The Biology of Pigmentation

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Abstract

Having roots in the earliest Mendelian experiments, the scientific examination of pigmentation offers the unique opportunity to better understand the contributions of genetics, signaling pathways, hormones, and the external environment on the phenotype of our body's largest organ system: the skin. Epidermal pigmentation is a product of the genetically determined melanin content, the cellular response to external stimuli, and the individual capacity for tanning. These processes are dependent upon a functional pigmentation pathway, which requires proper melanocyte migration, adequate melanogenic enzyme activity, and correct packaging and transfer of melanin to neighboring cells. Disruption of any of these processes leads to alterations in pigmentation. Although cutaneous pigmentation is most heavily focused upon, information about pigment patterning can also be gleaned from other pigmented tissues, including the hair and eyes. Numerous molecular signaling pathways and hormone systems converge to modulate pigment at the cellular level, which further contribute to the overall phenotype. These systems acquire greater importance when considered in

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the context of melanoma development, as these pathways are frequently found to be dysregulated.

Keywords
Pigmentation · Melanocyte · MC1R · MITF · Melanocyte stem cells · Tanning · Melanin · Ultraviolet

Introduction

Evolution is perhaps the greatest artist of all time. The natural world is ripe with masterpieces: from the iridescent blue and green plumage of the peacock to the blotchy dark spots scattered along the cream-colored fur of the cheetah, these pigmentary patterns all add to the beauty of the natural world. External coloration is an important evolutionary trait in the animal world – it confers a survival advantage via thermoregulation, camouflage, protection from solar radiation, and the facilitation of reproductive and social behavior. Most of our understanding of human pigmentation – the exterior coloration that absorbs or reflects light – is derived from studies on our animal counterparts.

Epidermal pigmentation is not a shared trait among all mammals. While many mammals don pigmented coats, their underlying skin is often surprisingly lacking in color. For these species, their pelage is an adequate form of defense against solar radiation. The appearance of cutaneous pigmentation in humans is thought to have coincided with the loss of body hair on the majority of the skin surface area. This evolutionary event was believed to take place when the *Homo erectus* population migrated to the African savannah (Maresca et al. 2015). Over countless generations, our ancestors developed an increased number of sweat glands and a reduced amount of body hair. Concurrently, hair became more heavily concentrated on top of the head. These changes were thought to develop in response to the hot environment, which created physiological stress and, via selective pressure over several generations, favored survival of those with improved thermoregulatory capacity. Hypotheses for the gradual pigmentation of skin are more variable. Some researchers argue that pigmentation developed as a protective mechanism against ultraviolet (UV)-mediated skin damage, whereas others argue that it developed because pigmented skin operates as a more effective barrier against the harsh environment. Over time, migration and dominion of new lands led to variations in skin phenotype. Those that migrated to Europe and Asia, where solar radiation is less intense, escaped the functional constraint on the gene(s) promoting dark pigmentation of the skin and developed more varied and lighter shades of pigmentation.

The scientific examination of pigmentation is not new. In the early 1900s, French biologist Lucien Cuénot performed one of the earliest mammalian genetic experiments, in which he crossed normally pigmented mice with unpigmented albino mice to demonstrate Mendelism applied to animals as well as plants. Although Cuénot demonstrated the important role of genetics in skin/fur phenotype, pigmentation is truly a result of a complex interplay between genetics, molecular signaling pathways, hormones, and the external environment. This chapter will cover the biology of pigmentation in depth and will touch upon the molecules and cells that act as building blocks of pigmentary units, the underlying signaling pathways that mediate pigmentation, and finally the relationship between pigmentation, solar radiation, and melanoma.

Part 1: Creating the Pigmentary Phenotype

Skin color results from reflected and absorbed light of unpigmented skin in combination with its constitutive pigments. The primary molecules that contribute to the coloring of human skin are melanin, hemoglobin, and carotenoids, although melanin is the greatest contributor to the resulting phenotype. When light hits the surface of the skin, it may be reflected back, scattered, or absorbed by molecules called chromophores, within the skin. The epidermal layers scatter light, while epidermal melanin granules absorb light and create brownish-black or reddish-yellow colors depending

on melanin type. Some light also reaches the dermis, where part of it is scattered and part of it is absorbed by collagen, creating a yellow color. Oxygenated hemoglobin produces a reddish tint, whereas reduced hemoglobin generates a bluish tint.

Skin pigmentation is primarily a trait of epithelial cells, in which most of the epidermal melanin resides after being synthesized and transferred from melanocytes. Epidermal pigmentation can be divided into two categories: constitutive and facultative. Constitutive pigmentation is the baseline color of the skin and is a function of the genetically determined melanin content (i.e., amount, type, and distribution). Facultative pigmentation is the result of the interplay between UV exposure, hormones, and the capacity for tanning. For instance, UV exposure in individuals with good tanning capability leads to an increase in the amount and type of melanin produced by melanocytes.

In 1975, Harvard dermatologist Thomas Fitzpatrick devised a numerical skin phototype classification system for use in clinical practice (see Table 1). The thereafter named Fitzpatrick scale ranges from phototypes I to VI and classifies persons by skin complexion and tolerance of sunlight. At the extremes, persons with phototype I (red-haired individuals) have light skin and eyes, always burn, and do not tan. Persons with phototype VI have dark-brown or black skin, never burn, and tan darkly. Although this scale is imperfect, it is a widely used metric both in research and in the clinic. Dermatologists most frequently utilize this tool to assess a patient's cancer risk.

Melanin, Melanocytes, and Melanosomes

Melanin

Melanins are a class of polymorphous biopolymers derived from the amino acid tyrosine. Two kinds predominate in the skin, hair, and eyes: eumelanin and pheomelanin (Thody et al. 1991). They differ both in their chemical composition and physical properties and thus respond disparately to light. Eumelanins are dark brown/black and highly polymerized, whereas the sulfur-containing pheomelanins are blond/red and less polymerized. Eumelanin is found in abundance in individuals with darker hair and skin. In contrast, pheomelanin predominates in individuals of skin types I and II but is also found together with eumelanin in darker phototype individuals. Hair concentrations of eumelanin and pheomelanin are higher than those in the skin.

As seen in Fig. 1, both eumelanin and pheomelanin share the first step of biosynthesis, in which tyrosinase catalyzes the conversion of tyrosine to dihydroxyphenylalanine (DOPA), which is then further oxidized by tyrosinase to dopaquinone. At this point, the pathways diverge. If available, cysteine or glutathione will rapidly conjugate with dopaquinone to form cysteinyldopa or glutathionyldopa before ultimately forming

Table 1 Fitzpatrick scale. The Fitzpatrick scale classifies persons to skin phototype according to both constitutive and facultative pigmentations. This chart demonstrates characteristics associated with each phototype, including sunburn and/or tanning history and minimal erythema dose (MED) (Table adapted from Astner and Anderson 2004)

Phototype	Constitutive color	Sunburn and tanning history	UVA-MED (mJ/cm^2)	UVB-MED (mJ/cm^2)
I	Ivory white	Burns easily, never tans	20–35	15–30
II	White	Burns easily, tans minimally with difficulty	30–45	25–40
III	White	Burns moderately, tans moderately and uniformly	40–55	30–50
IV	Beige-olive	Burns minimally, tans moderately and easily	50–80	40–60
V	Moderate brown	Rarely burns, tans profusely	70–100	60–90
VI	Dark brown-black	Never burns, tans profusely	100	90–150

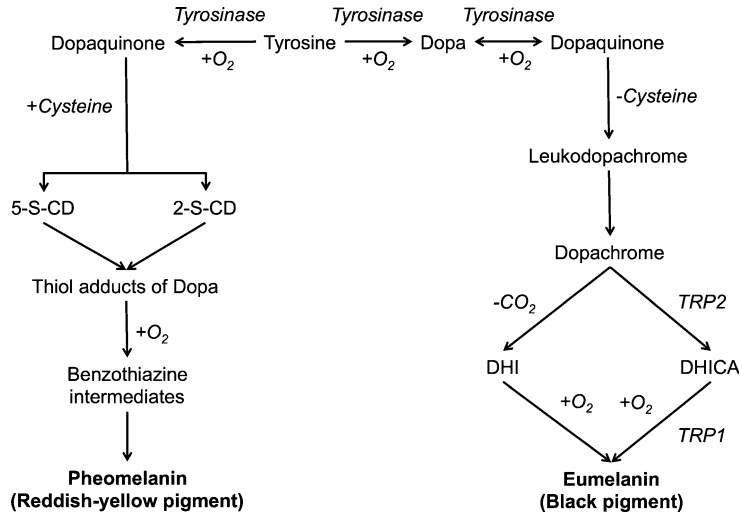

Fig. 1 Melanin synthesis. There are two separate biosynthetic pathways for eumelanin and pheomelanin synthesis (Image from Seo et al. 2007) (Adapted from Lamoreux et al. 2001)

pheomelanin. Otherwise, dopaquinone is transformed to leukodopachrome followed by a series of oxidoreduction reactions that result in the intermediates dihydroxyindole (DHI) and DHI carboxylic acid (DHICA), which become oxidized and polymerize to form eumelanin. Individual melanocytes can synthesize both eumelanin and pheomelanin, but only one pathway can remain active at a time since it is determined by the presence or absence of reduced thiols. The ratio of eumelanin to pheomelanin within the cell is a product of tyrosinase activity, as well as availability of tyrosine and sulfhydryl-containing reducing agents in melanosomes (the organelles responsible for pigment production, storage, and transport).

