Differential Expression and Activity of Melanogenesis-Related Proteins During Induced Hair Growth in Mice

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In C57 Bl-6 mice, melanogenesis is strictly coupled to the growth phase of the hair cycle (anagen). To further study this phenomenon of concerted developmental and pigmentary activity, we followed the sequence of tyrosinase (key enzyme of melanogenesis) expression and activity and the presence of the melanosomal protein gp 75 during the development of traumatically induced anagen follicles (days 0 = telogen, and days 1–12, after anagen induction studied). In addition to performing Northern and Western blots for tyrosinase, tyrosine hydroxylase activity (THA) and dopa oxidase activity (DOA) were measured. On day 0, DOA was undetectable, and THA was very low. On days 1 and 2, both activities were undetectable; starting from day 3, they increased rapidly, reaching a plateau on days 8 and 12. DO-positive proteins had apparent molecular weights (MW) of 66–68 kD (days 3–12), 72–74 kD (days 5–12), and 130 kD (days 8 and 12). Western blotting emphasized proteins of MW 66–68 kD (tyrosinase), and 73–75 kD (gp 75); tyrosinase was undetectable on day 0, but already present on days 1 and 2; it increased by day 5 and had reached a plateau on days 8 and 12; gp 75 was undetectable on days 0–2; it was present on day 3, increased by day 5, and reached a plateau on days 8 and 12. Northern blot analysis revealed high levels of tyrosinase mRNA on days 5 and 8, low levels on days 1–3, and none on day 0. These data suggest a highly regulated, time frame-restricted, differential pattern of tyrosinase transcription, translation, and enzyme activity during the different stages of the developing murine anagen follicle, possibly as a result of complex interactions between follicular melanocytes and their environment. *J Invest Dermatol 96:172–179, 1991*

Hair grows in a cyclic fashion, and is characterized by three developmental stages: resting (telogen), growth (anagen), and regression (catagen). During anagen, a metabolically highly active mini-organ with one of the highest mitotic rates found in mammalian tissues, the anagen follicle, develops in order to produce the hair shaft, formed by pigmented, keratinized epithelial cells [1,2].

Hair growth and the cyclic activity of the hair follicle are timed by a "biological clock" of unknown nature and are the result of as-yet poorly understood tissue interactions. For example, it is well-appreciated that development and function of the epithelial part of the anagen follicle (epithelial bulb) are critically dependent on interactions with its mesenchymal component, the dermal papilla [3–6]. However, because pigment production and transport by follicular melanocytes also are important features of anagen [7,8], hair growth is more than a phenomenon of epithelial-mesenchymal interactions, but instead requires the coordinated and programmed activity of epithelial, mesenchymal, and neuroectodermal cells. Studying pigmentation during the adult murine hair cycle thus addresses key questions relevant to pigment, hair, developmental and neurobiology.

The C57 Bl-6 mouse is a particularly useful model for studying the biology of hair growth [9–11] and the phenomenon of anagen-coupled melanogenesis. In these mice, all truncal melanocytes are confined to the hair follicle, and melanogenesis is strictly coupled to the anagen stage of the hair cycle [1,12,13]. In catagen, melanin formation is inhibited, whereas it ceases in telogen [7,13]. Murine models of hair growth offer the opportunity to study large numbers of biologically homogeneous follicle populations, because hair growth in mice is synchronized; in contrast to humans, hair follicles enter or leave anagen in waves covering large skin areas. Anagen C57 Bl-6 mice (grey to black skin) can easily be distinguished from telogen mice (pink skin) by observation of their skin color [1]. Telogen follicles can be induced to enter anagen by mechanical traumatization, such as depilation [9,11]. Thus, this mouse can serve as a model for studying the highly controlled interactions occurring between keratinocytes, melanocytes, and fibroblasts during hair growth.

In the melanocyte, melanogenesis is restricted to a specialized organelle, the melanosome [14], and is initiated by the enzymatic

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Abbreviations:
BIS: N,N′-methylene-bis-acrylamide
BSA: bovine serum albumin
DAB: diaminobenzidine
dCTP: deoxy cytosine triphosphate
DOA: dopa oxidase activity
L-dopa: L-dihydroxyphenylalanine
MW: molecular weight
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
PMSF: phenylmethylsulfonylfluoride
SDS: sodium dodecyl sulfate
SSC: citrate-buffered saline, pH 7.0
TBS: Tris-buffered saline, pH 7.5
THA: tyrosine hydroxylase activity
TTBS: Tris-buffered saline plus 0.05% Tween 20

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Table I. Differential Expression of Pigmentation-Related Parameters During Induced Hair Growth

Day After Anagen Induction	Melanin[a] Production	Tyrosinase Activity[b] THA	Tyrosinase Activity[b] DOA	Tyrosinase mRNA[c]	Tyrosinase Protein[d]	Dopa Oxidase Isozymes[e]	gp75[f]
Telogen	−	(+)	−	−	−	−	−
1	−	−	−	(+)	+	−	−
2	−	−	−	(+)	+	−	−
3	−	+	+	(+)	+	I	(+)
5	+	++	++	+++	++	I,II	++
8	++	+++	+++	+++	+++	I,II,III	+++
12	+++	+++	+++	ND	+++	I,II,III	+++

[a] Melanin production as judged by skin color.
[b] Activity represents tyrosine hydroxylase (THA) and dopa oxidase activity (DOA) of tyrosinase.
[c] mRNA transcripts detected by the Mty811C mouse tyrosinase cDNA probe [26].
[d] Protein detected by polyclonal anti-mouse tyrosinase antibodies [22].
[e] Isozymes of dopa oxidase represent proteins of MW(kD) 66–68 (I); 72–74 (II); 130–135 (III).
[f] gp75 represents protein detected with monoclonal anti-gp75 (Mel-5) antibodies [29].

hydroxylation of L-tyrosine to L-dopa and further oxidation of L-dopa to dopaquinone [15]. Both reactions are under the rate-limiting control of the copper-based enzyme tyrosinase (monophenol dihydroxyphenylalanine: oxygen oxireductase; EC 1.14.18.1) [15]. Pigment granules, the final product of this process, are transported to the surrounding keratinocytes in a phagocytic fashion [16,17].

The molecular basis for the interesting phenomenon of concerted developmental and pigmentary activity occurring during hair growth in C57 Bl-6 mice is unknown. As a first step towards its elucidation, we have followed the sequence of tyrosinase expression and activity and the expression of gp75 (melanosomal protein) during the development of traumatically induced anagen follicles in C57 Bl-6 mice. We report differential changes in tyrosinase transcription, translation, activity, and isozyme repertoire, as well as changes in the concentration of gp75 in mouse skin during the hair cycle between day (d) 0 (telogen) and d 12 after anagen induction.

