Biochemical Control of Melanogenesis and Melanosomal Organization

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Current knowledge on the regulation of mammalian pigmentation at the genetic and biochemical level, and constituents that participate in melanosomal organization, is summarized. Approximately 25% of the more than 80 genes known to regulate pigmentation in mammals have been cloned and characterized to date. Almost half of those encode proteins that localize, either specifically or nonspecifically, to melanosomes; mutations in those genes generally lead to phenotypic changes in pigmentation as well as in other pleiotropic changes. The expression and function of these proteins not only affects phenotypic appearance, but also the properties of melanins, especially their photoprotective characteristics. Because many of those melanosomal proteins also serve as melanoma-specific targets, regulation of their expression has dramatic implications for immune targeting of malignant melanoma. *Key words: melanin/melanoma/photoabsorption/pigmentation. Journal of Investigative Dermatology Symposium Proceedings 4:24–28, 1999*

Melanins are unique pigmented biopolymers synthesized by specialized cells known as melanocytes, dendritic cells that exist as relatively minor populations in the skin, hair, eyes and other locations (as reviewed by Spritz and Hearing, 1994; King *et al*, 1995, 1997; Hearing, 1997). Melanins are produced and deposited within discrete membrane-bound organelles known as melanosomes, and visible pigmentation depends on the size, number, shape and distribution of those melanosomes as well on the chemical nature of the melanins deposited within them. In the skin, melanocytes residing at the dermal–epidermal border propel melanosomes they produce into their dendrites as they mature and then transfer them to surrounding proliferating keratinocytes. Movement of these keratinocytes towards the surface of the skin, and the distribution of their ingested melanosomes, eventually yields visible skin color. Similarly, melanocytes responsible for hair pigmentation reside deep within hair bulbs in the dermis. Again, they are a relatively minor cell population that produce melanosomes and transfer them to the growing hair shaft; the processed and distributed melanosomes in the emerging hair shaft give it color.

Melanin has a number of important and distinct functions, ranging from its role in the determination of phenotypic appearance (Ortonne and Prota, 1993), to protective coloration (Jackson, 1991, 1993), to balance and auditory processing (Creel *et al*, 1983, 1990), to absorption of toxic drugs and chemicals (Lindquist, 1973; Larsson, 1995), and to neurologic development during embryogenesis (Proctor, 1976; King *et al*, 1985; Creel *et al*, 1986). With respect to the topic of this Symposium, however, melanin's most relevant function is protection from ultraviolet light, and thus prevention of UV-induced photodamage, photoaging, and photocarcinogenesis.

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Abbreviations: ASP, agouti signal protein; DHI, 5,6-dihydroxyindole; DHICA; DHI-2-carboxylic acid; MSH, melanocyte stimulating hormone; TRP, tyrosinase related protein.

It should be obvious from the comments above that many different types of cells interact to determine pigmented phenotype, and consequently that regulation of melanogenesis is a complex process. In fact, the number of genes involved in regulating mammalian pigmentation is quite large, and at least 80 genetic loci regulate melanogenesis either directly or indirectly. At this moment, 21 of those genes have been cloned, and in most instances, analogous genes have been cloned and at least partially characterized functionally in mice and in humans. So far, virtually all of them have comparable functions in both species, and in many instances, mutations in those genes have been shown to be associated with different human pigmentary diseases, including various forms of ocular and oculocutaneous albinism (OCA), piebaldism, Hirschsprung's disease, and Waardenberg's syndrome.

Genes that regulate mammalian pigmentation act at the tissue, the cellular, the subcellular, and/or the environmental level. To get appropriate pigmentation and patterning, melanoblasts must originate in the neural crest during embryologic development, and they must be given appropriate signals, first to mature and then to migrate in distinct fashions throughout the organism. Obviously, once melanoblasts have arrived at their intended destinations, they must receive the appropriate signal(s) to stop migrating and to then differentiate into melanocytes. Mature melanocytes must of course then be able to respond to environmental signals, and they must be competent to produce melanosomes and the melanins within them, and to then transfer them to keratinocytes. It is not surprising then that there are a relatively large number of genes involved in the regulation of these diverse processes, and mutations in genes important to each of those steps have been identified that interfere with normal pigmentation. Inherited mutations of those genes are responsible for many of the known clinical pigmentary abnormalities.

