulb melanocytes adopt a self-perpetuating, catagen-resistant strategy of de-differentiation during hair follicle regression and re-differentiation upon entry into a new anagen phase; however, this explanation remains problematic in the absence of evidence for de-differentiation/re-differentiation plasticity in most nonmalignant cell systems. ***Key words: tyrosinase/melanin/alopecia areata/C57BL/6/canities. Journal of Investigative Dermatology Symposium Proceedings 4:323–332, 1999***

Our understanding of the status of the mammalian hair pigmentary unit during the hair growth cycle has been advanced by the observation that important enzyme systems of follicular melanogenesis, such as tyrosinase and dopachrome tautomerase, are switched off long before the morphologically recognizable termination of anagen, i.e., in late anagen VI. Furthermore it has been reported that apoptosis accounts, at least in part, for the loss of hair bulb melanotic melanocytes during catagen. Resultant pigment incontinence is removed, in part, via the dermal papilla and outer root sheath by phagocytes, including macrophages and Langerhans cells, and by a subpopulation of outer root sheath keratinocytes. Furthermore, the effect of repopulating the hair bulb, during successive anagen phases, with melanocytes from the presumptive reservoir in the outer root sheath may be linked to melanocyte aging and ultimately canities. Finally, the pharmacologic manipulation of the hair cycle is discussed as a useful model for assessing the melanocyte response to cytotoxic drug injury *in situ*. This manuscript attempts to provide a brief overview of these and other recent data that may help to elucidate the factors that control the fate of the follicular pigmentary unit during the hair growth cycle.

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Abbreviations: CTS, connective tissue sheath; CYP, cyclophosphamide; DEX, dexamethasone; DP, dermal papilla; HF, hair follicle.

BACKGROUND

Integumental melanocytes originate as neural crest cells (Rawles, 1947) and migrate along stereotyped routes in the embryo (Serbedzija *et al*, 1990), involving, in part, the steel/c-kit and endothelin signaling pathways (Wehrle-Haller and Weston, 1995; Reid *et al*, 1996; Yoshida *et al*, 1996). By 7 wk in humans, these melanoblasts enter the epidermis via the dermis (Holbrook *et al*, 1988) and are detectable with antityrosinase-related protein-2 (TRP-2) antibodies (Steel *et al*, 1992). With the onset of hair follicle (HF) morphogenesis, some melanoblasts leave the epidermis and distribute randomly as dopa-positive and dopa-negative pigment cells in the forming HF and occasionally in the sebaceous gland (Chase *et al*, 1951; Hiraga, 1976).

In the mature HF, dopa-negative melanocytes distribute in the outer root sheath (ORS) (Staricco, 1959, 1960) whereas dopa-positive cells are detectable in the hair bulb matrix capping the dermal papilla (DP) and in the infundibulum. The hair bulb, however, is the only site of pigment production for the hair shaft, and contains both highly melanogenic melanocytes and a minor subpopulation of poorly differentiated pigment cells (Slominski and Paus, 1993; Tobin *et al*, 1995; Tobin and Bystryn, 1996). The restriction of melanogenically active melanocytes to the upper hair matrix of the anagen HF, just below the precortical keratinocyte population, correlates with the observation that melanin is transferred mostly to the hair shaft cortex, less so to the medulla, and, very rarely, to the hair cuticle (Mahrle and Orfanos, 1971). Ectopic melanin, however, may be found elsewhere in the HF under some pathologic circumstances (Slominski *et al*, 1996; Tobin *et al*, 1998).

Melanogenically active melanocytes in the hair bulb form functional units with neighboring immature precortical keratinocytes, which, as recipients of their melanized secretary granules, ultimately form the pigmented hair shaft. Bulbar melanocytes are

also likely to interact closely with the DP as evidenced by their anagen-long direct contact with the thin and permeable basal lamina that separates them from the mesenchymal DP. Thus, it is likely that the fate of all three cell types is mutually interdependent during the hair growth cycle. Less is known about the role of amelanotic hair bulb melanocytes in hair pigmentation, although it has been speculated that these cells may represent a pool of ''transient'' melanocytes that migrate from precursor melanocytes stores in the upper ORS (Staricco and Miller-Millinska, 1962; Staricco, 1963; Tobin *et al*, 1995; Horikawa *et al*, 1996; Slominski *et al*, 1996; Tobin and Bystryn, 1996).

Although HF and epidermal dopa-positive melanocytes share the epidermal environment prior to HF morphogenesis, these pigment cell sup-populations subsequently diverge in many important ways when distributed in their respective distinct, but open, compartments. For example, hair bulb melanogenic melanocytes are larger, have longer and more extensive dendrites, contain more developed Golgi and rough endoplasmic reticulum, and produce 2–4 times larger melanosomes than do epidermal melanocytes (Orfanos and Ruska, 1968). Additionally, in contrast to the degradation of melanin granules in the differentiating layers of the epidermis, hair melanin granules transferred into cortical keratinocytes are minimally digested (Roth, 1967), resulting in pigmentation of the entire hair shaft.

There are also significant differences in the nature of their respective melanocyte/keratinocyte functional units. The follicular melanin unit resides in the proximal anagen HF, an immunologically distinct region of the skin (Billingham and Silvers, 1971; Paus, 1997), and consists of one melanocyte to five keratinocytes in the hair bulb as a whole and 1:1 in the basal layer of the hair bulb next to the DP (Bell, 1967). By contrast, each melanocyte is associated with 36 ''viable'' keratinocytes (Frenk and Schellhorn, 1969; Quevedo, 1973) in the epidermal-melanin unit (Fitzpatrick and Breathnach, 1963). By far the most striking difference between these two melanocyte subpopulations, however, and one with significant implications for the regulation of hair pigmentation, is the observation that the activity of the hair bulb melanocyte is under cyclical control and that melanogenesis is tightly coupled to the hair growth cycle (Silvers 1979; Slominski and Paus, 1993; Slominski *et al*, 1993), whereas epidermal melanogenesis appears to be continuous.

MELANOCYTE DYNAMICS DURING THE HAIR GROWTH CYCLE

Hair grows in a cyclical manner, characterized by a finite period of hair fiber production (anagen), a brief regression phase resulting in the loss of up to 70% of the HF (catagen), and a relatively quiescent period (telogen) (**Fig 1**) (Chase, 1954). This sequence of events occurs in HF producing either pigmented or unpigmented hair fibers, although it has been reported that the caliber and growth rate of white anagen hair may be significantly greater than for pigmented adjacent follicles (Nagl, 1995). An important implication of this and other studies is that melanin transfer to precortical keratinocytes may promote terminal keratinocyte differentiation (Slominski *et al*, 1993; Nagl, 1995), perhaps by altering intercellular calcium levels in these cells (Drager and Balkema, 1987). In this manner, pigmented hair growth requires the coordinated and programmed activity of epithelial, mesenchymal, and neuroectodermal cells (Slominski and Paus, 1993; Paus *et al*, 1997).