MATERIALS AND METHODS

Animals Telogen C57 Bl-6 mice (female, syngeneic, 6–8 weeks old) were purchased from Charles River, Kingston, NY, housed in community cages in the Albany Medical College Animal Facility with 12-h light periods and fed ad libitum with water and rat/mouse chow 3000 (Agway, Syracuse, NY).

Anagen Induction Telogen mice (judged by their pink skin color) were anaesthetized (30 mg sodium pentobarbital/kg body weight) and stripped of hair using a warm beeswax/rosin mixture, 1:1. The melted mixture was painted over the whole back of the mouse and, after hardening, peeled off, thus inducing the resting follicles to enter anagen [11].

Skin Preparation Telogen mice (day 0), and anagen mice (days 1,2,3,5,8, and 12 after anagen induction) were killed by cervical dislocation under ether narcosis, and were shaved with an electrical animal clipper. The skin was washed briefly with 70% ethanol, all back skin dissected at the level of the subcutis, immediately transferred to liquid nitrogen, and stored up to 3 weeks at −80°C, or used directly for total RNA extractions. Three mice were used for each time point. In parallel fashion, skin samples from identical locations on the back were fixed in 10% formalin in phosphate-buffered saline, and processed for routine histology (paraffin embedded, hematoxylin-eosin stained sections [18]).

For enzyme assays and Western blots, frozen skin (see above) or frozen Bomirski Ma hamster melanoma tissue (passage 222; used as positive control for tyrosinase [19]), were pulverized in liquid nitrogen using a mortar. The pulverized tissues were further homogenized in lysis buffer (10 mM Tris, pH 7.4, 1% Triton X − 100, 1 mM PMSF, 0.01% aprotinin) at a ratio of 0.5 g tissue/4 ml of buffer using a polytron at maximum speed (30″). Tissue extracts were centrifuged at 15,000 × *g* for 30 min at 4°C, supernatants were aliquoted and frozen in −80°C for enzyme assays and western blot analyses. The pellets of skin extract preparations from different days were photographed and their melanin content compared. For the determination of melanin content, unlike for the other assays performed, the hair shafts of day 0 skin were removed by depilation after sacrificing the animal. Above manipulations were done on ice.

The protein content in the samples was estimated with the aid of a Bio-Rad protein assay kit, using bovine serum albumin (Sigma, St. Louis, MO) as a standard.

Tyrosinase Assays (THA, DOA) Tyrosine hydroxylase activity (THA) in the above supernatants was assayed by the Pomerantz method with 0.5 μCi of ^{3}H-L-tyrosine (55.7 Ci/mmol, New England Nuclear) plus 100 μM of unlabeled L-tyrosine and 100 μM L-dopa as cofactor in 0.1 M sodium-phosphate buffer, pH 6.8, at 37°C for 1 h as previously described [20]. Tyrosine hydroxylase activity was expressed in CPM of tritriated water produced during 1 h incubation per 1 mg protein.

For dopa oxidase (DOA) analyses, supernatants were suspended in loading buffer (0.063 M Tris, pH 6.8, 10% glycerol, 1% SDS, 0.001% bromophenol blue). The proteins were separated under nonreducing conditions according to a nondenaturating modification of the method of Laemmli [20,21]. Briefly, the samples (60 μg of protein), and high molecular weight standards (BRL), were subjected to SDS-polyacrylamide gel electrophoresis with gel containing SDS (0.1%), acrylamide (8%), and BIS (0.2%). The SDS gel electrophoresis was carried out at room temperature at pH 8.6 in Tris-glycine buffer containing SDS (0.1%). The gels were washed with PBS and stained with 5 mM L-dopa in phosphate buffer (0.1 M, pH 6.8) at 37°C. Alternatively, the proteins were blotted overnight from gels to Zeta Probe blotting membranes (Bio-Rad) at 4°C in 25 mM Tris, 192 mM glycine, pH 8.3 with constant current (30–35 mA). The membranes were washed briefly in phosphate buffer and stained with 5 mM L-dopa in 0.1 M sodium-phosphate buffer, pH 6.8, at 37°C.

Western Blot Analyses The samples (60 μg of protein) and prestained high molecular weight markers were separated under nonreducing conditions according to a nondenaturating modification of the method of Laemmli (see above). The proteins were blotted from the polyacrylamide gels to nitrocellulose membranes (Bio-Rad) overnight (see above). After blocking with 3% gelatin in 20 mM Tris, 0.5 M NaCl, pH 7.5 (TBS), the filters were washed in TBS plus 0.05% Tween 20 plus 1% BSA, and were probed with polyclonal anti-tyrosinase antibodies, 1:200 dilution (gift of Dr. Laskin; cf. [22]) and with nonimmune rabbit serum in TBS plus 0.05% Tween 20 plus 1% BSA (TTBS) overnight with shaking. After washing with 20 mM citrate, 500 mM NaCl, pH 5.5, plus 0.05% Tween 20, the membranes were incubated with protein

