# Hair Pigmentation: A Research Update

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**Hair is a uniquely mammalian trait with important functions, most easily appreciated in furred mammals. Our skin and hair color contribute very significantly to our overall visual appearance by highlighting striking variations between human sub-groups. Although melanins, hemoglobins, and carotenoids define the color perceived at the skin surface, our hair color relies only on the presence or absence of different melanins. The hair shaft's physical aspects provide only minor color modification. Various selective evolutionary pressures have determined that within the context of our specific ethnic backgrounds a bewildering array of natural shades are seen; ranging from yellows, reds, and browns to black and that harbinger of lost youth, gray/white hair. Skin/hair follicle melanins are formed in cytoplasmic organelles called melano-*somes* produced by neural crest-derived pigment cells called melano-*cytes* and are the product of a complex, phylogenetically ancient, biochemical pathway called melano-*genesis*. The following provides a review of research presented at the *4th Intercontinental Meeting of Hair Research Societies 2004* and so is not intended to represent a fully comprehensive overview of the subject—for that readers are directed to key references.**

Key words: hair follicle/melanocyte/pro-opiomelanocortin/hair cycle/nitric oxide synthase
J Investig Dermatol Symp Proc 10:275–279, 2005

## Development of the Hair Follicle Pigmentary Unit

By 7 wk estimated gestational age (EGA) melanocytes are already present in the human epidermis and remain there until hair morphogenesis begins, approximately 2 wk later (cf. Tobin and Paus, 2001). With the onset of hair follicle morphogenesis, some melanoblasts leave the epidermis and distribute randomly as dopa-positive and dopa-negative cells in the forming hair follicle and occasionally in the sebaceous glands. Melanogenic melanocytes can be detected in all stages of human hair morphogenesis from the hair germ stage onwards, usually confined to the peripheral regions of the hair follicle. Once the hair fiber has formed, melanocytes are noted in large numbers near the basal lamina around the apex of the follicular papilla. Although melanocyte mitosis is observed in the human epidermis at 14 wk EGA, mitosis of pigment cells is rarely observed in the hair follicles themselves.

Early work by Nishikawa *et al* (1991) and Yoshida *et al* (1996) demonstrated an important role for c-kit in melanocyte development. C-kit is required for melanocyte migration in the dermis (along with endothelin 3), but not just before their entry into the epidermis. C-kit signaling is again needed for their proliferation upon entering the epidermis (with the endothelin receptor B but not endothelin 3), but this is followed by a c-kit-independent stage upon entry into the developing hair follicles. C-kit is thereafter required for melanocyte activation during reconstruction of the hair follicle pigmentary unit life.

More recently it has been shown that melanoblasts expressing c-Kit migrate into the stem cell factor (SCF)-supplying hair follicle epithelium and that differentiated c-Kit-positive melanocytes target the bulb when "SCF positive" (Peters *et al*, 2002). C-Kit-negative melanoblasts invade the outer root sheath and bulge in the fully developed murine hair follicle. Postnatally, c-Kit is required during the hair growth cycle for activation of melanocyte, although the stem cell compartment appears to exhibit SCF/c-kit independence. The cutaneous expression of SCF (produced by fibroblasts, keratinocytes, and endothelial cells) and of its receptor c-Kit has recently been re-evaluated (Peters *et al*, 2003). Mast cells and melanocytes were thought to be the only cutaneous cell types that express c-Kit and so to respond to SCF. Recently, Peters and colleagues reported the rather unexpected and exciting finding that Kit expression in murine anagen hair follicles is not restricted to the pigmentary unit, but is also present in desmoplakin + /Trp-1-negative cells (identified as keratinocytes). Indeed, these c-kit-positive cells were also found in melanocyte-deficient hair follicles $Kit^{Sl}/Kit^{Sl\text{-}d}$ mice, and in Kit-neutralizing antibody-treated C57BL/6 mice. Moreover, $Kit^{Sl}/Kit^{Sl\text{-}d}$ mice unable to respond to SCF stimulation showed a significant retardation of anagen development compared with their wild-type littermates. Thus, expression of Kit in the most rapidly proliferating compartment of the hair follicle epithelium suggests intriguing, as yet unexplored, functions of Kit signaling in epithelial cell biology.