Several analyses of the regulation of HF melanogenesis have been conducted using the C57BL/6 mouse model of hair research (Paus *et al*, 1990; Slominski *et al*, 1991, 1994, 1996; Slominski and Paus, 1993; Plonka *et al*, 1995; Ermak and Slominski, 1997; Tobin *et al*, 1998), in large part because the mosaic nature of human hair growth precludes the systematic analysis of the human hair cycle, particularly its fairly elusive catagen phase. Another significant advantage of this murine model is that all melanogenically active truncal melanocytes in mice are confined to the HF, and as in human follicular melanocytes, melanogenesis is linked to the anagen stage of the hair cycle (Silvers, 1979; Slominski and Paus, 1993). The synchronous nature of hair growth in C57BL/6 mice, either spontaneous or induced by depilation, not only provides an opportunity to study large numbers of HF in the same phase of the hair growth cycle, but the intensely pigmented hair facilitates easy staging of anagen (gray to black skin) and telogen (pink skin) (Paus *et al*, 1990; Slominski and Paus, 1993). Cessation of hair growth (anagen VI-catagen transformation) is an appropriate starting point in an assessment of HF melanogenesis and its association to the hair growth cycle. Strikingly, towards the end of anagen, retraction of melanocyte dendrites and the suppression of melanogenesis are the earliest signs of imminent HF regression even before catagen-associated structural changes are apparent in the hair bulb (Slominski and Paus, 1993; Slominski *et al*, 1994).

Using electron paramagnetic resonance spectroscopy (EPR), an unexpectedly early decline in melanin synthesis is observed when HF were still in anagen VI (**Fig 2**) (Slominski *et al*, 1994). This is accompanied and followed by a decline in the activities of tyrosinase, dopachrome tautomerase (TRP-2), and gp75 (TRP-1) (**Fig 3**). The initiation of catagen itself is further characterized by a significant and rapid decrease in activity and concentration of tyrosinase and is accompanied by a second drop in TRP-2 some time before the cessation of keratinocyte proliferation (**Fig 3**). Thus, the switch-off of melanogenesis begins already in anagen VI (**Figs 2** and **3**) (Slominski *et al*, 1994). Because keratinocyte proliferation continues for some time after the follicular melanin unit ceases functioning, the most proximal portion of the hair in telogen follicles remains unpigmented. The functional relevance of this observation is unclear, although the processes involved in the switch-off of melanogenesis may also reduce dendricity, thereby restricting the transfer of melanin to precortical keratinocytes. Alternately, club formation itself may be facilitated in keratinocytes not containing melanin.

Fate of melanotic melanocytes during catagen? Melanogenically active melanocytes appear to be lost from the follicular epithelium during catagen (**Fig 1**). Their ''disappearance'' is not unheralded, however. Residual melanin generated during the preceding anagen can often be seen in the DP of catagen and telogen follicles, as the calling card of these ''disappearing'' melanocytes (**Fig 1**) (Chase, 1954; Sugiyama, 1979; Slominski and Paus, 1993; Slominski *et al*, 1994). Still, the fate of the hair bulb melanocytes during this phase of the hair growth cycle has remained elusive, and has long provided one of the fascinating enigmas of both HF and pigment biology. Not only is it important to know where these melanocytes go during catagen and telogen, but also where they originate from, when follicular melanogenesis is resumed during the next anagen phase. Answers to these questions may provide new insights into the physiologic control of melanocyte migration, differentiation, melanogenesis, proliferation, and death *in situ*. Furthermore, this may provide new clues as to what goes wrong during hair pigmentation disorders such as premature canities, poliosis, alopecia areata (Tobin *et al*, 1990, 1991; Paus *et al*, 1994a), vitiligo, and in hair shaft dyschromia after chemotherapy.

At least three possible scenarios can be envisaged for the pigmentary unit during this part of the hair cycle. One is that the hair bulb melanocyte system is self-perpetuating (Sugiyama, 1979). This view holds that melanocytes involved in the pigmentation of one hair generation are also involved in the pigmentation of the next. The proposed mechanism invokes melanocyte de-differentiation as evidenced by a de-activated melanocyte phenotype during catagen/telogen (e.g., retraction of dendrites and reduction of melanogenesis) and their subsequent re-differentiation during the subsequent anagen phase (Sugiyama, 1979); however, this would require a degree of cellular plasticity not seen in most nonmalignant cell systems and such cells would also need to survive/avoid the extensive apoptosis-driven regression of the hair bulb (Weedon and Strutton, 1981; Lindner *et al*, 1997) by actively suppressing apoptosis. Increasing evidence now