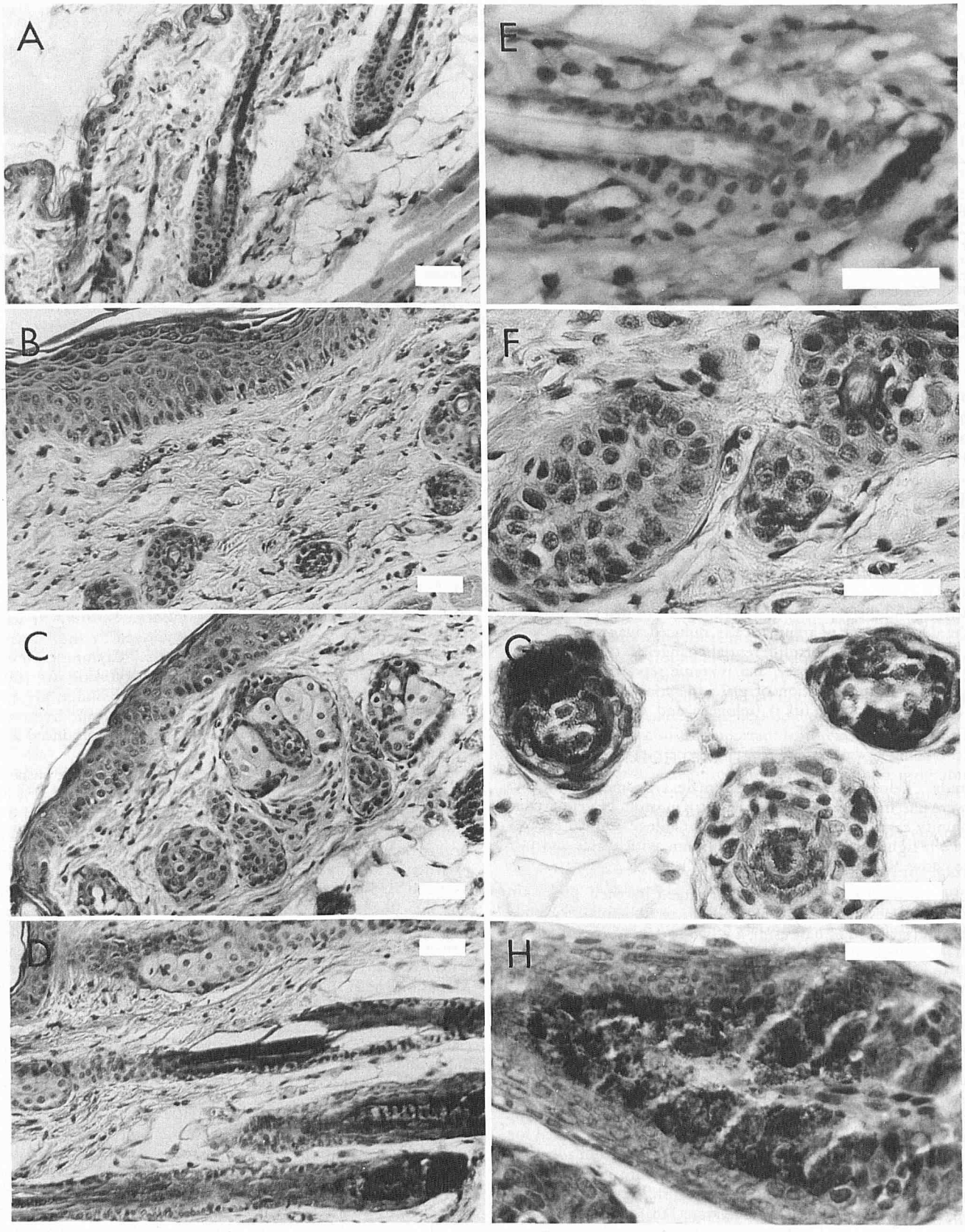

Figure 1. Photomicrographs of murine skin biopsies fixed in formalin and stained with hematoxylin and eosin. Telogen skin obtained at d 0 (*A* and *E*) and d 3 after induction of anagen phase (*B* and *F*) show the absence of melanin. However, epidermal thickening is seen at day 3 (*B*). Progressive melanin accumulation is found by d 5 (*C* and *G*) and by day 8 (*D* and *H*). *Bar,* 20 μm.

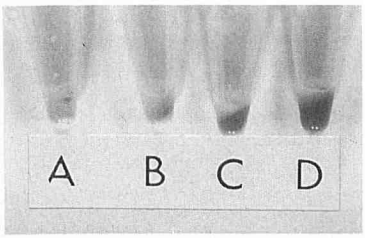

Figure 2. Photographs of murine skin pellets after buffer extraction. For the determination of melanin content, unlike for the other assays performed, the hair shafts of telogen were removed by depilation after sacrificing the animal. Note that there is an absence of melanin pigmentation of pellet in the telogen (*A*) and day 3 (*B*) of anagen skin. Increasing melanin pigmentation is seen on days 5 (*C*) and 8 (*D*) of anagen.

G-gold for 6–16 h in the dark (1:250 dilution, Bio-Rad). The membranes were photographed, or gold-stained immune complexes were enhanced in the dark, using the hydroquinone/silver lactate solution according to the Bio-Rad protocol, and photographed after fixation (Bio-Rad, Protein G-gold staining protocol). The enhancement and staining were performed on membranes probed with anti-tyrosinase antibodies and with nonimmune rabbit serum.

For probing with monoclonal antibodies against the melanosomal protein gp75 (Mel-5, Signet Lab, Inc., MA) the proteins were separated by PAGE (see above) and transferred to a Zeta Probe Blotting membrane (Bio-Rad). After blocking with 10% milk in TBS, the membranes were washed in TTBS, and probed with monoclonal antibodies Mel-5 (1:10 dilution) and non-immune mouse serum in TTBS overnight under continuous shaking. After washing with TTBS, the membranes were incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (BRL) for 6 h (1:200 dilution). After washing, the immune complexes were visualized by staining for peroxidase activity with diaminobenzidine (DAB, Sigma) and H_2O_2 (Sigma) as substrates [23], and photographed.

Northern Blot Analyses Three different RNA isolation protocols were performed. For protocol 1, skin preparations stored at −80°C were pulverized in liquid nitrogen using a mortar and the mRNA extracted using the "FAST track" mRNA isolation kit, according to the manufacturer's instructions (Invitrogen, CA). Alternatively (protocol 2), total RNA was isolated from pulverized tissues by a guanidinium thiocyanate method with subsequent ultracentrifugation of the lysate through a CsCl cushion and phenol-chloroform extraction [24]. In protocol 3, skin was processed immediately after harvesting, using the single-step method of RNA isolation by acidic guanidine thiocyanate-phenol-chloroform extraction, according to Chomczynski and Sacchi [25].

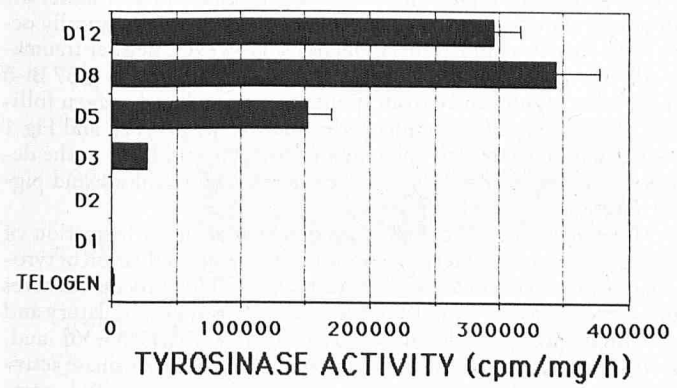

Figure 3. Tyrosine hydroxylase activity of tyrosinase in skin extracts. Tyrosine hydroxylase activity is very low in the telogen *(Telogen)*, undetectable on days 1 (*D1*) and 2 (*D2*) after induction of anagen, while starting from day 3 (*D3*) it increases rapidly, reaching a plateau on days 8 (*D8*) and 12 (*D12*). The enzyme activity is expressed as cpm of tritriated water produced during 1 h of incubation per 1 mg protein. The data represent the mean ± SD from four assays.