Abbreviations: BMP, bone morphogenic proteins; CRH, corticotrophin-releasing hormone; MC1-R, melanocortin 1-receptor; MSH, melanocyte stimulating hormone; NO, nitric oxide; NOS, NO synthase; POMC, pro-opiomelanocortin; SCF, stem cell factor

There is some evidence that fibroblast-like cells of the follicular dermal papilla may secrete more SCF than dermal fibroblasts and that follicular dermal papilla cells from beard hair follicles may secrete more SCF than androgen-insensitive scalp hair follicles (Hibberts *et al*, 1996). Two different splice variants for SCF encode for either a soluble or a membrane-bound form. Dr Tayyebeh Vafaee (Dr Valarie Randall's Group) reported at this workshop that human hair follicles, especially their lower part, express the genes for both the soluble and membrane-bound forms of SCF. These workers did not comment on the expression of the SCF isoforms at the protein level, and so it is not yet clear how this observation may affect the biology of the hair follicle including pigmentation. Other researchers have shown that soluble and membrane-bound SCF may even have opposite functional effects.

## Distribution and Antigen Expression of Hair Follicle Melanocytes

Although follicular melanocytes are derived from epidermal melanocytes during hair follicle morphogenesis, these pigment cell sub-populations diverge in many important ways as they distribute to their respective distinct compartments. Thus the follicular-melanin unit resides in the "immune privileged" proximal hair bulb (cf. Tobin and Paus, 2001) and consists of one melanocyte for every five keratinocytes in the hair bulb as a whole; the ratio is 1:1 in the basal epithelial layer next to the dermal papilla. Hair bulb melanotic melanocytes differ from epidermal melanocytes in that they are larger, with longer and more extensive dendrites, containing more developed Golgi and rough endoplasmic reticulum, and produce 2–4-fold larger melanosomes. Moreover, whereas melanin degrades almost completely in the differentiating layers of the epidermis, eumelanin granules transferred into hair cortical keratinocytes remain minimally digested.

Melanocytes in the fully developed anagen follicle can be assigned to five distinct anatomic compartments. Dopa-positive melanotic melanocytes are readily detectable in the outer root sheath of the infundibulum, basal layer of sebaceous gland, and around the upper dermal papilla. Dopa-negative amelanotic melanocytes are detectable in the mid-to-lower outer root sheath, and the amelanotic dopa-negative melanocytes are distributed in the periphery of the bulb and most proximal matrix. The melanocyte stem in murine hair follicles has been localized to the bulge region (Nishimura *et al*, 2002). Immature melanocytes can be clearly demonstrated in the adult hair follicle (Tobin and Paus, 2001; Slominski *et al*, 2004b). Immunostaining with NKI-beteb, a monoclonal antibody that detects the (pre)melanosome glycoprotein gp100, detects all dopa-positive cells and also highlights some dopa-negative melanocytes of the mid-outer root sheath. Although the dopa-oxidase activity of tyrosinase is not detectable in amelanotic hair follicle melanocytes, the protein itself may be detected in some of these cells. Similarly, whereas Kit and Bcl-2 reactive melanocytes are present in this hair follicle compartment, these amelanotic melanocytes do not express the melanogenic enzymes TRP-1 and TRP-2 (also called dopachrome tautomerase). 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, NKI-beteb$^+$, pigment cells (cf. Tobin and Paus, 2001; Slominski *et al*, 2004a, b). It has been speculated that amelanotic hair bulb melanocytes may represent a pool of "transient" melanocytes that migrate from precursor melanocytes stores in the upper outer root sheath. The restriction of melanogenically active melanocytes to the upper hair matrix of the anagen hair follicle, just below pre-cortical keratinocytes, correlates with the observation that melanin is transferred during anagen to the hair shaft cortex**,** less so to the medulla and rarely to the hair cuticle.