Time and space does not permit a full discussion of these genes and their functions, but briefly, pigment genes that function at the tissue level affect the eventual distribution of melanocytes in tissues; they typically encode transcription factors or growth factors (or their receptors) important to melanoblast function. Genes cloned to date that fall into this category include the *splotch*, *dom*, *white-spotting*, *steel*, and *patch* loci. A more thorough discussion and references to original articles of the pigment related genes can be found in the following

Table I. Genes encoding melanosome proteins/associated diseases[a]

Mouse locus	Human disease	Encoded protein
Genes encoding melanosome specific proteins		
albino	OCA1	tyrosinase – melanogenic enzyme
brown	OCA3	TRP*1*/Tyrp*1* – melanogenic enzyme
MART1	Vogt-Koyanagi–Harada syndrome	MART*1* – melanosome membrane protein
*OA*1	ocular albinism – type 1	OA1 – melanosome membrane protein
pinkeyed-dilution	OCA2	p protein – membrane transporter?
silver	hair silvering?	Pmel17 – melanosome matrix protein
slaty	OCA4?	TRP2/Dct – melanogenic enzyme
Genes encoding lysosome/platelet/melanosome specific proteins		
ashen	unknown	RAB-related GTPase
beige	Chediak–Higashi syndrome	LYST lysosomal membrane protein
dilute	Griscelli disease	MYH*12* myosin type V
mocha	unknown	δ subunit AP-3 complex
mottled	Menkes disease	ATP7 A copper transporter
pale ear	Hermansky–Pudlak syndrome	HPS lysosomal membrane protein
pallid	platelet storage pool disease	protein 4.2 pallidin
pearl	unknown	β subunit AP-3 complex

[a]cf. Spritz and Hearing (1994), King *et al* (1997), and Hearing (1998) for full references.