Three μg of poly (A) + RNA or 30 μg of total RNA were electrophoretically separated through formaldehyde/1% agarose gels by standard procedures [24]. RNA was transferred to Zeta Probe blotting membranes (BioRad) by capillary transfer overnight. The membranes were photographed under UV and dried for 2 h at 80°C under vacuum. The membranes were prehybridized in 50% deionized formamide, 7% SDS, 5 × Denhardt's solution, 5 × SSC, 20 mM NaH_2PO_4, pH 7.0, and 100 μg/ml denatured salmon sperm DNA at 42°C for 12 h. The heat-denatured, random prime-labeled tyrosinase cDNA probe (specific activity 6×10^8 cpm/μg) was added to the prehybridization buffer at 42°C and hybridization performed for 12–24 h. After hybridization, the membranes were washed for 5 min with 2 × SSC plus 0.5% SDS and 15 min with 2 × SSC plus 0.1% SDS at room temperature, then with 0.2 × SSC plus 0.1% SDS at 68°C for 2 h. A final 5-min rinse in 0.2 × SSC plus 0.5% SDS was performed before exposing the hybridized blots to Kodak XAR film at −70°C.

Random prime-labeled probes were produced with the aid of the Boehringer-Manheim kit and alfa-^{32}P-dCTP (Dupont, NEN, Boston, MA), using the 2-kb mouse tyrosinase cDNA clone Mty811C (gift of Dr. Kwon, cf. [26]).

RESULTS

Macroscopically, no melanin production was apparent in telogen skin and between d 1–3 after anagen induction (pink skin), whereas on day 5 the skin turned grey, and on day 8 and day 12, black. Figure 1 demonstrates that this pattern of visible pigmentation changes during anagen development corresponds histologically to the absence of pigment granules between day 0–3 and the appearance of dark brown melanin granules by day 5, with several mature, fully pigmented follicles developed by day 8–day 12.

Note in Fig 1 the dramatic anagen-associated morphologic changes affecting the skin as a whole during the development of anagen follicles. From previous studies, it is well appreciated that the epidermis wins additional cell layers, whereas dermis and subcutis increase significantly in thickness (cf. [10,11,27,28]). Figure 2 shows the increase in melanin production during anagen by comparing the pigmentation of pellets from skin extracts of day 0, 3, 5, and 8.

Table I summarizes the changes observed when the melanogenesis-related proteins tyrosinase and gp75 were studied. Tyrosine hydroxylase activity (THA) of tyrosinase was very low in telogen skin, undetectable on day 1 and 2 of anagen skin, whereas starting from d 3, it increased rapidly, reaching a plateau on days 8 and 12 (Fig 3). Dopa oxidase activity (DOA) of tyrosinase was undetectable in telogen skin and on d 1 and 2 of anagen skin (Fig 4*a*). On gels stained with L-dopa, it appeared on day 5 as a protein of molecular weight (MW) of 66–68 kD and its activity increased on days 8 and 12 (Fig 4*a*). Note that in the control lane (hamster melanotic melanoma), dopa oxidase activity appeared as proteins of MW of 66–68 kD, 72–74 kD, 85 kD, and 130–135 kD. On nylon membrane, dopa oxidase activity appeared already on d 3 of anagen development as a protein of MW 66–68 kD, reaching a plateau on days 8 and 12 (Fig 4*b*). Also, two other DOA-positive proteins with MW of 72–74 kD and 130 kD appeared on days 5 and 8, respectively (Fig 4*b*). This difference between L-dopa stain of gels and nylon membranes is most likely due to a higher sensitivity of the latter method.

Western blots, employing previously characterized polyclonal anti-mouse tyrosinase antibodies [22] emphasized a protein of approximate MW 66–68 kD (Fig 5*A*), which corresponded to a 66–68 kD DOA-positive protein shown in Fig 4*a, b*, and was absent in control blots stained with nonimmune serum (Fig 5*b*). This protein was undetectable in telogen skin, whereas already present on days 1 and 2, slightly decreased on day 3, increased by day 5, reaching a

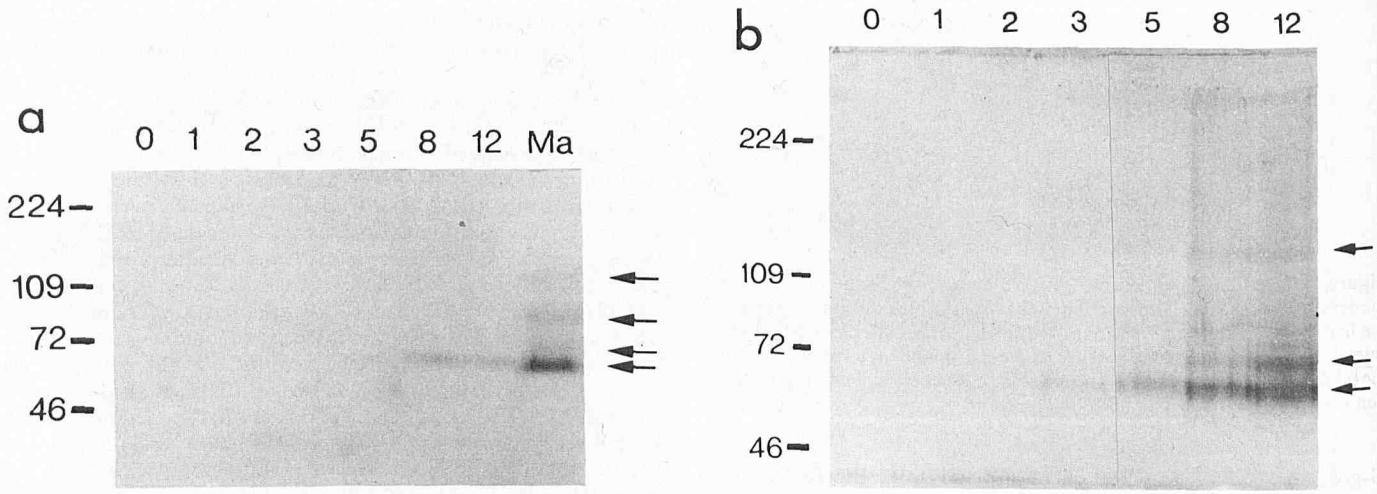

Figure 4. Dopa oxidase activities (DOA) of tyrosinase in skin extracts. DOA is undetectable in the telogen (0) and days 1 (*1*) and 2 (*2*) of anagen. On gels stained directly with L-dopa (*a*), it appears on day 5 (*5*) as a protein of 66–68 kD and its activity increases on days 8 (*8*) and 12 (*12*). In the control line (Ma), DOA of hamster melanoma appears as proteins of 66–68 kD, 72–74 kD, 85 kD, and 130–135 kD. On western blots stained with L-dopa (*b*), DOA appears sequentially as proteins of 66–68 kD on day 3 (*3*), of 72–74 kD on days 5–12 (*5,8,12*) and of 130–135 kD on days 8 (*8*) and 12 (*12*). *Arrows* show dopa oxidase positive proteins. *Left,* MW markers (kD).