Several enzymes play a key role in producing pigment and are thus named melanogenic enzymes. These include tyrosinase, the tyrosinase-related proteins TRP1 and TRP2 (also commonly known as DCT), melan-A (MLNA), the P protein, and premelanosome protein (PMEL) (Hirobe 2011). Tyrosinase is a copper-containing enzyme that catalyzes three reactions in the pigment-producing pathway: (1) the hydroxylation of tyrosine to DOPA, (2) the oxidation of DOPA to dopaquinone, and (3) the oxidation of DHI to indolequinone. TRP1 and TRP2 share approximately 40% homology with tyrosinase (Lin and Fisher 2007). Both TRP1 and TRP2 are thought to stabilize the enzymatic activity of tyrosinase. Mutations in these enzymes can result in oculocutaneous albinism (OCA1-4), in which melanocytes are intact but have altered ability to produce pigment (see Table 2). Specifically, defects in the *TYR* gene, which encodes tyrosinase, lead to tyrosinase-negative OCA1. OCA2 results from mutation of the *OCA2* gene encoding P protein, OCA3 is due to mutation of *TRP1*, and OCA4 is a product of mutation of the gene encoding membrane-associated transport protein (*SLC45A2*).

In humans, eumelanin is the primary determinant of dark eyes, hair, and skin color. Additionally, eumelanin has been established as a potent photoprotective agent due to its broadband absorption spectrum. Eumelanin may partially shield organisms from intense sunlight, through dissipating a percentage of UV energy as heat in a nanosecond or less. Despite the ubiquity of melanins in nature, the underlying physical structure has been surprisingly elusive to researchers. The structure of melanin is difficult to study outside of living organisms, as, once it is isolated, it loses its structure and may transform into an amorphous mass. More than 100 variations of eumelanin composition have been noted to exist. Interestingly, a group of researchers has recently uncovered a unique optical property of eumelanin in that eumelanin has geometric disorder in addition to previously recognized chemical disorder (Chen et al. 2014a). Geometric disorder

Table 2 Genetics of oculocutaneous albinism. The TYR, OCA2, TRP1, and MATP genes are all implicated in oculocutaneous albinism (Image adapted from Grønskov et al. 2007, available under a CC BY 2.0 license. URL: http://ojrd.biomedcentral.com/articles/10.1186/1750-1172-2-43. © Grønskov et al.; licensee BioMed Central Ltd. 2007)

Gene	Chr. localization	Size	Disease name	Prevalence
TYR	11q14.3	65 kb (529aa)	OCA1	1:40,000
			OCA1A	
			OCA1B (yellow alb.)	
OCA2 (p gene)	15q11.2–q12	345 kb (838aa)	OCA2 (brown OCA in Africans)	1:36,000 (white Europeans), 1:3,900 (Africans)
TRP1	qp23	17 kb (536aa)	OCA3 (Rufous OCA)	Rare (white Europeans, Asians), 1:8,500 (Africans)
MATP	5p13.3	40 kb (530aa)	OCA4	Rare (white Europeans), 1:85,000 (Japanese)

is a result of randomly oriented and randomly sized molecules forming the aggregate structures. The interplay of geometric order and disorder of eumelanin aggregate structures generates random excitonic couplings among the molecules. These couplings broaden the absorption spectrum.

Eumelanin and pheomelanin respond differently to UV radiation (UVR). Eumelanin, as a heterogeneous polymer of DHI, DHICA, and their derivatives, acts as a UV filter and also scavenges for UV-induced free radicals. Additionally, the melanin precursor DHICA has been shown to inhibit lipid peroxidation as well as stimulate the antioxidant defense systems and differentiation of keratinocytes. Pheomelanin is a poor UV filter and has also been found to increase formation of reactive oxygen species (ROS). Within melanosomes containing dark/eumelanin pigment, it has been noted that the initial (deepest) melanins are pheomelanin, but these become overlaid or "caged" by layers of eumelanin which may protect the cell from ROS production by the underlying pheomelanin. Absorption of radiation by various melanin species, and particularly pheomelanin, can generate radicals that are strong oxidants. Furthermore, melanin synthesis involves a series of highly reactive quinone intermediates that promote ROS and oxidative DNA damage.

Melanocytes

Melanin is the most important molecule in producing skin pigmentation, and proper functioning of melanocytes is vital for appropriate melanin production. As seen in Fig. 2, melanocytes are found within the human skin dispersed along the dermal/epidermal border. There, they can interact with the underlying fibroblasts of the dermis and the surrounding keratinocytes in the dermis. However, melanocytes are not merely pigment producers. They release a diverse array of signaling peptides (e.g., melanocortin peptides, catecholamines, serotonin, eicosanoids, and nitric oxide) that tightly connects them with both the neural and immune systems. Because of this, melanocytes are thought to play an important role in general epidermal homeostasis.

Cutaneous melanocytes are derived from pluripotent neural crest cells. Neural crest cells also give rise to neurons, glial cells, the adrenal medulla, cardiac cells, and craniofacial tissue. Upregulation of microphthalmia-associated transcription factor (MITF) by paired-box 3 (PAX3), the Wingless-type (WNT) signaling pathway, and sex-determining region Y (SRY)-box 10 (SOX10), in combination with downregulation of forkhead box D3 (FOXD3) and SRY-box 2 (SOX2), is important in creating committed melanocyte lineage cells. Melanoblasts, the melanocyte precursors, proliferate and migrate dorsolaterally from the dorsal portion of the neural tube to populate the basal epidermis and hair follicles. An additional pathway of melanocyte development from Schwann cell precursors has recently been found to be a significant contributor to cutaneous pigment cell formation (Mort et al. 2015). Human melanocytes can be detected in the

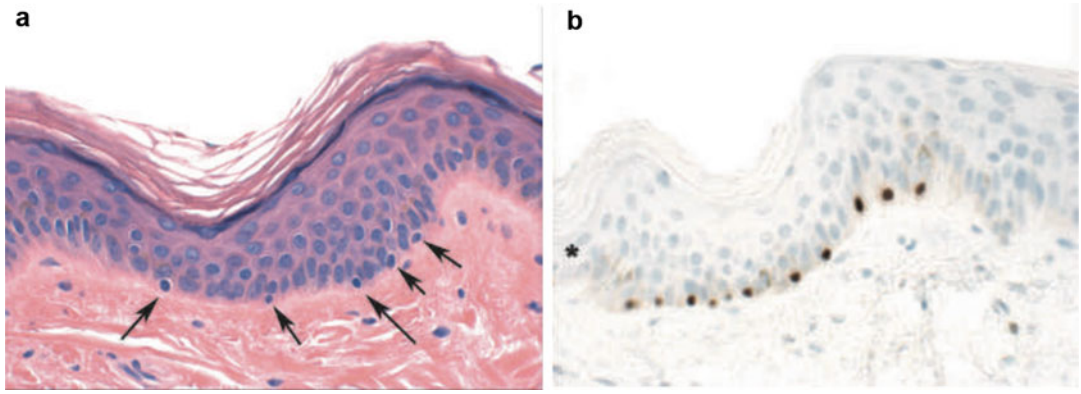

Fig. 2 Skin histology. (**a**) Hematoxylin and eosin staining of normal human skin tissue. Hematoxylin stains nuclei purple and eosin stains cytoplasm and collagen pink. The arrows point to melanocytes within the basal layer of the epidermis. (**b**) Immunohistochemistry of the same tissue. The melanocytes are stained brown by the antibody D5 (antimicrophthalmia-associated transcription factor) (Image from Lin and Fisher (2007) with permission from S. R. Granter)

dermis and epidermis by 7 weeks of estimated gestational age. Defects in melanoblast migration result in unpigmented patches of the skin.

Many signaling pathways and transcription factors provide input for proper melanocyte migration and proliferation. Integration of spatial and temporal signals from these pathways allows for precise control of melanocytes. Signals important in homing of melanocytes to the skin include the KIT proto-oncogene receptor tyrosine kinase (KIT) and its cognate ligand KITLG, as well as endothelin and its receptor B (EDNRB). Furthermore, the appropriate migration of melanoblasts and melanocytes is dependent upon integrins, cadherins, and extracellular matrix molecules. For example, mouse studies show that at embryological day 11.5 in mice, most dermal melanoblasts are E- and P-cadherin negative, but over the next 48 h during migration from the dermis to the epidermis, the majority of melanoblasts become E-cadherin high/P-cadherin low (Mort et al. 2015).

Overall, absolute melanocyte numbers are not the main drivers in pigmentary differences between races. Rather, it is the overall cellular activity, the type of melanin produced, and the size, number, and packaging of melanosomes that determine pigmentation. Variation in melanosome size can be seen between different skin types. Individuals with dark skin types have larger melanosomes that are packaged as single units, which limit their degradation in keratinocytes and provide greater visible pigmentation. Lighter-skinned individuals have smaller melanosomes that are packaged in groups, making them more vulnerable to degradation.

There is high variability in melanocyte population densities both within individuals and between individuals. For instance, there are twice as many melanocytes in the head and forearm skin compared to elsewhere on the body. The density of melanocytes within the skin is a function of UVR and stimulatory factors secreted by neighboring cells. Studies have suggested that after reaching 30 years of age, a person will lose approximately 10–20% epidermal melanocytes per decade. However, it is also possible that a reduction in pigmentation may be due to a reduction in melanogenic enzyme activity rather than complete cell loss. Aging additionally causes changes in melanocyte morphology and proliferative capacity, as well as a reduction in melanogenic enzyme activity. Terminally differentiated melanocytes are characterized by an accumulation of cyclin-dependent kinase inhibitors (e.g., $p16^{INK4A}$), hypophosphorylation of retinoblastoma protein (pRB), and decreased levels of cyclin D1. After time, terminally differentiated melanocytes also suffer from high levels of ROS due to a reduction in catalase activity and downregulation of anti-apoptotic factor BCL2.

Reduced activity of the mitogen-activated protein kinase (MAPK) pathway also leads to a reduction in melanocyte proliferation.