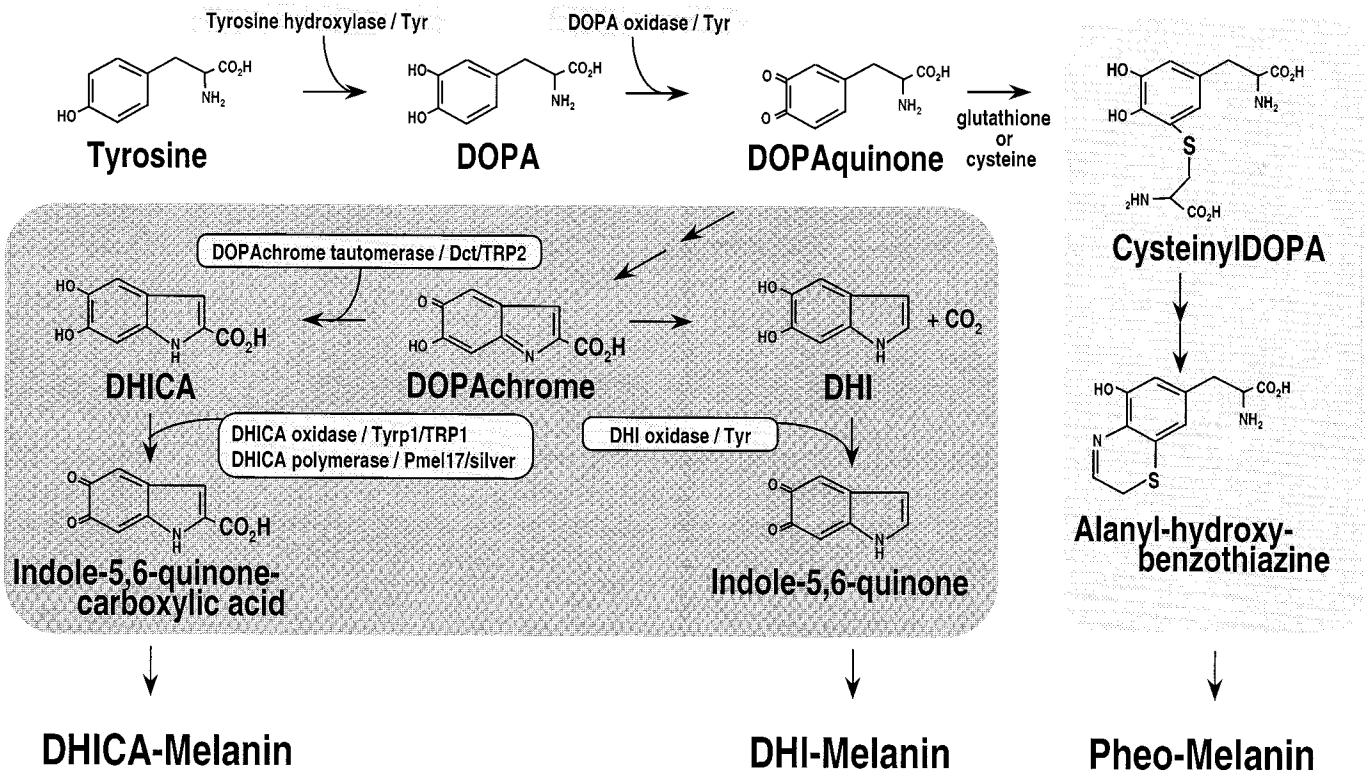

Figure 1. The reaction cascade of the melanogenic pathway. cf. Hearing, 1998 for complete references.

review articles (Bennett, 1991, 1993; Spritz and Hearing, 1994; King *et al*, 1995, 1997; Hearing, 1998). It must be borne in mind, however, that the ultimate distribution of melanin particles in the upper layers of the skin are critically important to the efficacy of photoprotection of that tissue. Although the various types of melanins as discussed below are chemically determined and synthesized in melanocytes, it is their transfer and processing in keratinocytes *en route* to the surface of the skin that ultimately provides effective photoprotection from UV light (Kaidbey *et al*, 1979; Prota, 1994; Jimbow *et al*, 1995; Kobayashi *et al*, 1998).

Pigment genes that function at the cellular level include genes that affect whether melanocytes survive and/or proliferate once in place. Several genes have now been cloned that act at this level, either in events that determine the initial differentiation of melanoblasts to melanocytes, or in their subsequent survival and proliferation. Typically such genes include those that encode factors (e.g., endothelin or steel factor) that stimulate melanocyte differentiation or survival, such as the *microphthalmia*, *piebald lethal*, and *lethal spotting* loci. Other genes in this category encode proteins required for pigmentation, but not necessarily restricted to pigmentation, e.g., those that encode subcellular organelle membrane proteins (i.e., proteins functional in organelles of the melanosome/lysosome/platelet lineage). Genes that fall into this category include those at the *ashen*, *beige*, *dilute*, *mocha*, *mottled*, *pale ear*, *pallid*, and *pearl* loci. Mutations in these genes typically lead to abnormal pigmentation because of the dysfunctional melanosomes produced, but also lead to pleiotropic effects due to malfunction of other related organelles (such as lysosomes and/or platelets) that also require those gene products for their function.