plateau on days 8 and 12 of anagen (Fig 5*A*). Proteins of higher MW were detected both by anti-tyrosinase and nonimmune serum (Fig 5). They may represent IgG present in the skin extracts, as they were detected when probed only by antimouse IgG (Fig 5*B*), and were absent when membranes were stained only for peroxidase activity (not shown).

Transblot immunostains with monoclonal anti-gp75 (Mel-5) antibodies detected the specific protein of apparent MW 73–75 kD first on day 3 with an increased concentration on day 5, reaching a plateau on days 8 and 12 (Fig 6). This protein was not found in telogen and early anagen (days 0–2).

Northern blot analysis, using the Mty811C mouse tyrosinase cDNA probe, revealed low but detectable levels of tyrosinase mRNA transcripts on days 1 and 2, and high levels of transcript on days 5 and 8 (Fig 7). Tyrosinase transcript was undetectable in telogen skin.

DISCUSSION

In these studies, we report a differential pattern of expression and enzyme activity of tyrosinase and of gp75 concentration during the different stages of anagen follicle development in adult mice.

First, tyrosinase mRNA and a protein of MW 66–68 kD precipitated by anti-tyrosinase antibodies were detected as early as on days 1 and 2 of anagen development. Second, tyrosine hydroxylase activity was first detected on day 3 and reached high levels (plateau) on days 8 and 12. Third, dopa oxidase activity appeared sequentially as proteins of MW of 66–68 kD (day 3), of 72–74 kD (days 5–12) and of 130–135 kD (days 8 and 12). Fourth, pigmentation-specific protein (gp75) [29] was first detected on day 3 and reached high levels (plateau) on days 8 and 12.

It is generally held that melanogenically active melanocytes are absent during telogen and the first 3 d after anagen induction (anagen stage I–II), whereas from the fourth day on (anagen III–IV) their number and activity increases, reaching a peak on days 8–12 (anagen V–VI) [7,13,30]. This opinion has been challenged by Sugiyama [31], who demonstrated in ultrastructural studies that undifferentiated melanocytes in telogen and early anagen contain unmelanized premelanosomes and are indeed dopa oxidase positive. Our results are in partial agreement with those findings; we provide evidence that tyrosinase transcripts and translational products appear as early as day 1 after anagen induction, with tyrosinase activity demonstrable by day 3 (see Figs 3–5).

Our findings of sequential appearance of the dopa oxidase-positive proteins along anagen development (protein of MW 66–68 kD, day 3; 72–74 kD, day 5; 130–135 kD, day 8) are in agreement with the study by Burnett et al [30], describing the presence of three isozymic forms of dopa oxidase in anagen IV–VI. Although due to non-specific binding (Fig 5*B*), we could not demonstrate immunoprecipitation of high MW forms of tyrosinase, we suggest that dopa oxidase proteins of 72–74 and 130–135 kD are tyrosinase. This suggestion is based on previous studies demonstrating high MW forms of tyrosinase both by dopa stain and immunoprecipitation in B 16 [22,32] and in hamster melanomas ([20]; see Fig 4), as well as in follicular melanocytes of C3H-HeAvy mice [33]. The differential pattern of dopa oxidase repertoire and tyrosinase activity observed in our experiments may correspond to the demonstration that, in C3H-HeAvy mouse skin, tyrosinase activity is dependent both on enzyme synthesis and posttranslational modification [33].

The very low THA observed in telogen skin (day 0, see Fig 3) is probably not due to active tyrosinase in follicular melanocytes at this time, but rather represents residual tyrosinase activity in telogen hair shafts not entirely removed during protein extract preparation from telogen skin (the hairs were only shaved, not depilated; see *Materials and Methods*).

Because our data were generated using traumatically induced anagen follicles, which might differ from spontaneously developing anagen follicles (cf. [9,34]), it remains to be established whether the findings reported here are representative of the physiologically occurring anagen-coupled melanogenesis. However, neither traumatically nor pharmacologically induced anagen follicles in C 57 Bl-6 mice differ significantly from spontaneously induced anagen follicles macroscopically or under light microscopy [10,11], and Fig 1 clearly demonstrates that plucking of telogen skin leads to the development of anagen follicles with normal morphology and pigmentation.

Melanogenesis is a complex process that requires formation of melanosomes in the melanocyte and delivery and activation of tyrosinase in the intramelanosomal milieu [17]. This process involves interactions between multiple genes as well as their regulatory and structural products, including tyrosinase [7,13,17,35,36] and, according to Halaban and Moellmann, gp75 [37]. Tyrosinase activity can be regulated at the level of gene transcription [26], posttranscriptional processing [38], posttranslational modification [17,32,39,40], and enzyme activation [17,41]. In addition, a regulation on the translation level has been proposed [42,43]. Our results showing a pattern of unparalleled appearance of tyrosinase mRNA and protein, enzyme activities, dopa oxidase isozymes, gp75, and pigmentation suggest that all of the above mechanisms may operate