Melanosomes

Within melanocytes, there are large, specialized pigment-producing organelles (measuring up to 500 nm in diameter) called melanosomes, which are responsible for melanin synthesis, storage, and transport. Melanosomes are a type of lysosome-related organelle and protect the rest of the melanocyte from the toxic by-products of melanin synthesis. Melanosomes are synthesized in the perinuclear region of the melanocyte. Melanosome development occurs in four morphologically distinct stages; the two earliest stages are associated with little to no pigment, whereas melanosomes are pigmented by the later stages. Stage I premelanosomes are formed by an outpouching of a smooth membrane from the rough endoplasmic reticulum. At stage II, PMEL is sorted into intraluminal vesicles and undergoes proteolytic cleavage to form the fibrillar matrix. The melanogenic enzymes tyrosinase and TRP1 are delivered to the organelle, inducing pigment synthesis. In stage III, melanin pigment is deposited onto the fibrillar matrix. In stage IV, the melanosome matures and is fully melanized (Slominski et al. 2004). This stepwise process can become dysregulated under pathological conditions like melanoma. In such states, tyrosinase can become activated by stage I of melanosomes synthesis, and melanin can be deposited without an underlying matrix.

For melanosomes to be transferred to surrounding epithelial cells, they must travel from the perinuclear region to the tips of dendrites within the melanocyte. Transport to the melanocyte dendrite is mediated by microtubules and microtubule-associated motor proteins (kinesins and cytoplasmic dyneins). Once at the tip, melanosomes are captured for migration to nearby keratinocytes. Myosin VA (MYO5A) is involved in the capture of melanosomes at the tip. Ras-related protein Rab-27a (RAB27A) assists in phosphatidylserine addition to synaptotagmin-like protein2-a (SLP2A), which docks melanosomes to the protein membrane.

Pigment transfer from melanocytes to keratinocytes has been extensively studied but remains incompletely understood. There are currently four non-mutually exclusive models for melanin transfer: (1) phagocytosis of melanocytic dendrites by keratinocytes, (2) melanosome transport by membrane nanotubules, (3) melanosome exocytosis of a polymerized melanin extracellularly followed by keratinocyte internalization, and (4) shedding by melanocytes of plasma membrane-enclosed melanosome-rich packages that are then phagocytosed by keratinocytes (Wu and Hammer 2014). The third and fourth models have gained considerable traction due to supporting evidence from experimental studies (Tarafder et al. 2014). More studies are required for the underlying mechanism to be fully elucidated. Once transferred to keratinocytes, the melanosomes are positioned over the superficial/top (sun-exposed) side of the nucleus, potentially shielding DNA from the deleterious effects of UVR.

Melanosomal disorders include Chédiak-Higashi syndrome (CHS), Hermansky-Pudlak syndrome (HPS), and Griscelli syndrome (GS). These disorders involve aberrant melanosome biogenesis. They are often accompanied by immunodeficiency and neurological dysfunction due to parallel roles of their genetically encoded mediators, which regulate melanosome-like vesicular structures in other cellular compartments. Other disorders include macromelanosomes and autophagic giant melanosomes, which can be seen in complexes of nevocellular nevi, lentigo simplex, and malignant melanoma.

Pigmented Lesions

Pigmented lesions arise on the skin from alterations in melanocyte cellular activity or increases in melanocyte number. One of the most focused upon pigmented lesions in the dermatology clinic is the melanocytic nevus, more commonly known as a mole. Striking in appearance, most people have a few dozen melanocytic nevi on their body, with many fair-skinned people having more. Nevi may either be congenital, developing in utero, or acquired during a person's lifetime, which is the more common presentation.

Typically found in sun-exposed sites, the common acquired melanocytic nevus can appear within the first 6 months of life, reaching its largest diameter in young adulthood and regressing with advancing age. Dermatology patients are provided with the mnemonic ABCDE (asymmetry, border irregularity, color variation, diameter, and evolutionary change) to assist in at-home monitoring of their nevi. Nevi meeting any one of these criteria should be pointed out to a dermatologist for appropriate clinical evaluation, which may include a biopsy and histopathologic analysis.

A melanocytic nevus is defined as a local proliferation of melanocytes in contact with each other, forming nests. Melanocytic nevi can be further subclassified into junctional, compound, or intradermal nevi based upon the histologic location of melanocytic nests. Nevi can progress from junctional to compound to intradermal locations as they migrate deeper into the skin, evolving from a flat macule to a raised papule.

Although benign, melanocytic nevi are formed by an activating mutation in an oncogene, causing proliferation until the onset of senescence which is thought to limit further growth. Although higher numbers of melanocytic nevi are associated with an increased risk of melanoma formation, only about one in four melanomas is derived from an apparent preexisting melanocytic nevus. Congenital and acquired melanocytic nevi vary in their oncogenic drivers. Congenital melanocytic nevi are thought to form from an error in neuroectodermal development and migration. Mutations in both neuroblastoma RAS viral oncogene (*NRAS*) and B-raf proto-oncogene (*BRAF*) have been found in CMN, with *NRAS* mutations being the primary driver in giant congenital melanocytic nevi. Acquired melanocytic nevi are thought to be a result of UV exposure and carry *BRAF* mutations in 50–70% of cases.

Other pigmented lesions that are commonly seen are ephelides (also known as freckles), lentigines (also known as age spots), and café au lait spots. Ephelides are small brown macules that arise on sun-exposed skin. They are seen most frequently in fair-skinned people, especially those with red hair. After UV exposure, increased melanin production occurs within melanocytes, which then transfer the pigment to neighboring keratinocytes. Accumulation of melanin in a localized group of keratinocytes creates the brown macular appearance of an ephelis. Typically, ephelides will fade during the winter months. Lentigines are brown macules that commonly arise in middle age, often due to sun damage. They are most frequently found on the face and hands and are generally larger and more defined than ephelides. They persist for a long time and do not fade in the winter months like ephelides. They are a localized proliferation of melanocytes and can be distinguished from melanocytic nevi by the absence of melanocyte nests. Café au lait spots are hyperpigmented lesions caused by an increase in melanin content in combination with giant melanosomes. They often develop in isolation, but a large number of them are suggestive of neurofibromatosis type 1 (NF1). Interestingly, café au lait spots of NF1 patients have increased melanocyte density and higher levels of KITLG than in those of individuals without NF1.

Pigment-Recipient Phenotype + Pigment Patterning

The Pigmentary Unit

Although melanocytes are the primary focus of pigment biology, epithelial cells play an equally important role. Indeed, the skin and hair are made up almost entirely of keratinocytes, while melanocytes are much fewer in number. Melanocytes may be the pigment generators, but proper skin color patterning ultimately relies on a finely controlled communication network between melanocytes and the surrounding epithelial cells. The underlying process by which this coloration happens has been named the "pigment-recipient phenotype." In this model, melanocytes act as the pigment donors, and dedicated epithelial cells behave as pigment recipients. Together they comprise what is called a "pigmentary unit," in which the melanocyte makes dendritic connections to a defined group of epithelial cells (typically there is one melanocyte interacting with 30–40 keratinocytes). Four major classes of pigment recipients have been identified in humans:

(1) keratinocytes of the basal layer of the epidermis, (2) keratinocytes of the first suprabasal layer of the epidermis, (3) progenitors of the hair cortex, and (4) precursors of medulla follicular cells.

In the epidermis, melanocytes sit above the basement membrane. From there, they communicate with basal and first suprabasal layers of epithelial cells. Via this arrangement, melanocytes are able to deliver pigment to the least differentiated keratinocytes with the greatest proliferative capacity. After melanin acquisition, epidermal keratinocytes concentrate the pigment on the apical side of the nucleus, forming a nuclear cap that functions as a parasol to shield the nucleus from sunlight.

Clues that keratinocytes may influence melanocyte behavior arose in early in vitro experiments. When isolated human melanocytes are placed into a culture dish with epidermal keratinocytes, the melanocytes localize to the basal layer, just as they do in the human epidermis. Thus, it appears that the epidermal cells provide vectorial signals to the melanocytes that assist in their positioning. Keratinocytes can also influence melanocyte survival, proliferation, melanogenesis, and dendricity by production of paracrine growth factors and cell adhesion molecules. Factors secreted by the keratinocyte include α-melanocyte-stimulating hormone (α-MSH), endothelin 1, KITLG, basic fibroblast growth factor (FGF2), nerve growth factor (NGF), prostaglandins, and granulocyte macrophage colony-stimulating factor (GM-CSF).

Epithelial Cell Targeting

As the skin develops, some epithelial cells become pigmented, while others do not. What drives this difference? Central to this question is whether epithelial cells are simply passive recipients of melanin or are self-driven to actively recruit melanin from melanocytes. Several studies have suggested the latter may be the case, and the major molecules thought to activate the pigment-recipient phenotype will be covered here. Two molecules that have been heavily studied in the interactions between epithelial cells and melanocytes are the transcription factor Forkhead box protein N1 (FOXN1) and its target FGF2. Both of these molecules are expressed in epithelial cells that are pigment recipients.

In humans, FOXN1 is present in the differentiating hair cortex, first suprabasal layer of the epidermis, and small portions of the basal layer of the epidermis. *FOXN1* is most highly expressed in these areas during cellular transitions from proliferation to differentiation. Humans with a nonsense mutation in *FOXN1* suffer from T cell immunodeficiency, congenital alopecia, and nail dystrophy. Mutation of *Foxn1* in mice, a gene that is 86% identical to the human gene, results in a nude phenotype. Mouse studies by Weiner and colleagues have suggested that *Foxn1* appears to have a role in pigmentation directioning (Weiner et al. 2007). FOXN1 is thought to cause keratinocytes to release FGF2, which is detected by melanocytes and allows them to recognize FOXN1-positive epithelial cells as pigment targets. Thus, through FOXN1 and the release of FGF2, epithelial cells appear to engineer their own pigmentation. This means that pigment recipients, like pigment donors, are also specialized cells dedicated to a pigmentary function. However, humans do not have FOXN1-mutation associated abnormalities, and thus, more research must be done to clarify its role in human pigmentation.