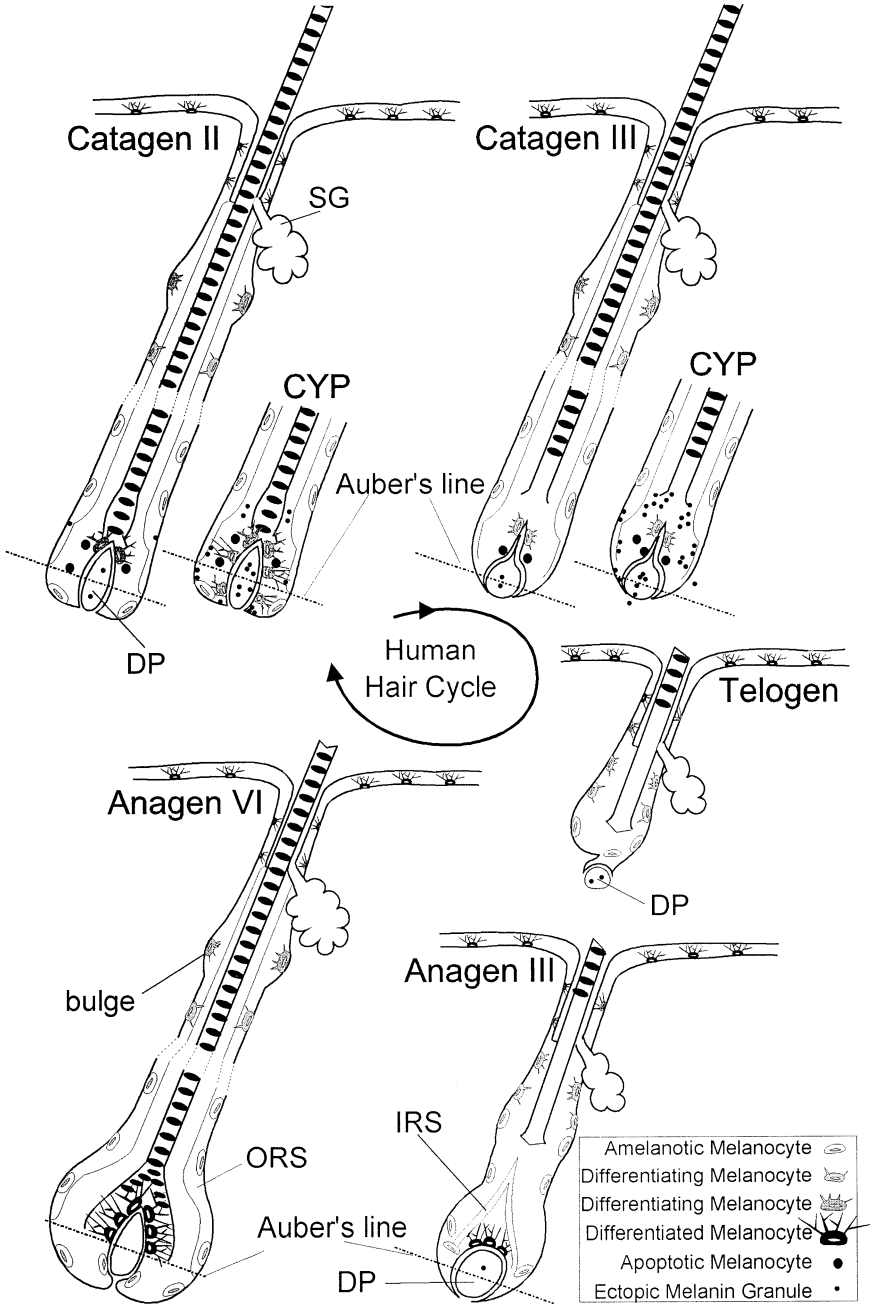

Figure 1. Schematic representation of the status of the "hair follicle melanin unit" during the hair growth cycle in normal human scalp and after cyclophosphamide treatment in C57BL/6 mouse back skin. The figure depicts five selected hair cycle stages, showing early regressing follicles (catagen II and III), a resting HF (telogen), a HF in early phase of active growth (anagen III), and a mature anagen VI HF. CYP-induced catagen in C57BL/6 mice are also depicted in early catagen. Note the distribution of melanocytes in the HF; mature melanotic melanocytes are located primarily in the human epidermis, upper infundibulum, and hair bulb. Melanocytes in less mature stages of differentiation are also located in the ORS and perhaps also in the matrix.

suggests that this is unlikely. The altered phenotype of melanocytes observed in late anagen/early catagen hair bulb melanocytes (**Figs 1**, **4*a***) reflects their less active state, and is perhaps due to reduced keratinocyte support within the follicular melanin unit. It is possible that these so-called "re-differentiating" melanocytes correspond to newly recruited immature melanocytes derived from a melanocyte reservoir located in the upper, so-called, permanent ORS, and not the re-activation of pre-existing hair bulb melanocytes that were melanogenically active in the previous anagen phase (**Fig 1**). This view is supported by the observation that immature melanocytes are located very close to the secondary epithelial germ of the telogen club (**Figs 1**, **4*d***) (Silver *et al*, 1969, 1976). These cells are commonly small, have high nuclear to cytoplasmic ratios, and have inactive cytoplasm with very few organelles; however, rare (pre)melanosomes may occasionally be present (**Fig 4*d***, *inset*), suggesting either the possibility of some low level melanogenic activity in these cells (Sugiyama, 1979; Sugiyama *et al*, 1995), or that these cells may indeed derive from a population of catagen-surviving post-melanogenically active cells. Morphologically, these presumptive, amelanotic melanocytes can be distinguished from other cell types by their lack of epithelial cell specializations or features of macrophages (e.g., phagolysosomes), and by the observation that lymphocytes and Langerhans cells are not detectable by immunohistochemistry in this region of the HF (Paus *et al*, 1998).

Using the C57BL/6 mouse model, we have recently shown that at least some, if not most, melanotic hair bulb melanocytes survive for just a single hair growth cycle (Tobin *et al*, 1998). Deletion of individual melanotic melanocytes by apoptosis (**Fig 4*b***) was confirmed using well-described morphologic features (Wyllie, 1981), TUNEL staining (Lindner *et al*, 1997), TUNEL/TRP-1 double staining (Tobin *et al*, 1998), and by the observation that melanocyte death occurred in the absence of an inflammatory response, as one would expect in necrosis (Searle *et al*, 1982). The melanocytic identity of these cells undergoing apoptosis was further confirmed by the presence of melanosomes (in all stages of maturation) distributed free in the cytoplasm. By contrast, melanization of cortical keratinocytes results from the incorpora-

tion of mature stage IV melanosomes, but not premelanosomes, within phagolysosomes (Prunerias, 1969; Cesarini, 1990), and keratinocytes are further identifiable by their epithelial specializations, including tonofilament bundles. Furthermore, the delivery of immature or unmelanized melanosomes (e.g., stage I/II premelanosomes) to keratinocytes is restricted to disease states, e.g., albinism (Parakkal, 1967). That some, if not all, hair bulb melanocytes are indeed lost during catagen is supported by the observation that the vast majority of cells within catagen hair bulbs are desmosomally linked. This is indicative of their epithelial origin, and contrasts strikingly with the 1:1 MC/KC ratio in the epithelial basal layer at the matrix/DP junction (Bell, 1967) in the anagen HF. The colocalization of TUNEL and TRP-I positivity in the same cells (**Fig 4*e*, *f***) and the observation that TRP-1 positivity is restricted to stage I/II premelanosomes[1] provides further evidence that these apoptosing cells in the regressing hair bulb are indeed melanogenic melanocytes.

A loss of hair bulb melanocytes has also been observed in light (*Blt/*) mice and silver (*si/si*) mice during mid-anagen. Here, hair bulb melanocytes degenerate and become dislocated from the bulb and finally incorporated into the hair shaft (Quevedo *et al*, 1994); however, a full complement of functional melanocytes re-populate the subsequent anagen hair bulb until they, too, suffer the same fate.

These observations strongly suggest that melanocyte loss during catagen is replenished from an undifferentiated melanocyte pool in the "permanent" portion of the HF or from another, as yet unidentified, precursor pool.

Given the strict dependence of mature melanocytes on keratinocyte-derived signals like FGF-2 and neurotrophins (Yaar *et al*, 1994; Pincelli and Yaar, 1997), it is also conceivable, though much less likely, that melanogenically active melanocytes leave the regressing follicular epithelium and migrate into the DP or the perifollicular mesenchyme after having switched off melanogenesis. These cells may then re-enter the follicular epithelium only after keratinocytes of the newly developing anagen hair bulb secrete appropriate inductive signals.