## Hair Follicle Melanogenesis Is Coupled to the Hair Growth Cycle

Activity of the hair bulb melanocyte is under cyclical control and melanogenesis is tightly coupled to the hair growth cycle (cf. Slominski *et al*, 2004a, b), in contrast to epidermal melanogenesis, which appears to be continuous. Hair grows cyclically through a finite period of hair shaft formation (anagen $\sim$ 3–5 y in human scalp), a brief regression phase resulting in the apoptosis-driven resorption of up to 70% of the hair follicle (catagen $\sim$ 2 wk in human scalp), and a relatively quiescent period (telogen $\sim$ 3 mo in human scalp). Even before catagen-associated structural changes, towards the end of anagen VI, the earliest signs of imminent hair follicle regression become apparent, e.g., retraction of melanocyte dendrites and attenuation of melanogenesis. Keratinocyte proliferation, however, continues for some time and the most proximal telogen hair shaft remains unpigmented. The melanogenically active melanocytes of the anagen phase are no longer detectable during catagen. The hair bulb melanocyte system has long been viewed as self-perpetuating, whereby melanocytes involved in the pigmentation of one hair generation are also involved in the pigmentation of the next. But melanocytes would need to survive/avoid the extensive apoptosis-driven regression of the hair bulb. Recently we have found that many highly melanotic hair bulb melanocytes do not survive catagen (cf. Tobin and Paus, 2001; Slominski *et al*, 2004a, b). Thus, it now appears that "re-differentiating" melanocytes in early anagen are more likely to correspond to newly recruited immature melanocytes derived from a melanocyte reservoir or stem cell pool (Tobin *et al,* 1999; Nishimura *et al*, 2002) located in the upper, "permanent," outer root sheath (Commo and Bernard, 2000). This view is supported by the observation that immature melanocytes are located very close to the secondary epithelial germ of the telogen club where they are commonly small, have high nuclear to cytoplasmic ratios, and inactive cytoplasm with very few organelles.

Melanin synthesis is not detected by histologic or histochemical examination in telogen hair follicles, but very low tyrosine hydroxylase activity of tyrosinase can be detected; this disappears on days 1 and 2 after anagen induction in mice (cf. Slominski *et al*, 2004a, b). Undifferentiated melanocytes/melanoblasts of the telogen germ are stimulated

at the start of anagen and respond by increasing their cell volume. 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 pre-cortical keratinocytes. Melanocytes in the S-phase of the cell cycle occur as early as anagen II and significant proliferation is clearly apparent by anagen III (Commo and Bernard, 2000). Mitosis is also observed in melanogenically active cells, indicating that melanocyte differentiation does not preclude mitotic activity.

In the murine telogen hair follicle some melanocytes/melanoblasts from the secondary germ are immuno-histochemically positive for TRP-2; of these, a subpopulation also expresses c-Kit. All telogen melanocytes are mitotically quiescent as assessed by Ki67 immunostaining. During anagen I, tyrosinase mRNA and tyrosinase protein become detectable, but barely [and melanin] TRP-1, and tyrosine hydroxylase and dopa oxidase activities of tyrosinase remain undetectable at the biochemical level. TRP-2-positive melanocyte precursor cells proliferate extensively at the onset of follicle development and thereafter assume a more differentiated status that is associated with the onset of TRP-1 expression and with the reduction of their proliferation (Botchkareva *et al*, 2003). At the same time, melanocytes residing at the site of the presumptive stem cell reservoir in the infundibulum remain TRP-1 negative, whereas some cells expressing TRP-1 or TRP-2 together with c-Kit may show proliferative activity (Botchkareva *et al*, 2001).