KITLG plays a clear and important role in melanocyte development. Mutations in KITLG (or KIT receptor itself) result in piebaldism: patches of unpigmented hair. KITLG exists both in diffusible and cell-bound forms, which are produced by alternative splicing of the same RNA transcript. The diffusible form is thought to mediate chemotaxis, whereas the cell-bound form is thought to direct cell positioning and promote proliferation and survival. KITLG activates the MAP kinase pathway in melanocytes through the KIT receptor, which is a receptor tyrosine kinase. KITLG may help identify or activate pigment recipients, either in conjunction with FOXN1 or in place of it. Other factors that may play a role in the recipient phenotype are noggin (NOG), epidermal growth factor receptor (EGFR), F2R like trypsin receptor 1 (F2RL1), and derivatives of proopiomelanocortin (POMC).

This division of activities – one cell producing pigment, another cell using it – is specific to the skin and carries unique advantages: specificity in pigmentary interactions and the creation of a finely mapped template for pigmentation.

Pigmentation in Other Tissues

Hair Pigmentation

The hair is one of the defining characteristics of mammals. In contrast to most mammals, humans grow long, thick hair on the scalp, with relatively short, thin hairs on the remainder of the body. The biological and evolutionary significance of this is uncertain, but purported theories range from UV protection on the scalp to serving as a method of expelling built-up toxic chemicals via melanin binding (Tobin and Paus 2001). A hair shaft is comprised of compact terminally differentiated keratinocytes known as trichocytes. Hair shafts grow from a follicle at a rough rate of 1 cm per month. Humans have different types of hair, including terminal, vellus, and androgenic hairs, which serve different biological purposes. Vellus hair is generally more lightly pigmented, fine, and short in length, whereas terminal and androgenic hair are thicker, darker, and longer.

The color of the hair is a function of the ratio of eumelanin to pheomelanin. Black hair follicles have melanocytes with a large number of eumelanosomes with a fibrillar matrix. Brown hair is associated with smaller bulb melanocytes. Blonde hair has poorly melanized melanosomes, such that only the melanosomal matrix is visible. Red hair is associated with pheomelanosomes that contain a vesicular matrix and irregularly deposited melanin. Most of the human population has dark hair. This was thought to arise evolutionarily as a selective advantage in tropical UV-intense climates. It is also believed that persons with a loss-of-function melanocortin 1 receptor (*MC1R*) mutation, which gives rise to the red hair phenotype, might have escaped this evolutionary pressure, explaining the wider variety of hair colors within Northern European populations. Interestingly, hair and skin pigmentations do not always perfectly correlate. Melanin is processed differentially by recipient hair cortical keratinocytes and epidermal keratinocytes. Melanin transferred to epidermal keratinocytes is partially digested. In contrast, melanin processing in hair keratinocytes is minimal. These variable degrees of melanin processing are thought to account for seemingly unrelated hair and skin phenotypes, such as dark hair and light skin.

The hair follicle is considered to be a mini-organ. It is tightly connected to many cell types, including keratinocytes, fibroblasts, nerves, and immune cells, all of which influence the hair growth cycle. The hair follicle is made up of concentric cylindrical layers of cells: the innermost layers make up the hair shaft, and the intermediate layers make up the inner root sheath. Stem cells at the hair bulb, which forms the base of the follicle, generate the cells forming the hair shaft and inner root sheath. These segments are displaced up and through the surrounding outer root sheath. Another important structure is the bulge region of the outer root sheath, which is the site of arrector pili muscle attachment.

The hair follicle pigmentary unit is tightly coupled to the hair growth cycle, whereas the epidermal pigmentary unit experiences continuous melanogenesis. It is comprised of follicular melanocytes, matrix keratinocytes, and dermal papilla fibroblasts. Hair becomes actively pigmented by proliferating melanocytes during anagen, the first phase of the hair cycle (see Fig. 3). During the subsequent two phases, catagen and telogen, melanogenic enzymes are downregulated before melanocytes ultimately undergo apoptosis. Melanogenesis and pigmentation then begin anew during the next cycle (Schneider et al. 2009).

Hair pigmentation develops via a coordinated sequence of events: the melanogenic activity of follicular melanocytes increases followed by transfer of melanin granules into cortical and medullary keratinocytes, ultimately producing a pigmented hair shaft. Melanocytes achieve this specific targeting of pigment transfer by extending their dendrites upward along the columns of cortical and medullary cells. Within the hair follicle, melanocytes exist in a 1:5 ratio with keratinocytes, which is more balanced than the

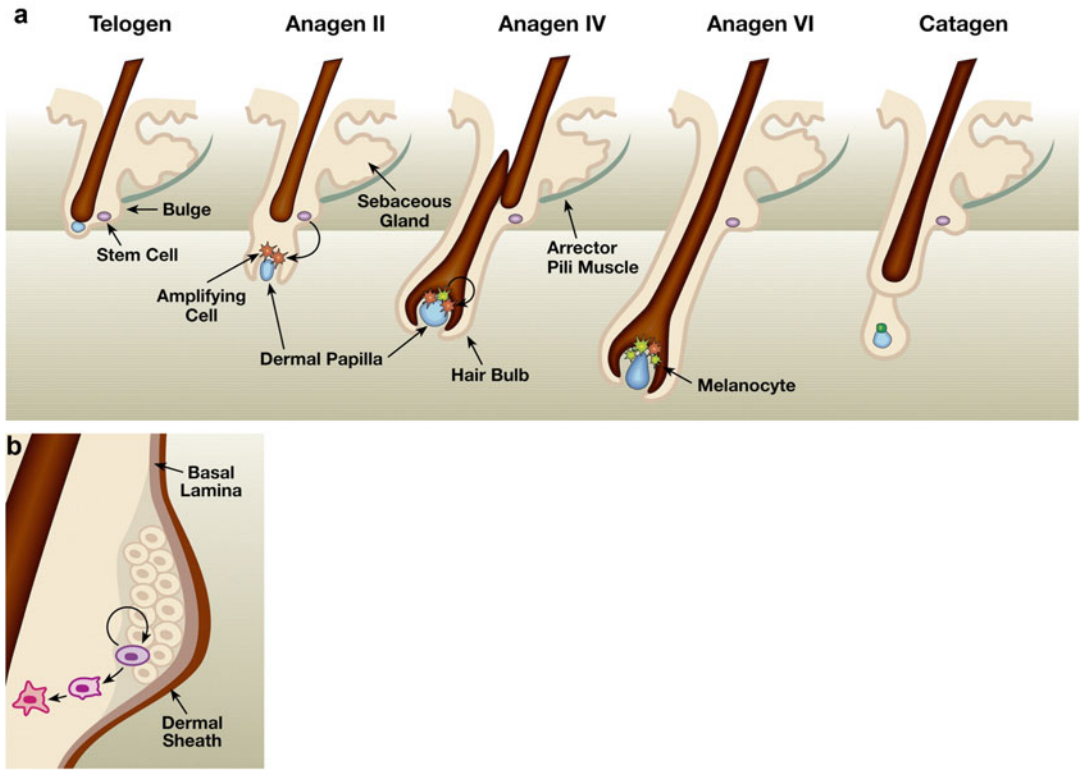

Fig. 3 Melanocyte stem cells and the hair cycle. (**a**) Shown here are the different stages of the hair cycle, including telogen, anagen, and catagen. At the beginning of anagen, melanocyte stem cells (purple) in the hair bulge give rise to amplifying cells (red) that migrate to the dermal papilla. Once there, they differentiate into melanocytes (green). (**b**) Melanocyte stem cells give rise to two types of daughter cells: those that self-renew (purple) and those that differentiate (red) (Image from Steingrímsson et al. 2005) (Adapted from Nishimura et al. 2002)

ratio within the epidermis. Melanin production within the anagen hair bulb is thought to mediate cellular stress caused by production of ROS through the free radical scavenger properties of melanin.

Like the rest of cutaneous melanocytes, hair follicle melanocytes are derived from neural crest cells. Homing to the follicular pigmentary unit during embryogenesis is thought to be directed by two receptor-ligand pairs that have been previously discussed: (1) receptor tyrosine kinase KIT and its ligand KITLG and (2) endothelin-3 and its receptor EDNRB. Melanoblasts expressing KIT migrate into the KITLG-positive hair follicle epithelium. Once there, differentiated KIT-positive melanocytes travel to the bulb when it is KITLG-positive. KIT-negative melanoblasts migrate to the outer root sheath and bulge region within the hair follicle. After homing is complete, active melanocytes are found within the basal layer of the infundibulum, the upper dermal papilla, and the basal layer of the sebaceous gland.

Melanocyte Reservoirs and Aging

Observations of vitiligo patients led to the hypothesis that collections of melanocyte stem cells might exist. Vitiligo is a skin condition characterized by loss of groups of melanocytes mirrored by patchy loss of skin pigmentation. Repigmentation in patients with autoimmune vitiligo following immunosuppressive UV therapy manifests as tiny islands of color centered upon hair follicles. This was suggestive of a stem cell reservoir within hair follicles. Early scientific studies supported this idea, as the presence of DOPA-negative amelanotic melanocytes was observed within the

outer root sheath and bulge areas of the hair follicle. Under normal conditions, these melanocytes did not produce pigment. However, after stimulation by UVR or epidermal wounding, these melanocytes could be induced to produce pigment. These observations led researchers to believe that this pool of amelanotic melanocytes might represent a reservoir of melanocyte stem cells (Steingrímsson et al. 2005).