Pigment incontinence during catagen The cessation of HF melanogenesis occurs *prior* to the cessation of hair growth (see **Fig 1**) and explains why the hair cortex formed at the end of anagen is not usually pigmented. Pigmented melanocytes, albeit with reduced melanogenic activity, can be detected in early catagen HF; however, by then it is too late for their cargo of performed melanin to be incorporated into the hair shaft, as active melanin transfer to keratinocytes is halted. It is likely that the dispersal of this "excess" pigment from the proximal HF involves melanocyte death by apoptosis, followed by removal of the indigestible melanin to the exit ports of the regressing follicle.

Remnants of melanin granules generated during the preceding anagen are commonly seen in the DP and connective tissue sheath (CTS) of catagen and telogen HF (**Fig 1**) (Chase, 1958). The mechanism(s) behind formation and removal of this pigment "incontinence" is currently unclear. At least some of the many "melanophages" in the catagen/telogen DP are likely to be fibroblasts, based on morphologic criteria (Grinnel and Geiger, 1986). This does not explain, however, how the melanin is removed from the epithelium to the mesenchyme – a journey that requires passage through a thickening basal lamina. Although this barrier can be crossed by migratory and indeed resident cells, direct incontinence of pigment granules is more difficult to observe. To our knowledge there are no reports showing that intact bulbar melanocytes shed melanin directly into the interstitial space in the regressing matrix, and

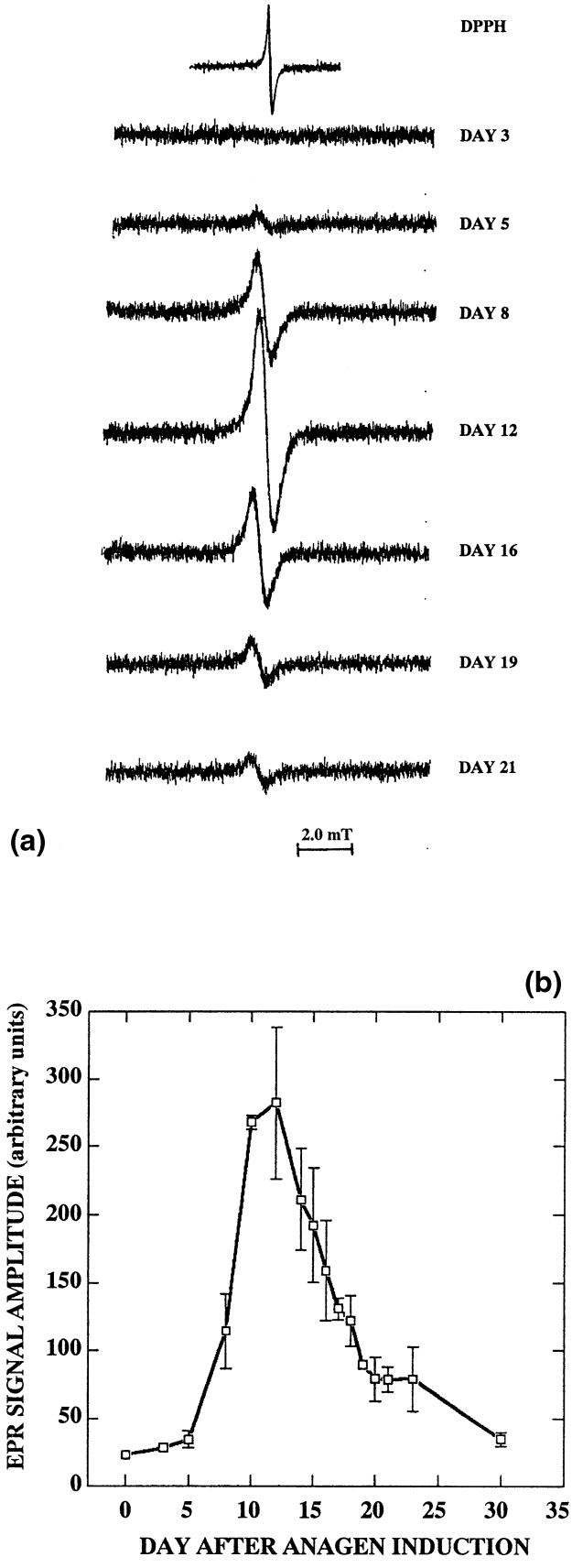

Figure 2. Changes in relative melanin content in the skin during depilation-induced hair cycle in the C57BL/6 mouse. Changes in eumelanin content in shaved skin were measured by electron spin resonance spectroscopy (EPR) as described previously (Slominski *et al*, 1994, 1996). (*a*) Representative EPR spectra of murine skin; (*b*) summary showing changes in EPR melanin signal during different hair cycle stages. Data are presented as means ± SE from 3–4 shaved skins.

[1]Jimbow K, Gomez PF, Chen H, Matsusaka H, Miura S: Involvement of rab 7 and phosphatidyl inositol 3-kinase for vesicular transport of TRP-1 between TGN and melanosome. *J Invest Dermatol* 110:473, 1998 (abstr)

that these granules make their way into the DP via the basal lamina. This may occur during melanocyte degeneration during catagen, although it is unlikely as these cells undergo the ''neater'' apoptosis rather than the more ''untidy'' necrosis. Likely candidate cells involved in the movement of melanin from the regressing hair bulb during catagen include the phagocytes, particularly macrophages. Macrophages are associated with the HF in increased numbers during HF regression (Parakkal, 1969), although it is unclear whether they have any role in driving hair follicle regression or provide a purely scavenger function during the process of tissue resorption (Westgate *et al*, 1991; Paus, 1997). Very rare macrophages, undetectable by immunohistochemistry, may be detected in the human (but not murine) hair bulb matrix during late anagen, where they can be seen to ingest melanin, presumably from degenerating hair bulb melanocytes (Christoph *et al*, in press). Unlike in ''melanophages'', melanin granules in macrophages appear to be digested within secondary lysosomes.