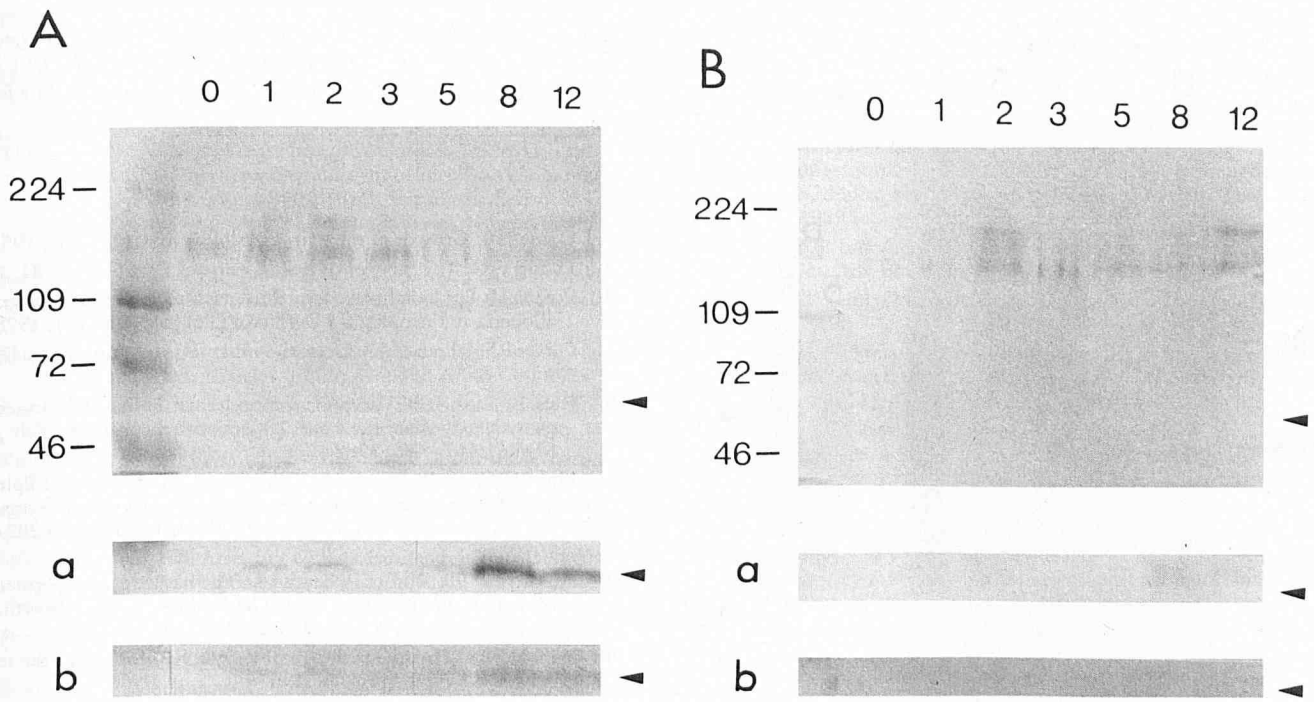

Figure 5. Tyrosinase visualized on Western blots with anti-tyrosinase antibodies. Western blots probed with polyclonal anti-tyrosinase antibodies (*A*) produce the specific protein of 66–68 kD (*arrowhead*), which is undetectable in the telogen (*0*), present on days 1–5 (*1,2,3,5*) and abundant on days 8 (*8*) and 12 (*12*) after induction of anagen (see *lower panel*). This protein is absent in control blots stained with nonimmune serum (*B*). *Top panels,* filters stained with protein G-gold; *lower panels,* enhanced gold-stained immune complexes (*a*, first experiment; *b*, second experiment). *Left,* MW markers (kD).

along hair growth in a time frame-restricted fashion, and that this regulation may involve interactions with other melanogenesis-related proteins such as gp75. Therefore, the C 57 Bl-6 mouse model utilized here allows us to dissect and study separately the different molecular mechanisms governing melanocyte activity in a physiologic environment (skin), and serves as an attractive alternative to studies using isolated melanocytes in vitro.

Whether the induction of melanogenesis by the plucking of telogen follicles is due to a direct effect on follicular melonocytes, or whether this is mediated indirectly via other skin cells affected by the traumatic stimulus, is currently unknown (cf. [44]). It is well-established that keratinocyte proliferation and differentiation in the anagen epithelial bulb are strictly mesenchyme dependent, with the dermal papilla cells [4] and possibly extracellular matrix molecules, such as fibronectin and collagen type IV [6], as critical components. It is equally well appreciated that neural crest cells, some of which differentiate into melanocytes, migrate along pathways marked and modified by the mesenchyme [45], that local factors produced by the mesenchyme are essential for the induction of melanoblast differentiation in avian neural crest cells [46] and that mesenchyme-derived factors may affect the development of melanoblasts in mice [47]. The initiation of melanogenesis during anagen might, therefore, be mediated via the mesenchyme.

Another aspect of anagen-coupled melanogenesis is an epithelial-neuroectodermal cell interaction. On the one hand, it is generally accepted that keratinocytes can regulate melanocyte proliferation and melanogenesis [48–50]. On the other, pigment granules, which are transferred to surrounding keratinocytes [16,17], are a

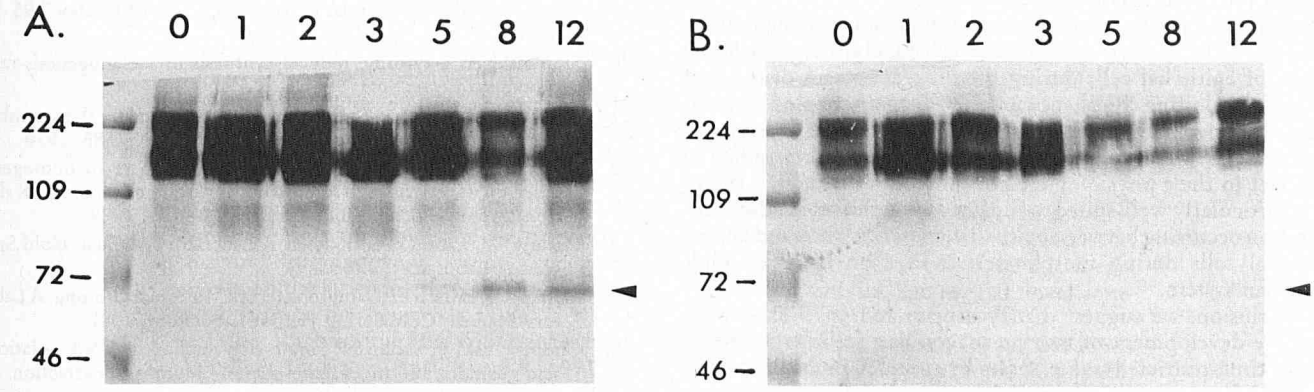

Figure 6. Gp75 protein visualized on Western blots with Mel-5 antibodies. Western blots probed with monoclonal Mel-5 antibodies (*A*) produce the specific protein of 73–75 kD (*arrowhead*) on day 3 (*3*) with increased concentration on day 5 (*5*), reaching plateau on days 8 (*8*) and 12 (*12*) after induction of anagen. This protein is not found in the telogen and days 1 (*1*) and 2 (*2*) of anagen. It is also absent in control blots stained with nonimmune mouse serum (*B*). *Left,* MW markers (kD).