Confirmation that this population was actually a pool of melanocyte stem cells was achieved through mouse studies. Addition of anti-KIT antibodies to mice was observed to induce hair graying via depletion of KIT-dependent melanocytes. However, later hair cycles produced normally pigmented hair, suggesting that the hair bulb melanocytes were replenished by a KIT-independent melanocyte source. In another experiment, when an anti-KIT antibody was given to deplete replicating melanoblasts from neonatal *Dct-LacZ* transgenic mice (historically used to monitor early melanoblast development), LacZ-positive cells were observed predominantly in the bulge area of hair follicles (Nishimura et al. 2002). This, combined with the observation that mice injected with anti-KIT antibody produced gray hair during the first hair cycle before again growing normally pigmented hair, was strongly suggestive of the presence of a resting melanocyte precursor population in the bulge region.

The proposed mechanism of melanocyte stem cell involvement in the hair cycle is that once the hair cycle moves from telogen to anagen, both the expression of *TRP2* and cell size increase in melanocyte stem cells, leading to cell division (see Fig. 3). One cell remains in the bulge region, while the other migrates to the hair matrix, divides further, and differentiates into a pigment-producing melanocyte. It was later shown through transgenic mouse models that the bulge melanocyte stem cells could act a source of melanocytes in the epidermis (under certain conditions) by melanoblasts traveling from the hair bulge to the surrounding epidermis. Maintenance and regulation of the melanocyte stem cell pool within hair follicles depend on the transcription factors PAX3 and MITF.

Improved knowledge of follicular melanocyte stem cells paved the way for better understanding of hair graying. Most humans begin to show gray hair at around 35 years of age. It was long thought that hair graying was a result of long-term toxicity from melanin biosynthesis, causing eventual degradation of melanocytes. However, it was later shown that hair graying is instead a product of melanocyte stem cell reservoir depletion (Nishimura et al. 2005). Two proteins play a particularly active role in the maintenance of hair pigmentation. Specifically, BCL2 protects melanocytes from apoptosis. Mice with a null mutation for *Bcl2* can only produce pigmented hair for a single hair cycle before the hair turns gray. Between postnatal days 6 and 8 (early–mid anagen cycle), a sudden loss of all bulge melanocytes occurs despite a normal number of differentiated melanocytes within the hair bulb. MITF also plays an important role in maintenance of the melanocyte stem cell niche. Premature graying is seen in mice with the $Mitf^{vit}$ mutation. This mutation caused melanocytes to differentiate prematurely (or become "aberrantly pigmented") within the bulge region, causing them to permanently lose their stem cell properties and as a result preventing proper migration to the bulb. Thus, MITF is thought to be important for self-renewal.

Eye Pigmentation

Eye color is a function of both iris pigmentation as well as the scattering of light by the stromal medium. The iris is comprised of five layers: the anterior border layer, stroma, muscular layer, anterior pigment epithelium, and posterior pigment epithelium. Iris pigmentation is dependent upon the concentration of melanin within both the iris pigment epithelium (IPE) and the stroma and the cellular density of the stroma. In the brown iris, there is a large quantity of brownish-black melanin within the anterior border layer and stroma that effectively absorbs light. In contrast, there is very little melanin in the blue iris. Interestingly, there is also no blue pigment present.

Rather, the blue color is a result of optics: as light traverses through the melanin-free layers, longer wavelengths are transmitted, while the shorter blue wavelengths are reflected via scattering by collagen fibrils. This is known as the Tyndall effect. Thus, the blue iris represents structural color. Patients with severe albinism lack pigment in the back of the iris, allowing light from inside the eye to escape through the iris to the front. The only color seen in eyes from these individuals is from hemoglobin in the capillaries, resulting in a reddish-pink eye.

There are three major classes of eye color: brown, blue, and green hazel. The number of melanocytes does not differ between eye colors. Darker eyes have a greater ratio of eumelanin to pheomelanin, whereas lighter eyes have more pheomelanin. Lighter iris colors are found almost exclusively in persons of European descent. The majority of babies of European descent have light-color eyes when they are born, which may later change to darker colors by the age of 1. This is due to an increase in melanin production driven by sympathetic neuronal stimulation. Eye color has also been noted to change in later stages of life, such as during puberty and after trauma. Also of note, half the adult population has iris nevi, which can appear on the surface of the anterior border layer when a group of melanocytes increases their melanin production.

Although eye color is a complex polygenic trait, it has been estimated that 74% of variation in human eye color can be attributed to a portion of the genome containing the *OCA2* gene (Sturm and Larsson 2009). Regulation of *OCA2* gene expression via epigenetic pathways is thought to determine blue-brown eye color in European populations. A single base change, rs12913832 T/C within intron 86 of the upstream *HERC2*, appears to play a role in this process. This SNP has been suggested to be a target site for the SWI/SNF family member helicase-like transcription factor (HLTF). In the proposed mechanism, as shown in Fig. 4, the *HERC2-OCA2* locus initially exists in a closed heterochromatin packaging state. When the rs12913832*T allele is active, HLTF recognition can occur, causing chromatin unwinding and exposure of the regulatory sequences recognized by MITF and lymphoid enhancer binding factor 1 (LEF1), permitting the *OCA2* promoter to be available for transcription factor regulation. Expression of the *OCA2* gene results in brown eye color. In contrast, the rs12913832*C allele prevents HLTF binding, keeping chromatin in a closed state that prevents transcription of the *OCA2* locus. This results in blue eye color due to the inability to form mature/eumelanotic melanosomes.

Extracutaneous Melanin

In addition to the epidermis, hair follicles, and eyes, melanocytes can be found in less suspected areas. They may be found in small numbers throughout the dermis and subcutis, blood vessel walls, and even within the muscle, nerves, and sebaceous glands. Melanocytes have even been detected within lymph nodes in a benign form. It has been suggested that these melanocyte collections are instead due to an error in embryological migration, and they may be seen in association with large congenital melanocytic nevi and blue nevi. Melanocytes present within lymph nodes can sometimes be troubling, as they can be mistaken for metastases.

Melanocytes may additionally be found within the heart valves and septa. Their function there is unclear, but they may contribute to atrioventricular valve function, as well as regulation of calcium and ROS levels. Melanocytes are also located within the stria vascularis of the cochlea and play a critical role in hearing due to maintenance of extracellular potassium in the endolymph. Indeed, the Waardenburg type II (WS2) phenotype resulting from an *MITF* mutation presents with sensorineural deafness that can range from mild to severe. Melanocytes are also present in the brain within the meninges overlying the medulla oblongata and upper cervical spinal cord. Notably, the dark melanin produced by dopaminergic neurons in the substantia nigra is neuromelanin and is believed to be an autooxidative product of dopamine synthesis rather than melanocytic in origin.

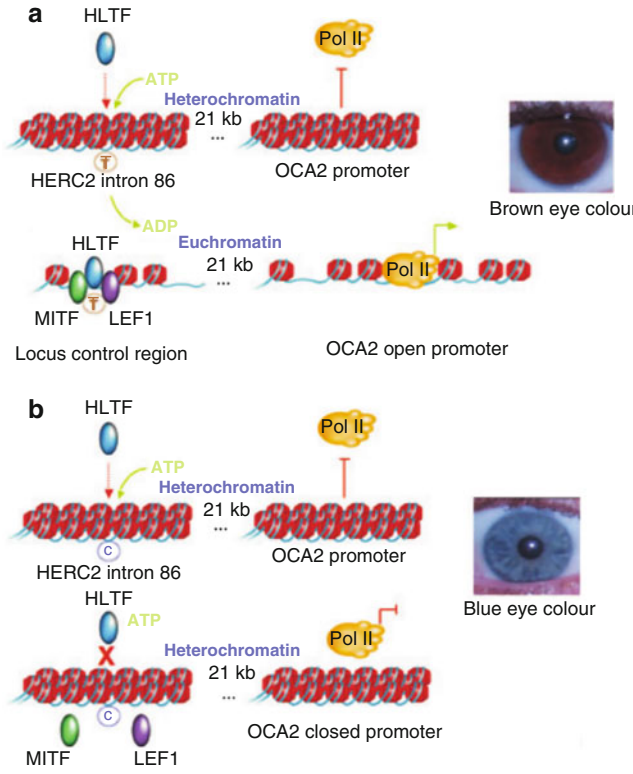

Fig. 4 A model of how regulation of *OCA2* gene expression determines blue-brown eye color. (**a**) Helicase-like transcription factor (*HLTF*), a member of the SWI-SNF family, is able to regulate genes by altering chromatin structure. Here, HLTF recognizes the evolutionary conserved element containing the SNP rs12913832*T within the HERC2 intron 86 region of a DNA molecule (DNA is represented as the blue coil, and nucleosomes are represented as the red spheres). This causes DNA to transform into a more relaxed state, which permits the transcription factors MITF and LEF1 to bind to the locus control region. As a result, RNA polymerase II (Pol II) can transcribe the *OCA2* gene, which stimulates eumelanogenesis and a resulting brown eye color. (**b**) If the SNP rs12913832*C is instead present, HLTF is unable to interact with the heterochromatin, which prevents MITF and LEF1 binding, as well as *OCA2* transcription. A resulting lack of melanin production leads to blue eye color (Image from Sturm and Larsson 2009)

Part 2: Molecular Control of Pigmentation

Multiple factors are involved in the control of pigmentation. More than 150 alleles spread over 90 loci are involved in the regulation of pigmentation, and they encode protein products such as enzymes, structural proteins, transcriptional regulators, transporters, receptors, and growth factors (Slominski et al. 2005). Keratinocytes and fibroblasts release POMC, growth factors, cytokines, and ROS. Hormones like corticosteroids and estrogens can also influence pigmentation. One of the most central interactions in the pigmentation is that between α-MSH and MC1R, which initiates cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling.