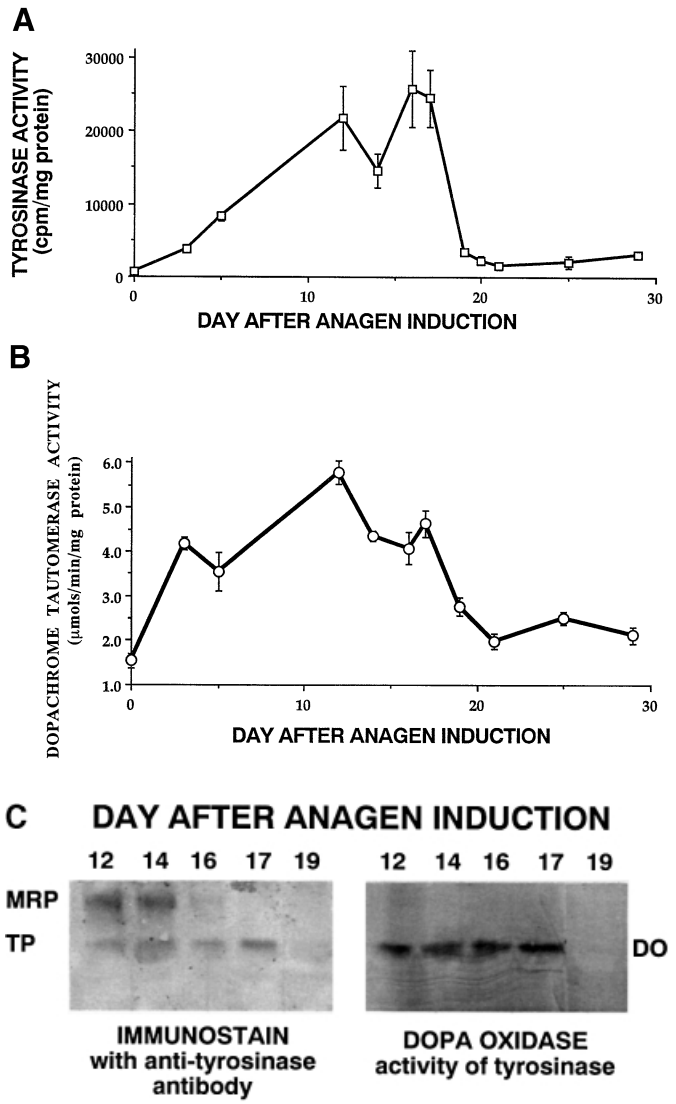

Figure 3. Changes in enzymatic activity and protein expression of melanogenesis-related proteins during induced murine hair cycle. (*A*) Changes in tyrosine hydroxylase activity of tyrosinase during the hair cycle (for details see Slominski *et al*, 1994); (*B*) changes in dopachrome tautomerase (TRP-2) activity during the hair cycle (for details see Slominski *et al*, 1994); (*C*) changes in expression of melanogenesis-related proteins during anagen-catagen transition. MRP (melanogenesis-related protein of 80–85 kDa) and TP (tyrosinase protein of 66–68 kDa) were detected using Pomerantz's antityrosinase antibody, whereas DO (dopa oxidase activity) was detected by staining filters in 5 mM L-DOPA (for details see Slominski *et al*, 1994).

The Langerhans cell is another phagocyte in the skin that may play a role in the removal of incontinent melanin (Tobin, 1998), although these cells are much weaker phagocytes than macrophages (Bartosik, 1991). Langerhans cells can be detected in large numbers in the HF infundibulum (Breathnach, 1963; Paus *et al*, 1998). They are very rare in the human, and absent in murine, anagen hair bulb (Jimbow *et al*, 1969; Paus *et al*, 1998). Interestingly, they are more commonly detected in the regressing normal catagen hair bulb of the human HF (Tobin, 1998), and in canities (Sato *et al*, 1973; Tobin and Cargnello, 1992) and alopecia areata (Niedecken *et al*, 1989; Tobin *et al*, 1990). Langerhans cells in the early catagen hair bulb may remove pigment from the regressing hair matrix to the DP (**Fig 5*a***). Ingestion of both (pre)melanosomes appears to occur via endocytosis involving their characteristic Langerhans granules, in addition to phagocytosis (**Fig 5*b***).

Supporting evidence for this process can be seen under experimental conditions, where these cells undergo receptor-mediated endocytosis via the cytomembrane-derived Langerhans granule system and transport ingested materials to the endosomal compartment for processing (Hanau *et al*, 1987; Bartosik, 1991; Bacuna *et al*, 1992). Removal of epidermal pigment by Langerhans cells has also been observed in Leopard syndrome (Fryer and Pope, 1992) and Werner's syndrome (Lazarov *et al*, 1995), although here the cells contained large membrane-bound accumulations of melanin granules that were not associated with LG. Langerhans cells in Chediak–Higashi syndrome, however, may contain giant membrane-bound granules that involve part of the Langerhans granule (Carrillo-Farga *et al*, 1990). Langerhans cells in the normal epidermis may also contain melanosomes within phagosomes, although no reference was suggested of any association with Langerhans granules (Bartosik, 1991).

In addition to the distribution of melanin to DP and CTS melanophages, matrix Langerhans cells, and rare matrix macrophages, melanosome complexes may also be detected within the phagolysosomes of a very low number of keratinocytes in the ORS of C57BL/6 catagen stage III/IV HF (**Fig 4*c***) (Tobin *et al*, 1998). These cells were located adjacent to the CTS and did not exhibit any direct association with the hair canal, suggesting that these keratinocytes migrated along this route. The location of these cells in the mid- and upper ORS strongly suggests that these cells are migratory with ultimate removal of the melanin from the skin via the infundibulum and/or epidermis.

Initiation of hair follicle melanogenesis The relatively quiescent telogen hair germ contains precursors for all the cell types that go to make the fully developed anagen VI HF (Silver and Chase, 1970), and whereas no melanin production is macroscopically visible in telogen HF (Slominski *et al*, 1991), they clearly contain cells that develop into pigment-producing melanocytes during anagen III. These cells appear as rare, clear quiescent cells (1:10 ratio with keratinocytes; Silver *et al*, 1976) in the telogen secondary germ (**Fig 4*d***) and may or may not contain rare (pre)melanosomes (Sugiyama, 1979). Furthermore, the expression of melanogenesis-related proteins such as tyrosinase or TRP-1 is below detectability by western and northern blot analyses (Slominski *et al*, 1991). Undifferentiated melanocytes/melanoblasts of the telogen germ are stimulated during the commencement of anagen and respond by increasing their cell volume (Silver *et al*, 1969). This anagen-associated signal predates the melanogenic stimulus delivered during anagen III and is followed by active melanogenesis and subsequent transfer of mature melanosomes into keratinocytes of the precortical matrix.