During murine anagen II tyrosine hydroxylase and dopa oxidase activities of tyrosinase, tyrosinase mRNA, protein, TRP-1, and melanin are readily detectable, to increasing rapidly during anagen IIIc (day 5 after anagen induction) and reaching their highest levels during anagen V and early anagen VI (days 8–12) (cf. Slominski *et al*, 2004a, b). By anagen IV, when the hair pigmentary unit becomes fully functional with respect to melanin synthesis, melanocytes distribute into discrete locations throughout the hair follicle. In murine hair follicles, melanocytes localized to the HF bulge (site of presumptive reservoir) express only TRP-2, lacking TRP-1, c-Kit, and Ki67 immunoreactivities. Melanocytes located in the elongating outer root sheath are TRP-2 + and c-Kit + and in some cases are also positive for the proliferation marker Ki67, but express little TRP-1 and no tyrosinase (Botchkareva *et al*, 2001). Only melanocytes distributing to the hair follicle melanogenic zone, i.e., the matrix above the dermal papilla, express TRP-1, TRP-2 and tyrosinase, c-Kit, and also Ki67 in the majority of cells. Both activity and concentration of tyrosinase remain steady during mid-to-late anagen VI (days 12–17), and decrease rapidly during the anagen VI/catagen transition phase to become undetectable or very low in catagen. Melanocytes from the fully developed anagen VI murine hair follicles express TRP-2 only when located in the hair bulge, TRP-2 and c-Kit only when located in the outer root sheath, and all three proteins, together with c-Kit, when located in the hair bulb matrix. Melanocyte proliferation, however, ceases by this sub-stage of anagen. The physiologic decrease in follicular melanogenesis at the end of anagen may reflect an exhaustion of an active signaling system that stimulates melanogenesis (late anagen VI), and/or production of inhibitors of melanocyte activity. Active depletion of differentiated hair bulb melanocytes via apoptosis also contributes to termination of melanogenesis during late anagen/catagen transition phase (cf. Tobin and Paus, 2001; Slominski *et al*, 2004b).

Null mutations and deletions at the loci for tyrosinase and TRP-1 phenotypically affect coat color because of absence of enzyme activity or because of intracellular mislocalization. Mutations (although not null mutations) have been described at the TRP-2/Dct locus, which also lead to a pigmentation phenotype. A forced TRP-2 null mutation has been reported to result in a diluted coat color phenotype with reduced melanin content. Although tyrosinase is absolutely required for melanogenesis, other melanosomal proteins, such as TRP-1, TRP-2, and gp100, play important roles in regulating mammalian pigmentation. For example, whereas TRP-1 can oxidize 5,6-dihydroxyindole 2-carboxylic acid (DHICA) in mice but not humans, TRP-1 may play an important role in stabilizing tyrosinase in both species.

During this workshop Dr Stéphane Commo (Dr Bruno Bernard's Group) reported the rather unexpected finding that, in contrast to mice, melanogenic melanocytes of the human anagen hair bulb (at least of those hair follicles producing eumelanic, i.e. brown and black hair fibers, irrespective of ethic origin) do not express TRP-2 at the protein level. Moreover, whereas only a faint mRNA signal was detected in hair bulb material, Dr Commo, like Tobin and colleagues, demonstrated TRP-2 expression in hair follicle-derived melanocytes *in vitro*. By contrast, melanogenic melanocytes located in the epidermis of these donors expressed TRP-2. Whether TRP-2 expression is present in hair follicle melanocytes producing predominantly pheomelanic fibers is not clear. One possible implication of this finding is that the TRP-2 associated rearrangement of L-dopachrome to DHICA to produce DHICA melanins is less critical here than in the epidermis, or that in hair bulb melanocytes may exhibit the spontaneous rearrangement of L-dopachrome leads to 5,6-dihydroxyindole alone. Moreover, the absence of TRP-2 in this compartment may make hair bulb melanocytes less resistant to the cytotoxic action of decarboxylated indoles. The basis for the species difference (i.e. mouse *vs* human) in TRP-2 expression in hair bulb melanocytes is not immediately clear, although it is interesting to note that the levels of TRP-2 in extracts of human melanocytic cells have previously been described as being relatively low compared with those in murine melanocytes (Kameyama *et al*, 1995).

## Hormonal Regulators of Hair Follicle Melanogenesis

The search for local regulators of follicular melanogenesis has generated significant interest among pigment cell biologists, particularly those that fluctuate during the hair cycle and are coupled to the early anagen onset of melanogenesis in bulbar melanocytes. Pro-opiomelanocortin (POMC) products have been proposed as the main regulators of