MC1R and the Pigment-Type Switching System

Melanocortin 1 Receptor

Much of the attention has been focused on establishing the link between pigmentation phenotype and genetic polymorphisms involving the

MC1R locus. So far, *MC1R* is the only gene identified that can account for the large phenotypic variation in pigmentation. MC1R acts as a regulator of both constitutive and facultative pigmentation. The *MC1R* gene encodes a seven-transmembrane G-protein-coupled receptor (GPCR), which upon activation leads to production of eumelanin within melanocytes. Mutations at this locus can alter the ratio of eumelanin/pheomelanin production within melanocytes and lead to widely variable coloring in the human eyes, skin, and hair.

More than 100 gene polymorphisms have been reported for *MC1R*. *MC1R* variants contain changes in ligand binding, receptor function, or a complete loss of function. In particular, loss-of-function polymorphisms result in the red hair, freckling, and fair skin phenotype, in which individuals have a decreased ability to tan. *MC1R* gene sequence variants are present in over 80% of individuals with red hair or poor-tanning skin (Roider and Fisher 2016). In contrast, variants are found in fewer than 20% of individuals with brown or black hair and in less than 4% of individuals with good tanning ability. Loss-of-function *MC1R* variants are linked to an increased risk of developing both melanoma and non-melanoma skin cancer.

The MC1R receptor is activated by α-MSH, a peptidic hormone derived from POMC in the pituitary and skin, and is inhibited by agouti signaling protein (ASIP). Thus, α-MSH is thought to promote eumelanin production, whereas ASIP induces pheomelanin synthesis. Once activated by α-MSH, MC1R signals through the G-protein α-subunit (Gαs) to increase intracellular levels of the second messenger cAMP via adenylate cyclase, leading to phosphorylation of cAMP responsive element-binding protein (CREB) and transcription of MITF. This leads to generation of eumelanin via transcription of genes for melanogenesis (*TYR*, *TRP1*, *TRP2*), melanosome biogenesis (*PMEL*), and melanosome transport (*RAB27A*). Additionally, α-MSH stimulation of MC1R leads to the creation of even more MC1R proteins. When MC1R is inhibited, cAMP levels remain low, and pheomelanin production is preferred. Adrenocorticotropic hormone (ACTH) can also induce pigmentation through interactions with MC1R.

The Pigment-Type Switching System

Melanocytes possess a pigment-type switching mechanism in which they can individually alternate between eumelanogenic and pheomelanogenic states. Upon binding to MC1R, α-MSH activates the eumelanogenic pathway, whereas ASIP provides an inhibitory signal and promotes pheomelanin synthesis (Walker and Gunn 2010). ASIP is a soluble protein secreted by dermal papilla cells of the hair bulb that competitively antagonizes α-MSH at MC1R and actively suppresses MC1R activity. This inhibits melanogenic enzymes and promotes pheomelanogenesis. Additionally, ASIP can exert effects even in the absence of α-MSH, presumably by down-regulating a degree of ligand-independent MC1R signaling activity.

Mice exhibit one of the clearest examples of pigment switching. In the wild-type "agouti" mice, there is a transient switch between eumelanin and pheomelanin production in the hair follicle, before once again reverting to eumelanogenesis. This manifests as a subapical yellow band on a background of dark hair (see Fig. 5). Agouti banding is a common phenomenon seen in many mammalian species, although it is not observed in humans. Pigment-type switching is absent in certain coat-color mutants. Mice with a null mutation for *ASP* (the murine orthologue to *ASIP*) have a completely black coat color with no yellow banding, indicative of continuous MC1R activity (this is the origin of the "black" designation in the C57Black6 mouse strain). Similarly, mice bred with a continuously active *somber* allele ($MC1r^{E\text{-}So}$) also have completely black coats. In contrast, mice engineered to express *ASP* continuously via a heterozygous lethal yellow allele (A^{Y}) demonstrate a completely yellow coat. This is reflective of ASP inhibition of MC1R. An inactivating mutation in the receptor ($MC1r^{e}$) also produces a similar yellow phenotype. Furthermore, mice with loss-of-function mutations for both *MC1R* and *ASP* exhibit completely yellow coat fur, suggesting that ASP signaling is dependent upon MC1R functionality. This

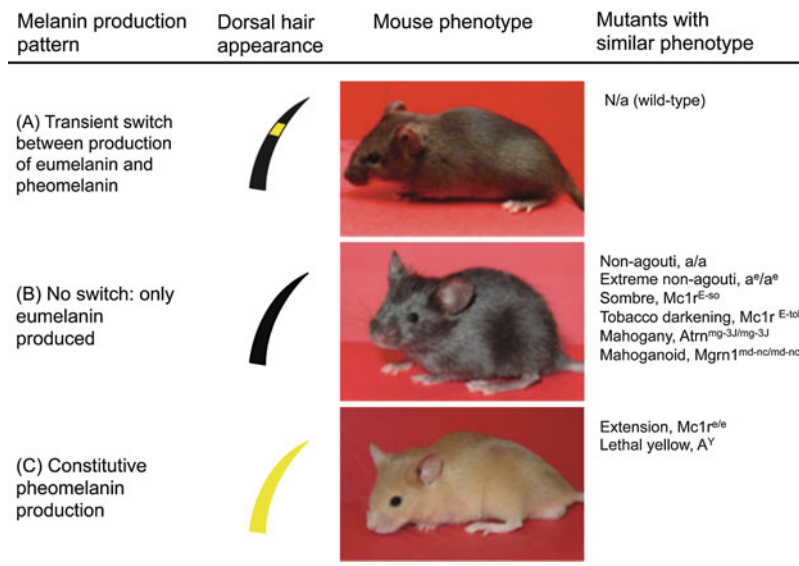

Fig. 5 Pigment-type switching in mice. There are three basic colors of melanin pigmentation in mice: agouti, black/brown, and red. (**a**) Agouti (wild-type mice) is a result of a transient switch to pheomelanin production during a baseline eumelanin-producing state. This creates a yellow band on a black background. (**b**) Black/brown is a result of continuous eumelanin production. This is a result of gain-of-function (*GOF*) *Mc1r* mutations or loss-of-function (*LOF*) mutations in agouti, Atrn, or Mgrn1. (**c**) Yellow fur is a result of continuous pheomelanin production. This is a result of LOF in Mc1r or GOF in agouti (Image from Walker and Gunn 2010)

strengthens the understanding of ASP as a ligand for MC1R, as MC1R is epistatic to ASP. An important caveat is that while increased ASP signaling results in greater pheomelanin production, defective MC1R signaling does not necessarily increase the amount of pheomelanin. Thus, ASP may be necessary to induce pheomelanogenesis. ASIP has two accessory proteins: attractin (ATRN), thought to be an obligatory accessory receptor for ASIP, and mahogunin (MGHN1), an E3 ubiquitin ligase. In vitro studies have suggested that ASIP may signal through MC1R to activate these proteins via a cAMP-independent pathway (Hida et al. 2009). Mutations in these proteins affect the functionality of ASIP. In humans, the larger variety of *ASIP* genetic variations makes understanding its role more difficult.

Interestingly, a third ligand involved in the pigment-type switching system has been identified: β-defensin 103. This ligand was discovered in canine coat-color gene mapping experiments performed by Candille and colleagues to better understand what alleles determined the dominant black coat color in dogs (Candille et al. 2007). Additionally, mice that transgenically express the black canine allele have black coats with small areas of agouti-banded hairs. β-defensin 103 competes against α-MSH binding at MC1R but does not activate the cAMP pathway. Rather, it seems the resultant black coat phenotype is a result of either inhibition of ASP binding to MC1R or interference with an ASP co-receptor by β-defensin 103.

MITF

MITF is widely known as the master regulator of melanocytes, as it has a central role in directing melanocyte development, function, and survival. MITF is a basic helix-loop-helix leucine zipper (bHLHZip) transcription factor encoded by the *MITF* locus that is active in lineage-specific pathway regulation of melanocytes, osteoclasts, and mast cells. Mutation in *MITF* leads to defects in these cell types. MITF is a member of the MIT family and can heterodimerize with related bHLHZip transcription factors, including the transcription factors E3, EB, and EC (TFE3, TFEB,

TFEC). Together with these transcription factors, MITF binds to DNA as a dimer at the E-box motif (CANNTG).

As shown in Fig. 6, the *MITF* gene has at least nine different promoter-exon units for each of the MITF isoforms (Levy et al. 2006). These isoforms differ primarily in their first exon, which encodes the transcriptional activation domain, thus giving amino terminus specificity. All isoforms share the carboxy-terminus, encoded by eight downstream exons, which contains the bHLHZip structure that is used for dimerization and DNA recognition. The melanocyte-specific exon 1 (exon1M) is transcribed exclusively in melanocytes and gives rise to the MITF-M isoform. This exclusive expression is due to its unique melanocyte-restricted promoter enhancer. *MITF-M* transcription is upregulated by several transcription factors that can bind to its promoter region, including SOX10, CREB, PAX3, LEF1, and MITF itself (see Fig. 7). The specificity of the MITF-M isoform to melanocytes is enhanced in part due to the obligate cooperativity between cAMP and SOX10, which is only expressed in cells of neural crest origin. WNT and α-MSH activate pathways responsible for driving activity of the *MITF*-M promoter. For example, in the WNT pathway, WNT proteins bind to Frizzled receptors, initiating interaction between β-catenin and the LEF1 transcription factor, and ultimately induction of the *MITF*-M promoter. Thus, multiple signals converge to induce expression of the *MITF*-M promoter.