Although the marked nuclear heterochromatin in these cells indicates very low levels of gene transcription (Bouteille *et al*, 1983), combined dopa-reaction cytochemistry and electron microscopy have revealed rare dopa-positive Golgi complexes in some of these cells, thereby providing evidence of low levels of tyrosinase activity (Sugiyama, 1979; Sugiyama *et al*, 1995). Tyrosinase and TRP-1 (gp75) were undetectable by western and northern blotting of telogen hair follicle extracts. Tyrosinase

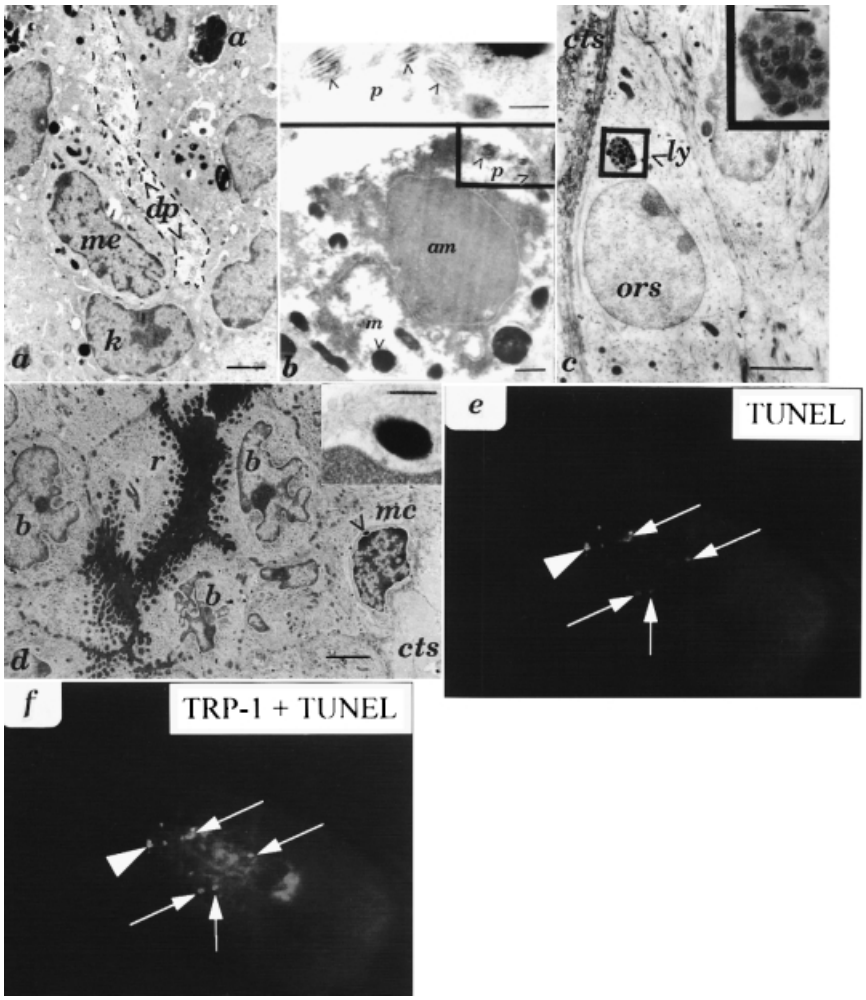

Figure 4. Melanocyte morphologic status in catagen and telogen hair follicles. (*a*) Alteration of melanocyte (mc) phenotype in early catagen. Note re-alignment of cell parallel to growth direction, retraction of dendrites, and marked reduction in melanogenic activity. (*b*) Melanocyte apoptosis (am) in spontaneous early catagen. Note typical morphologic features of apoptosis including cell shrinkage and nuclear condensation. The affected cells contain melanocyte-specific premelanosomes (p). (*c*) Melanin incontinence within lysosomes (ly) in distal ORS keratinocytes. (*d*) Clear cell in presumptive melanocyte reservoir close to bulge cells (*b*). Note lack of epithelial cell specializations (e.g., desmosomes), presence of rare melanosome (>) and its close association with the glassy membrane and CTS. (*e*, *f*) Double immunostaining with TUNEL (*e*) and TRP-1 (*f*) of cryostat sections of HF in early stages of spontaneous catagen (for details see Tobin *et al*, 1998). *Large arrowhead*, TRP-1 IR; *small arrowhead*, TUNEL expression; *arrows*, colocalization of TRP-1 and TUNEL. dp, dermal papilla; m, melanosome; k, keratinocyte; a, apoptosis. *Scale bars*: (*a*) 20 μm; (*b*) 0.5 μm (inset 0.25 μm); (*c*) 2 μm (inset 5 μm); (*d*) 2 μm (inset 0.25 μm); (*e*, *f*) 50 μm.

protein and mRNA, however, are already present 1 d postdepilation, whereas TRP-1 was not detected until anagen II at day 3 postdepilation (Slominski *et al*, 1991). Dopa oxidase and tyrosine hydroxylase activities of tyrosinase, as well as dopachrome tautomerase activity and expression of protein, are readily detected in anagen II and are significantly raised during anagen, correlating with the first morphologically detectable melanogenically active melanocytes in the HF. Hair melanin production itself, as judged by murine skin color, was evident 4–5 d after anagen induction (Burchill *et al*, 1986; Slominski *et al*, 1991). There was also a sequential appearance of dopa oxidase protein(s) with different molecular weights during anagen and in this murine model system this may reflect the importance of post-translational modification on tyrosinase activity. Significantly, these events coincide with the formation of the hair cortex to which melanin is transferred for hair shaft pigmentation.

Although melanocytes in the S-phase of the cell cycle have been reported as early as anagen II (Sugiyama *et al*, 1995), significant proliferation is clearly apparent in anagen III (Staricco, 1960; Jimbow *et al*, 1975). Mitosis is also observed in melanogenically active cells (**Fig 6*a*, *b***), indicating that melanocyte differentiation does not preclude mitotic activity (Jimbow *et al*, 1975). Ultrastructural changes in bulbar melanocytes that accompany the maturation of the HF from anagen III–VI include: (i) increased dendricity; (ii) development of Golgi and rough endoplasmic reticulum; (iii) increased size/number of melanosomes (Sugiyama and Kukita, 1976); and (iv) transfer of mature melanosomes to precortical keratinocytes. The process of melanosome synthesis, maturation, movement to the dendrites, and transfer to keratinocytes is the subject of intense research effort in both epidermal and HF pigmentation biology. Premelanosomes are synthesized in the peri-nuclear region of the cell and are shuttled toward the dendrites, as they mature through distinct morphologic stages characterized by increasing melanin deposition (Fitzpatrick *et al*, 1958). Melanosome movement to the dendrites occurs through the motive influence of myosin V and microtubule motors (Rogers and Gelfand, 1998). Myosin V (encoded by the *dilute* gene) can be upregulated by α-MSH, and has also been proposed as the molecular motor involved in dendrite outgrowth in mammalian melanocytes (Nascimento *et al*, 1997). Significantly, mutations at the dilute locus are associated with dilution of hair color (Wei *et al*, 1997).