follicular pigmentation (Tobin and Paus, 2001; Kauser *et al*, 2004; Slominski *et al*, 2004b). Studies in guinea-pigs have shown that α-melanocyte stimulating hormone (MSH) increased the proportion of black to gray hairs when administered intramuscularly. But the injection of both α-MSH and a potent synthetic analog [Nle$^4$,*D*-Phe$^7$]-α-MSH into human skin results in increased melanogenesis particularly of sun-exposed skin. No effect, however, was seen in hair follicles. We have recently found that the expression of α-MSH is very low to undetectable in pigmented hair bulb melanocytes *versus* epidermal counterparts both *in vivo* and *in vitro* (Kauser *et al*, 2005).[1] But it is likely that the melanocortin 1-receptor (MC1-R), the product of the extension (e) locus and cognate receptor of α-MSH, is an important positive regulator of hair pigmentation. This G protein-coupled membrane receptor is activated upon binding of POMC-derived ACTH and α-MSH peptides (cf. Slominski *et al*, 2004a, b). The resultant signal transduction cascade results in the activation of adenylate cyclase activity, subsequent cAMP production, and results in increased melanocyte proliferation, melanogenesis, and dendrite formation.

The expression and translation of POMC gene products are expressed in the skin in a hair cycle-dependent manner, being low at telogen and high during anagen development (cf. Slominski *et al*, 2004a, b). In murine skin, the expression of ACTH, α-MSH, β-endorphin, and MC1-R fluctuates during cycling, suggesting that these peptides may be involved in the reconstitution of the follicle pigmentary unit and perhaps also in the regulation of hair growth itself. There is particular interest in the pro-eumelanogenic peptide α-MSH because mutations in extension (e) [MC1-R] or agouti (a) [ASP] loci affect murine follicular melanocytes. Moreover, accumulation of POMC products in scalp outer root sheath follicular keratinocytes in the scalp and less so in the overlying epidermis suggests that the activity of the local POMC/MC1-R axis plays an important role in the physiologic regulation of anagen-associated hair pigmentation. It is noteworthy that MC1-R gene polymorphisms have been strongly linked to red hair and fair skin in humans (cf. Tobin and Paus, 2001; Slominski *et al*, 2004b).

The existence of a POMC complementary system, namely the β-endorphin/μ-opiate receptor system (see Tobin and Kauser in this issue, pp. x–x), has recently been shown to influence human epidermal and follicular melanocyte biology by inducing changes in melanocyte dendricity, proliferation, and melanogenesis (Kauser *et al*, 2004). Dr Söbia Kauser (Dr Desmond Tobin's Group) at this workshop presented a study examining the role of corticotrophin-releasing hormone (CRH)—the most proximal element of the hypothalamic–adrenal axis (HPA) and chief regulator of pituitary POMC gene and peptide expression—in human hair follicle biology and in hair pigmentation. She showed that CRH can modulate follicular melanocyte behavior and possibly also hair growth. There is increasing evidence to suggest that mammalian skin contains an equivalent of the HPA axis, composed of locally produced CRH that together with signaling via CRH receptors (CRH-Rs) 1 and 2 may regulate skin homeostasis (Ito *et al*, 2005; Slominski *et al*, 2004a). Dr Kauser presented evidence that specific CRH and urocortin analogs, designed to variably activate CRH-R1 or -R2, can have variable effects on melanogenesis, dendricity, and proliferation.[1]

Other positive regulators of melanogenesis include the bone morphogenic proteins (BMP). Originally discovered as an osteo-inductive extract derived from bone matrix, the BMP consist of a family of dimeric proteins (BMP-2–15) that are secreted as signaling molecules to regulate several aspects of cutaneous development and function via their participation in cell proliferation, differentiation, and death. BMP and their associated downstream transcription factors also influence neural crest-derived cell populations, including melanocytes, in several species. BMP-2 specifically targets tyrosinase gene expression in primary quail neural crest cultures resulting in increased melanin synthesis. Furthermore, BMP-4 may stimulate proliferation of normal human melanocytes in culture, likely via the proliferation-associated receptor, BMPR-IA. BMP-4 treatment of human cutaneous melanocytes reduces the expression of mRNA and protein for tyrosinase and the tyrosinase activator PKC-β. Production of BMP-4 is greater in melanocytes than keratinocytes, suggesting that this BMP may be an autocrine factor for melanocytes. At this workshop Dr Michael Fessing (Dr Vladimir Botchkarev's Group) reported hair pigmentation abnormalities in Noggin transgenic mice (Sharov *et al*, 2003). Overexpression of Noggin, an antagonist of BMP-2 and BMP-4, in agouti mice resulted in a wide range of phenotypes including accelerated hair follicle development, upregulation of keratinocyte proliferation and downregulation of apoptosis in the hair bulb. In addition, these mice exhibited increased eumelanogenesis in hair bulb melanocytes that replaced the normal agouti pheomelanogenesis that occurs in wild-type mice. Thus, the switch between eumelanogenesis and pheomelanogenesis may, in part, be regulated by the BMP signaling pathway. There is also some evidence that BMP-4 can stimulate proliferation and inhibit melanogenesis in cultured human epidermal melanocytes.[2]