MITF also undergoes numerous posttranslational modifications. MITF is phosphorylated by several kinases, including MAPK ribosomal s6 kinase (RSK) and glycogen synthase kinase 3β (GSK3β). Posttranslational modification often leads to MITF repression or degradation. For instance, KIT activation in melanocytes triggers the phosphorylation of two serines on MITF: Ser73 by extracellular signal-regulated kinase 2 (ERK2) and Ser408 by p90 RSK (p90RSK). Phosphorylation at Ser73 targets MITF for ubiquitin-mediated proteolysis. Another form by which MITF undergoes posttranscriptional modification is sumoylation. MITF can be sumoylated at two residues, one of which (E318K) is disrupted by a recurrent mutation in certain individuals with familial melanoma, thereby constitutively increasing MITF's activity.

Severe loss-of-function mutations in *MITF* cause serious autosomal dominant auditory-pigmentary disorders, including the autosomal dominant condition WS2a and Tietz syndrome

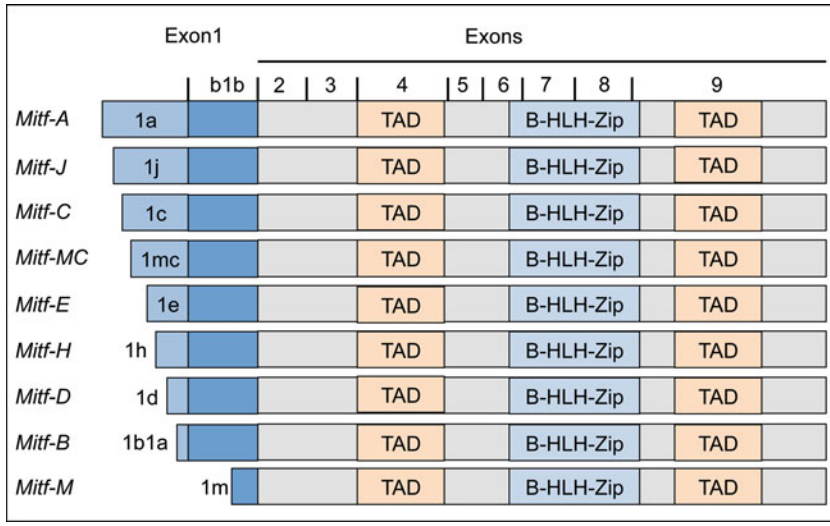

Fig. 6 MITF isoforms. Thus far, nine different promoters have been identified for MITF. MITF isoforms vary within the first exon but share exons 2–9, which contain common functional domains. The *Mitf-M* promoter is expressed only in melanocytes and gives rise to the isoform M (Image adapted from Levy et al. 2006)

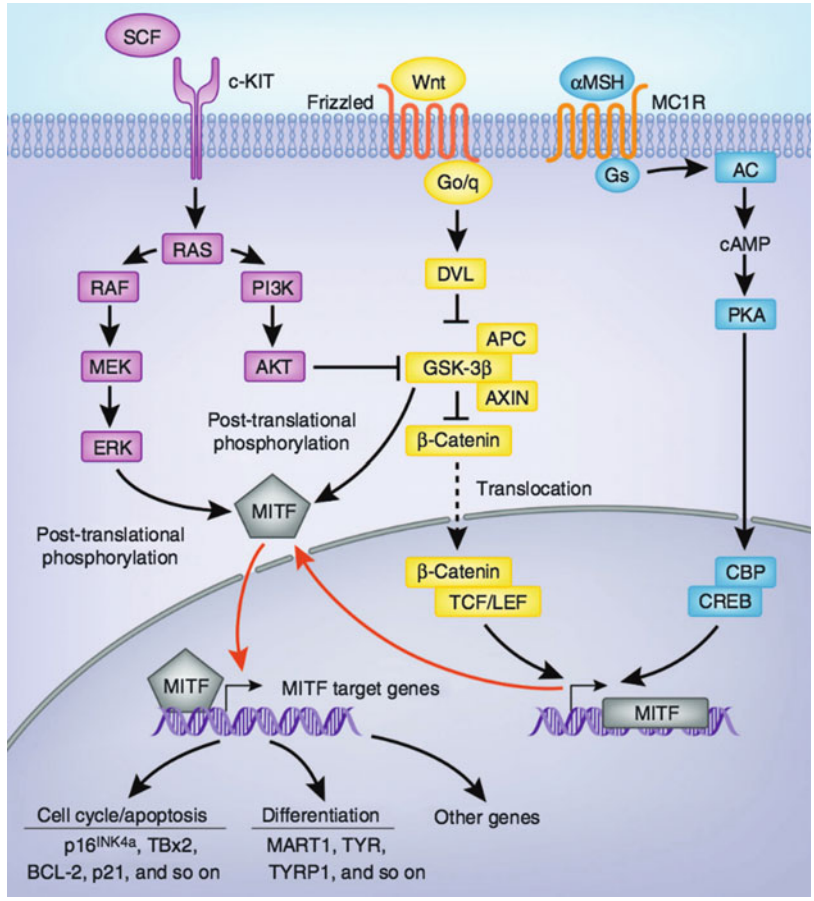

Fig. 7 MITF regulation and its target genes. MITF receives input from several upstream pathways, including c-Kit (purple), Wnt/β-Catenin (yellow), and α-MSH (blue). Activation of c-KIT by SCF activates RAS and the downstream MAPK and PI3K pathways, which influence MITF activity. In the Wnt/β-Catenin pathway, Wnt activates Disheveled (DVL), which inhibits degradation of β-catenin. β-catenin and LEF stimulate MITF expression. Binding of α-MSH to MC1R leads to PKA activation, the phosphorylation of CREB, and the recruitment of CREB-binding protein (CBP) to aid in activation of the MITF promoter (Image from Hocker et al. 2008)

(TS). More than 35 gene mutations have been identified in WS2a, and they range from producing a truncated MITF protein to altering the helix-loop-helix or leucine-zipper motif. These mutations result in disruption of dimer formation. Heterozygous individuals with WS2a are found to have a reduction of melanocytes that results in varying degrees of sensorineural deafness and a patchy distribution of cutaneous hypopigmentation. Mouse models of *Mitf* null mutations are similarly found to lack melanocytes and have white fur, microphthalmia, and deafness. The other types of WS provide additional insight into MITF regulation. WS is divided into four types depending on the presence or absence of additional symptoms. Types I and III are caused by *PAX3* mutations. These types are characterized by additional abnormalities in the facial musculoskeletal system. WS type IV is caused by an array of gene mutations, including those that encode the proteins SOX10, endothelin, and EDNRB.

TS is another manifestation of MITF mutation, in which a single amino acid within the basic domain is altered, resulting in a dominant negative protein as dimers with a wild-type MITF partner are unable to be transported to the cell nucleus to appropriately bind with DNA. The phenotype is one of complete penetrance and is characterized by deafness with light hair and skin color. In contrast, WS2 has a more variable appearance. TS may be considered a more severe form of WS2. Thus far, two *MITF* gene mutations in the basic motif region have been identified in people with TS. Few functional studies have been performed to understand the alterations in MITF signaling in these rare diseases. Depending on the location of the *MITF* mutation in

WS2-associated MITF, varying degrees of alterations in protein activity, DNA-binding ability, and cellular localization may be observed in vitro (Zhang et al. 2013). Mutations in the nuclear localization signal are believed to cause a more dramatic effect. Of note, the observed phenotypic effects in TS are thought to be due to haploinsufficiency rather than a dominant negative effect. Surprisingly, in one study, TS-associated MITF showed comparable in vitro activity to WT MITF, despite the severity of the TS phenotype.

MITF is involved in the expression of genes promoting melanocyte survival (*CDK2*, *p16INK4a*, *TBX2*, *CDKN1A*), motility (*MET*), differentiation and apoptosis (*BCL2* and *HIF1A*), and melanosome production (*TYR*, *TRP1*, *TRP2*, *SLC45A2*, *PMEL*, *RAB27A*). MITF also plays a central role in melanogenesis. Melanogenesis is regulated mainly through the α-MSH/MC1R interaction leading to activation of the cAMP/PKA signaling cascade to induce expression of *MITF*. Transcriptional targets of MITF include the melanogenic genes TYR, TRP1, and TRP2 and the matrix protein PMEL. MITF additionally promotes pigmentation by upregulating EDNRB. EDNRB activation by endothelins 1 and 3 activates MAPK, which phosphorylates MITF, stimulating MITF expression. Expression of DICER, a regulator of miRNA maturation, is also upregulated by MITF during melanocyte differentiation. DICER expression causes posttranscriptional processing of miRNA-17, causing downregulation of BCL2-interacting mediator of cell death (BIM) and thus promoting melanocyte survival.

MITF gene amplification or recurrent E318K mutations have been identified in melanoma. *MITF* amplification is more prevalent in metastatic disease. *BRAF* mutation and *p16* inactivation were found to co-occur with MITF amplification in melanoma cell lines. It was also found that ectopic MITF expression in the presence of a $Braf^{V600}$ mutation transformed human melanocytes into melanoma, establishing MITF's role as a melanoma oncogene (Garraway et al. 2005). Thus, targeting MITF in combination therapies may confer a greater survival benefit. MITF can thus be representative of a "lineage addiction" oncogene.

The Effect of pH on Melanogenesis

It has been noted that melanosomes of melanocytes derived from lighter human skin have lower tyrosinase activity and are more acidic compared to melanocytes from darker human skin, which have higher tyrosinase activity. Based on these observations, a link between pH and melanogenic activity has been suspected, but the mechanism is poorly understood. One of the molecules believed to be involved in pH regulation of melanogenesis is vacuolar (V)-ATPase (Kondo and Hearing 2011). cAMP upregulates the expression of V-ATPase subunits as well as acidification of melanosomes. Ion transporter proteins also regulate the pH of melanosomes and can affect pigmentation. For instance, SLC45A2 (MATP/AIM1) is a transporter protein localized to melanosomes that mediates melanin synthesis. Its mutation leads to OCA4. The P protein, a sodium/sulfate transporter that mediates melanosomal pH neutralization, similarly causes OCA2 when mutated (Ancans et al. 2001). Despite knowledge of these enzymes and membrane proteins, more research has to be done to understand the precise effect pH has on melanogenesis.