Pigment granule transfer to matrix keratinocytes begins during anagen IV (Slominski *et al*, 1991), a stage in hair growth characterized by the extension of the hair tip to the level of the sebaceous gland (Chase *et al*, 1951). It is still unclear how cortical keratinocytes incorporate pigment from the bulbar melanocytes, although this is considered to resemble the process of melanosomal transfer in the epidermis. At least four theories have been proposed explaining the mechanism involved in the passage of mature melanosomes from the bulbar melanocyte to the precortical keratinocytes: (i) the ''cytophagic'' theory, where the keratinocyte, as active partner, phagocytoses the tips of dendrites that contain stage IV mature melanosome (Mottaz and Zelickson, 1967; Garcia, 1979); (ii) the ''discharge'' theory, where mature melanosomes are discharged into the intercellular space and later internalized by adjacent keratinocytes; (iii) the ''fusion'' theory, where mature melanosomes pass from melanocyte to keratinocyte via fusion of their respective plasma membranes (Okazaki *et al*, 1976); and (iv) the ancient ''cytocrine'' theory, whereby melanocytes are thought

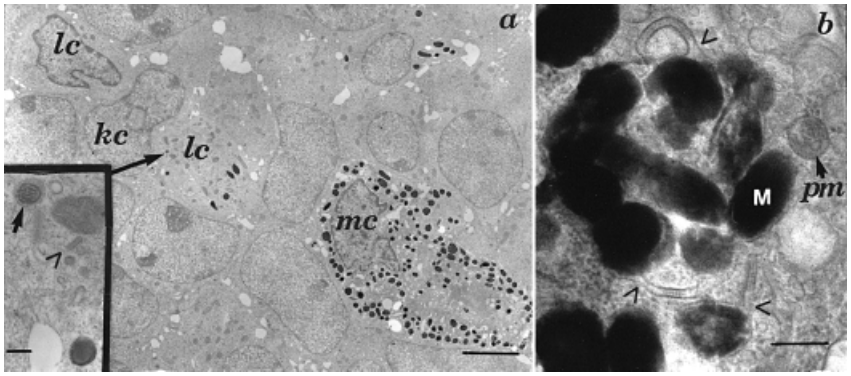

Figure 5. Removal of "incontinent" melanin from early catagen hair bulb by Langerhans cell. (*a*) Note the close proximity of the Langerhans cell (lc), keratinocytes (kc), and hair bulb melanocyte (mc); the later exhibits early signs of degeneration (e.g., vacuolation). The Langerhans cell (lc) contains both melanin granules, premelanosomes (→) and characteristic Langerhans granules (>). (*b*) Close association of Langerhans granules (>) with internalized melanosomes (M) in Langerhans cells. Note that the "heads" of the racket-shaped granules (>) apparently enclose the stage IV melanosomes (M). *Scale bars*: (*a*) 3 μm (inset 0.2 μm); (*b*) 0.25 μm.

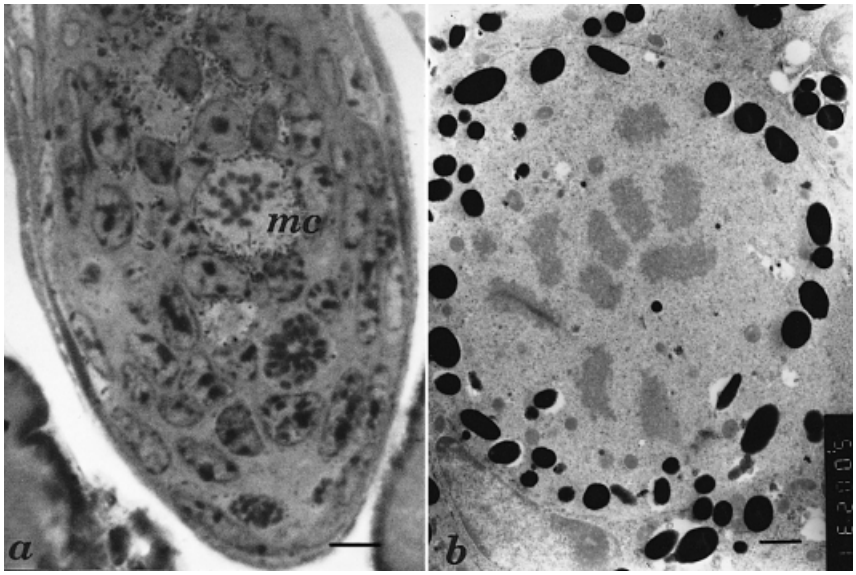

Figure 6. Melanocyte (mc) proliferation in anagen III hair follicles. *Scale bars*: (*a*) 4 μm; (*b*) 1 μm.

to inject melanin into recipient keratinocytes (Masson, 1948). Melanogenic activity in the hair bulb continues for the duration of anagen until the switching off of melanogenesis is initiated mid-way through anagen VI (Slominski *et al*, 1994), until ultimately melanocyte apoptosis is induced during early catagen (Tobin *et al*, 1998), thereby concluding a complete hair cycle.

AGE-RELATED FUNCTIONAL CHANGE IN THE FOLLICULAR MELANIN UNIT

The length of time hair bulb melanocytes remain melanogenically active, and thus the length of pigmented hair shaft they produce, depends on the duration of anagen for that given HF. It is also assumed that the same population of hair bulb melanocytes pigment the hair shaft from anagen III–VI. We have recently provided evidence that the full complement of melanogenically active melanocytes during anagen III–VI does not survive catagen (Tobin *et al*, 1998). Thus, the necessity to replenish the hair bulb with melanocytes from a reservoir, presumably located in the ORS, will be greater in HF with shorter anagen phases but producing hair of similar caliber. Given that scalp anagen can last from about 3–10 y (Kligman, 1959), the total number of anagen-associated melanocyte seedings to scalp hair bulbs will range from seven to 15 per average life-span, and may directly impact age-related factors of the follicular melanin unit. Canities therefore may reflect a genetically regulated exhaustion of the melanocyte reservoir's seeding potential or alternatively some defect in cell activation/migration.

The hair bulb melanocyte may also be more susceptible to age-related effects than melanocytes in the epidermis, most probably due to cyclic or intermittent activity of follicular melanocytes. That anagen, and its associated production of pigmented hair shaft, can last for up to 10 y in the human scalp (Kligman, 1959) attests to the phenomenal synthetic capacity of these melanin factories. This synthetic capacity is greatest during youth, during which time relatively few melanocytes can, in a single hair growth cycle, produce intensely pigmented hairs of up to 1.5 m in length. Although the onset of canities appears gradually, the reduction/cessation of pigment production may be abrupt in any one given HF. In these follicles partial reversal of canities may occur, whereby melanogenesis in de-activated bulbar melanocytes may be switched on again during anagen VI of the same hair growth cycle (Tobin and Cargnello, 1992).