Pigment genes that are expressed specifically in melanocytes fall into the group that function at the subcellular level; they are genes that encode typically melanosome-specific proteins – melanosomes being the specific organelle produced only by melanocytes. Seven genes that

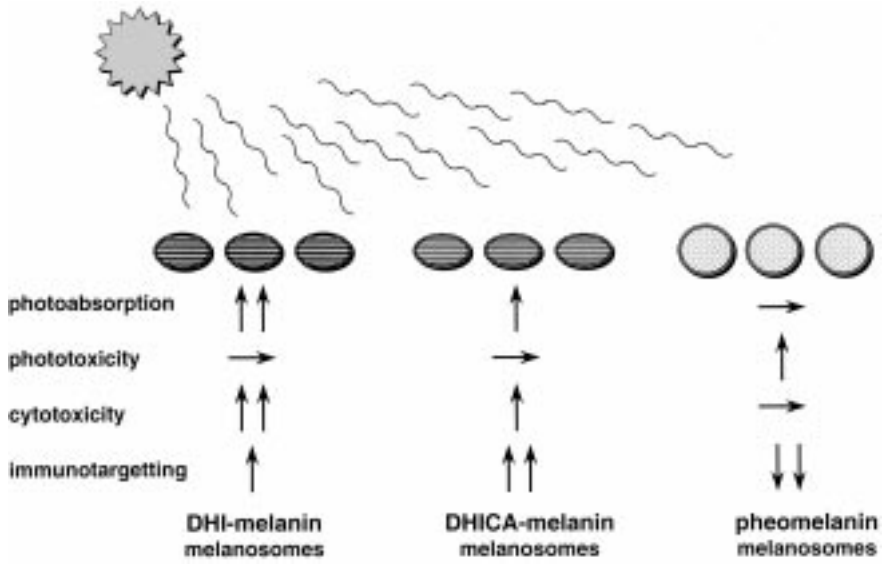

Figure 2. Properties of biologic melanins in ultraviolet photoabsorption. →, no change or no function; ↑, significant increase in function; ↑↑, very dramatic increase in function; ↓, significant decrease in function; ↓↓, very dramatic decrease in function.

function at this level have been cloned so far, and they encode melanosomal proteins that are functional enzymatically or structurally. These are discussed in more detail below, but include those at the *albino*, *brown*, *MART1*, *OA1*, *pinkeyed-dilution*, *slaty*, and *silver* loci. Genes that encode proteins found in melanosomes (either specifically or nonspecifically) are listed in **Table I**, along with their putative functions and associated pigmentary diseases.

In the final group are genes that regulate melanocyte function as environmental factors. These include the *extension* locus (which encodes the MSH receptor), the *agouti* locus (which encodes the agouti signal protein), and the *piebald lethal* locus (which encodes the endothelin-1 receptor). All of these play important roles in modulating melanocyte function and thereby affecting pigment production. Of course, gene encoding the ligands for those receptors should also be included on this list; however, this is by necessity a very incomplete list at this time because a wide variety of physiologic factors are known to affect melanocyte proliferation and/or differentiation and will surely be added in the future. Included among such factors that are currently not listed are a number of cytokines, lymphokines, prostaglandins, vitamins, and growth factors known to modulate melanocyte function (reviewed in Hearing, 1998). Many of those factors are produced by melanocytes themselves (in an autocrine fashion), or by other cells in the epidermis (such as keratinocytes or Langerhans cells) and/or in the dermis (such as inflammatory cells, endothelial cells, or mast cells). Some of those signals regulate the type of melanin that is produced and/or how much of it is produced, whereas others affect the growth, dendricity, migration, or survival of melanocytes and thereby exert their effects on pigmentation. It is obvious that the melanocyte is in an extremely interactive environment and responds to signals coming from virtually every source and direction.