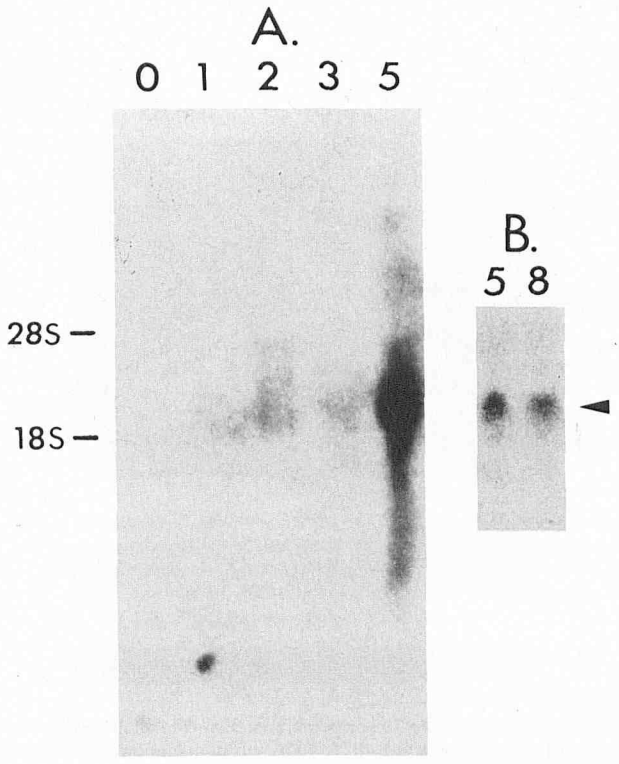

Figure 7. Tyrosinase mRNA probed with mouse tyrosinase cDNA clone Mty811C. Northern blot analysis of total RNA (*A*) shows that tyrosinase mRNA is undetectable in telogen skin (*0*), low on days 1–3 (*1,2,3*) and high on day 5 (*5*) after induction of anagen (*A*). Northern blot analysis of poly $(A)^+$ mRNA (*B*) shows that tyrosinase mRNA level reaches a plateau on days 5 (*5*) and 8 (*8*) after induction of anagen. *Arrowhead,* a transcript hybridized to tyrosinase cDNA.

rich source of divalent cations (cf. [51,52]), consume oxygen (cf. [17,54]), can act as buffering system for cytoplasmic calcium (cf. [51]), and reversibly bind several natural bioregulatory compounds such as serotonin, catecholamines, and prostaglandins (cf. [51,53]). Anagen-associated melanogenesis may, therefore, be part of a regulatory circuit coordinating complex interactions between bulb keratinocytes, melanocytes, and possibly other cell types, with melanocytes as regulatory and not only pigment-producing cells (cf. [54,55]. The activity of tyrosinase, the key enzyme of melanogenesis, should then be under stringent control during hair growth, which, in fact, was demonstrated above.

The C57 Bl-6 mouse model of anagen induction utilized in these studies allows to analyze and compare selectively the growth and function of epithelial cells during the different stages of the hair cycle [11]. Exploiting the phenomenon of anagen-coupled melanogenesis, we show here that, in addition, follicular melanocyte populations of defined and distinct biologic activities can be studied in this model in their physiologic environment. This model, therefore, is particularly well-suited to further dissect the molecular communication occurring between epithelial, mesenchymal, and neuroectodermal cells during morphogenesis in a physiologic, adult mammalian system.

In conclusion, we suggest that tyrosinase and gp75 expression during the development of murine anagen hair follicles are regulated in a time-restricted frame, set up by complex multidirectional interactions between follicular melanocytes and their environment. The C57 Bl-6 mouse model promises to serve as an excellent tool for further analysis of anagen-coupled melanogenesis as a model system for studying epithelial-mesenchymal-neuroectodermal tissue interactions under physiologic conditions.

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REFERENCES

1. Chase HB: Growth of the hair. Physiol Rev 34:113–126, 1954
2. Ebling FJG: The biology of hair. Dermatol Clin 5:476–481, 1987
3. Pinkus H: Epithelial mesodermal interaction in normal hair growth, allopetia and neoplasia. J Dermatol (Tokyo) 5:93–101, 1978
4. Oliver RF, Jahoda CAB: Dermal-epidermal interactions. Clin Dermatol 6:74–82, 1988
5. Paus R, Link RE: The psoriatic epidermal lesion and anagen hair growth may share the same "switch-on" mechanism. Yale J Biol Med 61:467–476, 1988
6. Link RE, Paus R, Stenn KS, Kuklinska E, Moellmann G: Epithelial growth by rat vibrissae follicles in vitro requires mesenchymal contact via native extracellular matrix. J Invest Dermatol 95:202–207, 1990
7. Fitzpatrick TB, Burnet P, Kukita A: The nature of hair pigment. In: Montagna W, Ellis RA (eds.). The Biology of Hair Growth. Academic Press, New York, 1958, pp 255–303
8. Parakkal PF: The fine structure of anagen hair follicle of the mouse. Adv Biol Skin 9:441–469, 1967
9. Argyris TS: Hair growth induced by damage. Adv Biol Skin 9:339, 1967
10. Paus R, Stenn KS, Link RE: The induction of anagen hair follicle growth in telogen mouse skin by cyclosporine A administration. Lab Invest 60:365–369, 1989
11. Paus R, Stenn KS, Link RE: Telogen skin contains an inhibitor of hair growth. Br J Dermatol 122:777–784, 1990
12. Billingham RE, Silvers WK: The melanocytes of mammals. Q Rev Biol 35:1–40, 1960
13. Silvers WK (ed.): The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction. Springer, New York, 1979
14. Seiji M: Subcellular particles and melanin formation in melanocytes. Adv Biol Skin 8:189–222, 1967
15. Lerner AB, Fitzpatrick TB: Biochemistry of melanin formation. Physiol Rev 30:91–125, 1950
16. Quevedo WC, Fitzpatrick TB, Szabo G, Jimbow K: Biology of melanocytes. In: Fizpatrick TB, Eisen AZ, Wolff K, Freeberg IM, Austen KF (eds.). Dermatology in General Medicine. McGraw Hill, New York, 1987, pp 224–251
17. Moellmann G, Slominski A, Kuklinska E, Lerner A: Regulation of melanogenesis in melanocytes. Pigment Cell Res (suppl) 1:79–87, 1988
18. Putt FA (ed.): Manual of Histopathological Staining Methods. J Wiley and Sons, New York, 1972
19. Bomirski A, Slominski A, Bigda J: Natural history of a family of transplantable melanomas in hamsters. Cancer Met Rev 7:95–118, 1988
20. Slominski A: L-tyrosine induces synthesis of melanogenesis related proteins. Life Sci 45:1799–1803, 1989
21. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277:680–685, 1970
22. Yurkow EJ, Laskin JD: Purification of tyrosinase to homogeneity based on its resistance to sodium dodecyl sulfate-proteinase K digestion. Archiv Biochem Biophys 275:122–129, 1989
23. Harlow W, Lane D: Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, 1988
24. Maniatis T, Fritsch EF, Sambrook (eds.): Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, 1987
25. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159, 1987
26. Kwon BS, Wakulchik M, Haq AF, Halaban R, Kestler D: Sequence analysis of mouse tyrosinase cDNA and the effect of melanotropin on its gene expression. Biochem Biophys Res Commun 153:1301–1309, 1988