Melanocytes in the epidermis express BCL-2, an anti-apoptotic oncogene associated with cell survival. Follicular melanocytes, especially those located in the outer root sheath, also express this survival factor. It has been proposed that BCL-2 inhibits cell death particularly in areas where reactive oxygen species are generated (e.g., melanogenesis) by regulating anti-oxidant pathways. Moreover, UV-induced apoptosis of melanocytes *in vitro* can be blocked via nerve growth factor-induced upregulation of BCL-2 (cf. Slominski *et al*, 2004a). Nitric oxide (NO) is a potential source of oxidative stress in skin. At this workshop Ms Heather Sowden (Dr Desmond Tobin's Group) presented a comparative assessment of NO synthase (NOS) expression in the pigmentary units of the human scalp epidermis and hair follicle during the hair cycle. NO, a lipophillic free radical, is synthesized from the oxidation of L-arginine by NOS. NOS include the constitutive isoforms eNOS and nNOS which produce nM amounts of NO to exert physiologic roles, e.g. vasodilation/neurotransmission, and the

[1] Kauser *et al* (submitted).

[2] Yaar M, Park HY, Botchkarev V, Stewart K, Panova I, Gilchrest BA: Bone morphogenetic protein 4 modulates melanocyte proliferation and melanogenesis. J Invest Dermatol 119:1;337, 2002 (abstr).

inducible iNOS that produces NO in mM amounts in response to stimuli like LPS and is associated with both beneficial and detrimental effects. Moreover, a common tissue marker of radical-mediated damage is nitro-tyrosine (reaction of NO with superoxide anion (peroxynitrite)) expression. Recently, a role for NO in cutaneous physiology has been advanced including in vasodilation, wound healing, and melanogenesis (cf. Slominski *et al,* 2004a). Ms Sowden showed that epidermal melanocytes *in situ* express both eNOS and bNOS (but not iNOS) whereas no eNOS or nNOS could be detected in hair bulb melanocytes during anagen (Sowden *et al*, 2005).[3] The constitutive NOS isoforms were expressed, however, in weakly pigmented and/or amelanotic melanocytes of the outer root sheath and sebaceous gland. Similar compartmentalization was also evident for nitrotyrosine expression. These opposing expression patterns in the epidermal and follicular pigmentary units may reflect UVR stimulation of melanocytes in the epidermis but not those in the proximal hair bulb. Interestingly, NO synthase and nitrotyrosine expression was modulated by the hair growth cycle. There are several potentially important implications of these findings. For example, there is evidence that eNOS-derived NO may protect against apoptosis, for example via the induction of bcl-2 expression. Thus, eNOS expression is highest in apoptosis-resistant epidermal melanocytes but low/undetectable in hair bulb melanocytes that can be susceptible to apoptosis. Thus, different compartments of the skin and hair follicle may have different regulation of the cutaneous NOS/NO system (Sowden *et al,* 2005).[2]

In conclusion, several difference aspects of hair follicle melanocyte behavior were featured in this very interesting workshop at the 2004 IMHRS meeting, which add to our steady dissection of differences between these two cutaneous pigment cell populations. It is likely that exploitation of these differences will enable us to differentially regulate activity of these melanocyte sub-types to influence pigmentation of the skin and hair follicle. The chairs would like to thank all the speakers for their stimulating presentations and the audience for their active participation.

DOI: 10.1111/j.1087-0024.2005.10117.x

Manuscript received September 20, 2004; revised November 22, 2004; accepted for publication December 2, 2004

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