The dilution of hair pigment in canities, however, is not due to the total loss of all follicular melanocytes, as ORS melanocytes are retained for some time in senile white HF (Kukita *et al*, 1971; Sato *et al*, 1973; Takada *et al*, 1992). The function of these remaining melanocytes in white HF is unclear. If not required for hair pigmentation, they would still be available for repigmentation/repopulation of the epidermis if necessary (Cui *et al*, 1991), Nevertheless, this suggests the existence of some other deficit in the inductive microenvironment necessary for bulbar pigment production. In fact, there is even anecdotal evidence of irradiation-induced repigmentation of senile canities, such as hair repigmentation after radiation therapy for metastatic carcinoma of the prostate (Shetty, 1995). Reversal of canities in this case is likely to have resulted from the radiation-induced activation of ORS melanocytes, and raises the attractive possibility that these ORS melanocytes may be induced to migrate and differentiate to naturally repigment greying HF.

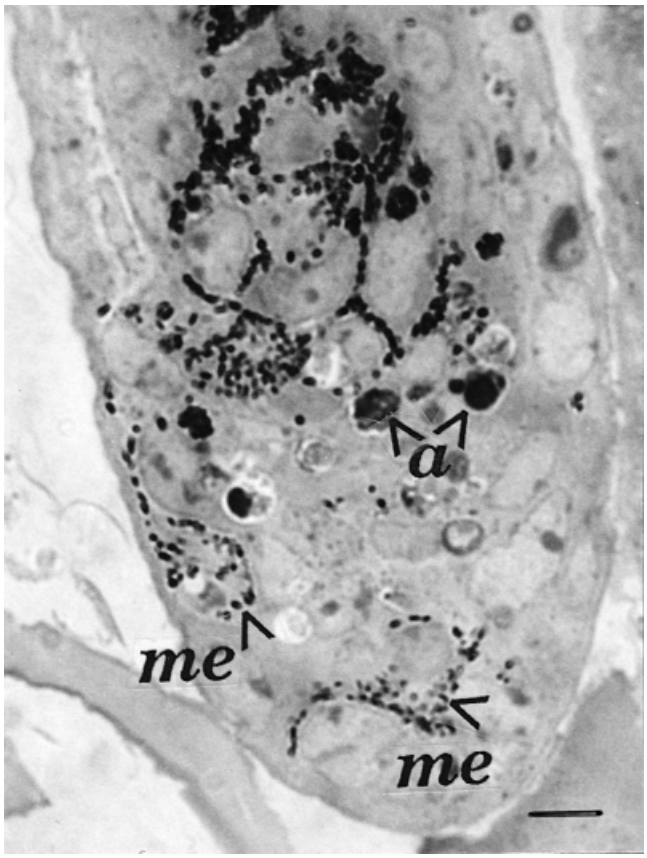

Figure 7. Melanocyte status in cyclophosphamide-induced early catagen. Re-distribution of melanogenic melanocytes (me) to the most proximal ORS. Note also the presence of widespread keratinocyte apoptosis (a), some in very early stages of programmed cell death 10 d after CYP-treatment initiation. *Scale bar*: 10 μm.

PHARMACOLOGIC MANIPULATION OF THE FOLLICULAR MELANIN UNIT

In vivo assays permitting the controlled induction and manipulation of the cyclical nature of HF melanogenesis need also, by implication, to involve manipulation of catagen. The mosaic nature of human scalp hair growth precludes the systematic analysis of the follicular melanin unit during catagen. This is further complicated by the brevity of catagen [2–5 wk in humans (Kligman, 1959), 2–3 d in mouse], providing possible access to only a very small proportion of HF in this stage at any one time (Parakkal, 1990). These, and the over-arching ethical considerations in using humans for such research, can, in part, be alleviated by studying fairly well-synchronized, spontaneous catagen development in mice (Straile *et al*, 1961), but even here the yield of catagen HF can be very low due to the relatively poor predictability of the exact time of onset.

One such assay is the topical application of the potent glucocorticosteroid dexamethasone (DEX) in C57BL/6 mice, which provides a rapid, highly reproducible and predictable pharmacologic induction of histologically normal-appearing catagen in almost all anagen VI HF (Paus *et al*, 1994b). DEX treatment induces the premature inhibition of tyrosinase expression/activity, and of the post dopa oxidase steps of melanogenesis, 3–4 d earlier than occurs in the normal anagen-catagen transition (Slominski *et al*, 1994). Although the histologic/ultrastructural features of hair bulb melanocytes in the treated HF are, in the main, very similar to those observed in HF after induction of spontaneous catagen, melanocyte apoptosis is more common than in spontaneous catagen (Tobin *et al*, 1998).

By contrast, pharmacologic catagen induced by cyclophosphamide (CYP) is accompanied by significant alterations of biochemical and biophysical markers of melanogenesis, and is associated with a massive disruption of the HF pigmentary unit. As in DEX-induced catagen, termination of melanogenesis by CYP is characterized by a rapid decrease in the tyrosine hydroxylase activity of tyrosinase and in the oxidation of dopa to melanin (Slominski *et al*, 1996) that occurs 3–4 d earlier than during the normal anagen-catagen transition (Slominski *et al*, 1994). Unlike DEX-induced catagen, however, TRP-2 activity increases and dihydroxyindole carboxylic acid-conversion factor activity remains unchanged compared with control HF, suggesting that tyrosinase is the most sensitive melanogenesis target for pharmacologic regulation (Slominski *et al*, 1996). CYP induces gross pigmentary defects including the disruption of melanosome transfer to keratinocytes resulting in the ectopic distribution of melanin in the inner root shealth, ORS, and even cuticle (**Fig 1**) (Paus *et al*, 1994c; Slominski *et al*, 1996; Tobin *et al*, 1998), rather than the precortex and medulla only as occurs during normal hair pigmentation (Cesarini, 1990). Notably, CYP damage to the HF is also associated with melanocyte displacement to the most proximal hair bulb epithelium. This re-location of melanogenic melanocytes to ectopic regions of the hair bulb (**Fig 7**) is likely to result in the transfer of melanin to noncortical keratinocytes. Despite such changes, histologically normal-appearing melanogenic hair-bulb melanocytes are observed in catagen II/III HF 6 d post-CYP injection, even though massive keratinocyte apoptosis occurred in their direct vicinity (Tobin *et al*, 1998).

Significant progress has been made in the last two decades in understanding the life-cycle of follicular melanocytes. The likely presence of a HF melanocyte reservoir that can replenish the bulbar melanin unit after apoptosis in catagen, provides intriguing perspectives for manipulation of this epithelial-mesenchymal-neuroectodermal interacting system (Slominski and Paus, 1993; Paus *et al*, 1997). The C57BL/6 mouse model offers an unrivaled study system for dissecting the controls that drive the rhythmic construction and deconstruction of the follicular melanin unit during each hair cycle.

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