The sum of those factors regulate the amounts and types of melanins produced by melanocytes, although the exact chemical structures and properties of different melanins are not yet completely known. Tyrosine is the physiologic substrate required for melanin production and the initial catalytic function of tyrosinase, i.e., the hydroxylation of tyrosine to DOPA, is the essential and rate-limiting activity for melanin formation. Once produced, DOPA can auto-oxidize and cyclize spontaneously to produce 5,6-dihydroxyindole (DHI) melanin (**Fig 1**); however, tyrosinase is not the only melanogenic enzyme involved in the pathway and there are at least three other melanosomal proteins that in part determine melanin production. The first of these is DOPAchrome tautomerase, also known as tyrosinase related protein 2 (TRP2 or Dct); TRP2 functions specifically to tautomerize DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). DHICA is a melanogenic intermediate that still contains the carboxylic acid group, which is spontaneously lost in the absence of TRP2 catalytic activity upon which DHI is produced. Melanins generated *in vitro* from DHICA are brown in color, poorly soluble, and of intermediate molecular weight, whereas those generated from DHI derivatives are black, completely insoluble, and of high molecular weight. Yet another melanogenic enzyme, known as tyrosinase-related protein 1 (TRP1 or Tyrp1), functions as a DHICA oxidase, and promotes further oxidation and polymerization of DHICA-melanins. The product of the *silver* locus, known as the silver protein (or Pmel17), also functions in melanogenesis as a polymerase, possibly by serving as a solid-phase substrate upon which melanin polymerization proceeds. The DHI- and DHICA-melanins are not only distinctly different visibly, but have other important differences in their properties as well, as for example in their photoprotective and cytotoxic properties (reviewed in Prota, 1992). The DHI- and DHICA-melanins produced downstream of DOPAchrome are termed eumelanins.

The other major diversion in the melanogenic pathway occurs upstream in the pathway immediately following the production of DOPAquinone from DOPA. It is not yet known how this switch to produce pheomelanin is regulated, but if a sulfhydryl donor, probably cysteine (Potterf *et al*, 1998), is available when DOPAquinone is generated, the latter will be stoichiometrically converted to cysteinyl-DOPA. Once this sulfur group has been incorporated into the melanin polymer, further oxidation, cyclization, and polymerization leads to the production of pheomelanin. Pheomelanins are yellowish-red in color, quite soluble, and of very low molecular weight. Mammals with yellow or bright red hair typically result from pheomelanin production, whereas brownish or black hair results from the production of eumelanins. It is important to realize that the melanocyte effectively determines, depending on whether cysteinylDOPA, DHI, and/or DHICA is made, which type of melanin is ultimately produced by further oxidation and polymerization, and this in turn dramatically affects the various physical properties of those melanins, including their photoprotective function (Jimbow *et al*, 1995; Pathak, 1995; Hill *et al*, 1997).

It is not yet completely clear how the switch to produce eu- or pheo-melanins is effected, although in mice it has been known for some time that interactions between MSH and ASP are critical to this (reviewed in Barsh, 1995, 1996). Conditions under which there is over-stimulation of MSH receptor function elicit eumelanin production and a black phenotype, whereas conversely, conditions under which function of the MSH receptor is abrogated or is overwhelmed by ASP overexpression, result in the production of pheomelanin. Recent studies (Valverde *et al*, 1995; Smith *et al*, 1998) have clearly shown that MSH and ASP function similarly in humans as they do in mice, although levels of response in human melanocytes may be muted compared with lower mammals.

Melanosomes have been known for some time to consist of 30 or more different proteins, some of them specific to this organelle, whereas

Table II. Melanosomal-specific proteins that elicit immune responses[a]

Disease (type of response)	gp100/Pmel17	tyrosinase	TRP1/gp75	TRP2/Dct	MART-1	B700
melanoma (humoral)				X		X
melanoma (cellular)	X	X	X	X	X	
vitiligo (humoral)	X	X	X	X		
Vogt-Koyanagi Harada (cellular)					X	

[a]cf. Hearing (1996) and Sakai *et al* (1997b) for pertinent references.

others are shared with other subcellular components such as lysosomes and platelets (Orlow, 1995). So far, genes encoding 15 such proteins have been cloned; these include the four melanogenic enzymes (tyrosinase, TRP1, TRP2, and silver) noted above. Three other melanosome-specific membrane proteins have also been cloned, encoded at the *MART1*, *OA1*, and *pinkeyed-dilution* loci; the functions of those proteins are still under investigation. A summary of currently cloned genes that encode melanosomal proteins (specific or nonspecific) is shown in **Table I**.