27. Chase HB, Montagna W, Malone JD: Changes in the skin in relation to hair regrowth cycle. Anat Rec 116:75–82, 1953
28. Hansen LS, Coggle JE, Wells J, Charles MW: The influence of the hair cycle on the thickness of mouse skin. Anat Rec 210:569–573, 1984
29. Thomson TM, Real FX, Murakami S, Cordon-Cardo C, Old LJ, Houghton AN: Differentiation antigens of melanocytes and melanoma: analysis of melanosome and cell surface markers of human pigmented cells with monoclonal antibodies. J Invest Dermatol 90:459–466, 1988
30. Burnett JB, Holstein TJ, Quevedo WC: Electrophoretic variations of tyrosinase in follicular melanocytes during the hair growth cycle in mice. J Exp Zool 171:369–376, 1969
31. Sugiyama S: Mode of redifferentiation and melanogenesis of melanocytes in mouse hair follicles. J Ultrastruc Res 67:40–54, 1979
32. Laskin J, Piccinini L: Tyrosinase isozyme heterogeneity in differentiating B 16/C3 melanoma. J Biol Chem 261:16626–16635, 1986
33. Burchill SA, Virden R, Thody AJ: Regulation of tyrosinase synthesis and its processing in the hair follicular melanocytes of the mouse during eumelanogenesis and phaeomelanogenesis. J Invest Dermatol 93:236–240, 1989
34. Silver AF, Chase HB, Arsenault C: Early anagen initiated by plucking compared with early sponateous anagen. Adv Biol Skin 9:265–286, 1987
35. Pawelek A, Korner A: The biosynthesis of mammalian melanin. Am Sci 70:136–145, 1982
36. Slominski A, Moellmann G, Kuklinska E: L-tyrosine, L-dopa, and tyrosinase as positive regulators of the subcellular apparatus of melanogenesis in Bomirski Ab amelanotic melanoma cells. Pigment Cell Res 2:109–116, 1989
37. Halaban R, Moellmann G: Melanocyte-specific catalase as positive regulators of pigmentation. J Cell Biol 109:57a, 1989
38. Muller G, Ruppert S, Schmidt E, Schutz G: Functional analysis of alternatively spliced tyrosinase gene transcipts. EMBO J 7:2723–2730, 1988
39. Halaban R, Moellmann G, Tamura A, Kwon BS, Kuklinska E, Pomerantz SH, Lerner AB: Tyrosinases of murine melanocytes with mutations at the albino locus. Proc Natl Acad Sci USA 85:7241–7245, 1988
40. Hearing V, Jimenez M: Mammalian tyrosinase—the critical regulatory point in melanocyte pigmentation. Int J Biochem 19:1141–1147, 1987
41. Wong G, Pawelek J: Melanocyte-stimulating hormone promotes activation of pre-existing molecules in Cloudman S91 melanoma cells. Nature (London) 255:644–646, 1975
42. Slominski A, Bomirski, Scislowski PWD, Zolnierowicz S: Effects of actinomycin D and cycloheximide on the increase in tyrosinase activity of hamster amelanotic melanoma cells cultured in vitro. Biosci Rep 4:1059–1064, 1984
43. Tamate HB, Hirobe T, Wakamatsu K, Ito S, Shibahara S, Ishikawa K: Levels of tyrosinase and its mRNA in coat-color mutants of C57BL/10J congenic mice: effects of genic substitution at the agouti, brown, albino, dilute, and pink-eyed dilution loci. J Exp Zool 250:304–311, 1989
44. Chase HB: Induced melanocyte deactivation and reactivation in hair follicles. Adv Biol Skin 8:503–507, 1966
45. Gilbert SF (ed.): Developmental Biology. Sinauer Assoc., Sunderland, MA, 1988
46. Campbell S: Melanogenesis of avian neural crest cells in vitro is influenced by external cues in the periorbital mesenchyme. Development 106:717–726, 1989
47. Mayer TC: Tissue environmental influences on the development of melanoblasts in Steel mice. In Slavkin HC, Greulich RC (eds.). Extracellular matrix influences on gene expression. Academic Press, New York, 1976, pp 555–560
48. Deluca M, D'Anna F, Bondanza S, Franzi AT, Cancedda R: Human epithelial cells induce human melanocyte growth in vitro but only skin keratinocytes regulate its proper differentiation in the absence of dermis. J Cell Biol 46:176–180, 1988
49. Halaban R, Langdon R, Birchall N, Cuono C, Baird A, Scott G, Moellmann G, McGuire J: Basic fibroblast growth factor from human keratinocytes is a natural mitogen for melanocytes. J Cell Biol 107:1611–1619, 1988
50. Prunieras M: Interaction between keratinocytes and dendritic cells. J Invest Dermatol 52:1–17, 1969
51. Drager UC, Balkema GW: Does melanin do more than protect from light? Neurosci Res Suppl 6:S75–S86, 1987
52. Horcicko J, Borovansky J, Duchon J, Prochazkova B: Distribution of zinc and copper in pigmented tissues. Hoppe-Seyler's Z Physiol Chem Bd 354:S203–204, 1973
53. Aula P, Kaila T, Huupponen R, Salminen L, Lisalo E: Prostaglandin F_2 binding to bovine ocular and synthetic melanins in vitro. Pharmacol Toxicol 65:100–103, 1989
54. Slominski A, Paus R: Are L-tyrosine and L-dopa hormone-like bioregulators? J Theor Biol 143:123–138, 1990
55. Steel KP, Barkway C: Another role for melanocytes: their importance for normal stria vascular is development in the mammalian inner ear. Development 107:453–463, 1989

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