Our laboratory has been studying the level(s) at which MSH and ASP exert their dramatic effects on melanin production. If MSH is used to stimulate pigmentation, tyrosinase expression is dramatically increased but there is little or no change in TRP1, TRP2, or silver expression or function. ASP, on the other hand, decreases almost all melanogenic gene expression (Kobayashi *et al*, 1995; Sakai *et al*, 1997a). We have found that these changes in gene expression are reflected at the protein level as well, and consistent with those observations, the catalytic functions of those enzymes are similarly affected, and the melanin contents are dramatically affected. These results predict dramatic increases or decreases in visible melanin production by melanocytes responding to MSH or ASP, respectively, and that is indeed found. How these transcriptional events are regulated has become an interesting target of study and a recent report has demonstrated that the responses are complex indeed, involving a number of transcription factors and structural proteins within melanocytes (Furumura *et al*, 1998).

UV stimulation of melanogenesis is remarkably similar to that of MSH treatment (Archambault *et al*, 1995; Gilchrest *et al*, 1996). Following chronic UVB exposure, there are dramatic increases, not only in the number and dendricity of melanocytes, but also in the amount of melanin produced in those melanocytes and transported to the surface of the skin. It has been proposed that the UV response may be elicited via increases in MSH receptor expression and function in melanocytes (Bolognia *et al*, 1994; Chakraborty *et al*, 1995).

Important questions remain about the implication(s) of making different types of melanins (**Fig 2**). Production of the darker and more polymerized DHI-melanins maximizes photoabsorption but also increases cytotoxic effects (but with no apparent phototoxic effect) (Jimbow *et al*, 1995; Schmitz *et al*, 1995). DHICA-melanins have somewhat reduced photoabsorptive properties (and are not very phototoxic), but have a dramatically decreased cytotoxicity towards the cell (Hochstein and Cohen, 1963; Pawelek and Lerner, 1978; Urabe *et al*, 1994). In contrast, pheomelanin provides little or no photoabsorption and has a relatively high phototoxic content, but with very low cytotoxicity (Chedekel and Zeise, 1988; Young, 1994; Chedekel, 1995; Kollias, 1995). A complication is added when considering malignant melanoma, because many of these differentiation proteins are targets of the immune system (**Table II**). Melanoma cells whose pigment production is downregulated, or perhaps even worse, switched to pheomelanin production, obviously have dramatically downregulated expression of these potential antigens, and thus are more obscure targets for the immune system (Soballe and Herlyn, 1994; Orlow *et al*, 1995; Meier *et al*, 1998). Because TRP2 is perhaps the most widely expressed melanocyte-specific antigen by melanomas of varying phenotype (Orlow *et al*, 1995, 1998; Reynolds *et al*, 1998), a switch to DHI-melanins implies a decrease in TRP2 expression and function and therefore further implies a decrease in immune-targeting potential. Therefore in light of all these considerations, the optimum type of melanin for melanocytes to be producing is probably derived from DHICA; these DHICA-melanins would provide a good compromise between maximizing photoprotection while minimizing cytotoxicity, and imply that all melanosomal antigens are being expressed. It is important to recognize the fact that the commitment to produce one or another type of melanin has varied consequences to the melanocyte and the skin in which it resides on many different levels.

In summary, mammalian pigmentation is a very complex process. It is regulated at different levels by many distinct factors. The genes and regulatory processes involved in melanin formation are now being identified rapidly; about 25% of the known genes have already been cloned and characterized, and this will probably increase to 50% or 60% over the next decade. Many of these gene products have been shown to be involved in various clinical pigmentary diseases, and many also play important roles in immune responses to malignant melanoma. This is exciting and new information because we are now rapidly achieving a better position to evaluate various clinical pigmentary conditions, and potentially to help such patients.

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