Part 3: UVR, Skin Phototype, and the Link to Melanoma

An Overview of UV, Tanning, and Sunburns

Properties of UVR

UVR has a wide array of effects on the skin, including tanning, photoaging, immune suppression, phototoxicity, and carcinogenesis (Armstrong and Kricker 2001). The human skin buffers this damage by thickening of the epidermis, DNA repair mechanisms, antioxidant enzymes, and apoptosis. UV light is an electromagnetic radiation with a wavelength shorter than that of visible light but longer than that of X-rays. Although UVR is invisible to humans, it can have a profound effect on our biology

and health. Common physical manifestations of UVR exposure include suntan, freckling, and sunburn. Long-term effects include melanoma and non-melanoma skin cancer. UVR also provides unseen protective health benefits such as the conversion of vitamin D into a usable form.

There are three regions of the UV spectrum: UVA (400–320 nm), UVB (320–280 nm), and UVC (280–100 nm) All three have been linked to an increased risk of skin cancer. The ozone layer differentially filters the different types of UVR. Sunlight is composed of approximately 94% UVA and 6% UVB. UVC is completely filtered by the ozone layer. Although UVA makes up a greater proportion of solar radiation, UVB delivers more intense dose response. UVA has the longest wavelength and can penetrate into the dermis. It is responsible for generating an immediate tan, premature skin aging, and wrinkles. Indoor tanning equipment typically emits UVA with a smaller proportion of UVB. UVB rays have shorter wavelengths. UVB penetrates the epidermis and is responsible for a delayed tan, sunburns, most skin cancers, and cataracts. UVB is also responsible for the photolysis step involved in vitamin D biosynthesis within the skin. Factors affecting UV delivery to the Earth include time of day, season, latitude, altitude, cloud cover, and reflection off surfaces.

Facultative Pigmentation and Photoproducts

Tanning is the most common form of acquired skin pigmentation and is believed to be a form of environmental adaptation in humans. UVR increases skin pigmentation by increasing active epidermal melanocytes, melanogenic enzymes, and melanocyte dendricity. Soon after UVR exposure, keratinocytes release pro-inflammatory cytokines. UVR induces DNA damage in keratinocytes, which stabilizes the *p53* tumor suppressor gene and activates transcription of POMC (see Fig. 8). POMC is enzymatically cleaved to produce α-MSH, which is then released by keratinocytes and binds to MC1R on melanocytes, ultimately leading to transcription of MITF via CREB.

Generation of ROS followed by depletion of cellular antioxidants also occurs. ROS produced include hydrogen peroxide, hydroxyl radical, singlet oxygen, and peroxyl radicals. The ROS go on to damage lipids, proteins, and DNA. Antioxidant enzymes in the skin (superoxide dismutatase, glutathione peroxidase, and catalase) actively neutralize ROS.

The skin contains several photosensitive molecules called chromophores that, when receiving photons from UVR, are raised to a higher energy state. After absorbing a photon, the chromophores can pass on the excited energy state to other molecules, causing a chain reaction. DNA and RNA contain strongly absorbing chromophores for UVB, as aromatic heterocyclic nitrogen bases absorb wavelengths at 260–265 nm. Although UV targets many epidermal cellular components, including nucleic acids, proteins, lipids, and other macromolecules, its effect on DNA is probably the most profound (Chen et al. 2014b). The premutagenic photoproducts cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (64PP) are commonly generated. These lesions alter the structure of DNA, inhibiting polymerases and arresting replication. CPDs consist of a four-membered ring arising from the coupling of carbon-carbon double bonds of pyrimidines. This structure interferes with base pairing during DNA replication and increases the rate of mutations. 64PP occur at only one-third of the frequency of CPDs but they are more mutagenic. Dimers can be repaired by photoreactivation or nucleotide excision repair. If left unrepaired, they can lead to highly specific mutations known as UVR fingerprint mutations (CC➔TT double-base substitutions and C➔T substitutions) at dypyrimidine sites. Xeroderma pigmentosum, a genetic disorder of nucleotide excision repair in which these types of mutations are left unrepaired, causing a fourfold risk of childhood melanoma.

Other Effects of UVR

Sunburns are understood as one of the greatest risk factors for cutaneous melanoma development. Although it is frequently cited that a history of severe sunburns during childhood is the greatest risk factor for cutaneous melanoma

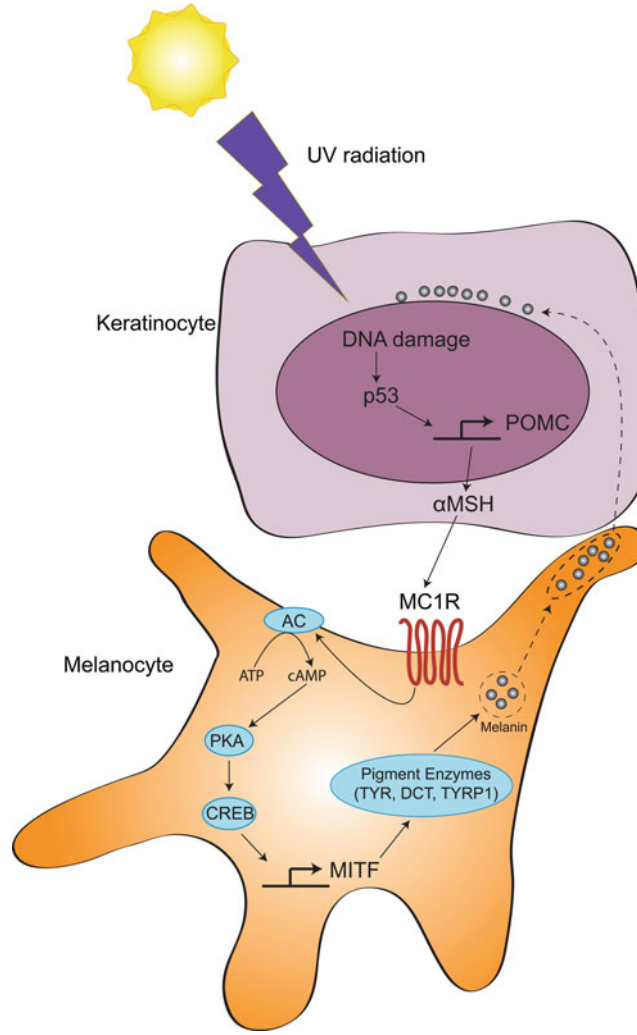

Fig. 8 UV-mediated tanning pathway. UVR causes DNA damage, which activates p53. The p53 protein promotes production of proopiomelanocortin (POMC), which can be processed into either adrenocorticotrophic hormone (ACTH), β-endorphin (β-end), or α-melanocyte-stimulating hormone (α-MSH). α-MSH binds to melanocortin 1 receptor (MC1R) on adjacent melanocytes and promotes melanogenesis and storage of melanin within melanosomes. Melanin is transported to keratinocytes and forms a cap over the nucleus to protect the cell's DNA from UVR (Image from Hsiao and Fisher 2014)

development, a recent meta-analysis taking into account dose-response effects has indicated that melanoma risk rises with increasing number of sunburns during all life periods, whether it be childhood, adolescence, or adulthood (Dennis et al. 2008).

UVB is known to more potently induce sunburns than UVA by about 1,000-fold. UVB-induced erythema can be detected within several hours after UVB exposure and can fade within a day. However, in fair-skinned individuals, the erythema may last significantly longer. At the cellular level, erythema is classically associated with the presence of apoptotic keratinocytes, known as sunburn cells.

As stated above, UVB induces vitamin D production in the skin. Vitamin D plays an important role in calcium metabolism and likely multiple other signaling/regulatory pathways. Use of UV as the sole source of vitamin D synthesis is unlikely to offer stable maintenance of healthy vitamin D-related metabolism, given that UV is an unpredictable source of vitamin D synthesis. Its dose response is dependent upon numerous variables, including latitude, time of year, time of day, phototype of the person, amount of skin exposed, and duration of exposure. Of course, UV as a source of vitamin D also brings concurrent carcinogenic risk. For these reasons it is strongly recommended that individuals define their

vitamin D blood levels using routine blood testing and obtain oral supplementation (which is readily and inexpensively available) to obtain stable, predictable circulating vitamin D.

UVR also causes immunosuppression both in the skin microenvironment and systemically. In particular, Langerhans cells are depleted from the skin following UV exposure. The evolutionary function of this response is unclear but has been suggested that it is a means of limiting immune reactions following DNA damage from UVR. Evidence shows that UV-induced immunosuppression may be a mechanistic contributor to UV-induced tumor development.

Redefining the Roles of UVR and Melanin in Melanomagenesis

Melanoma incidence rates are continually rising and have increased over 30-fold within the last century. Stratospheric ozone depletion will only worsen these numbers, as decreased ozone layer protection results in greater UV delivery to the Earth. Intense intermittent, rather than chronic, UVR exposure is known as the major risk factor for melanoma. Much is still being learned about the relationship between UV, melanin, DNA damage, and cancer risk. Although UVA and UVB have an established role in their contribution to non-melanoma skin cancer, their roles in melanomagenesis have been less clear. However, recent research has begun to unravel the precise contributions of both types of UV to the onset of melanoma formation.

UVR and Melanomagenesis

UVA and UVB cause distinct alterations to the genome and, as a result, on skin pigmentation. Traditionally, UVA has been known to cause oxidative damage, which generates ROS that can damage DNA and increase photoaging of the skin. UVB causes direct DNA damage in the form of CPDs and 64PP almost instantaneously. Although UVA was once thought to primarily induce skin aging, research over the past decade is now implicating it as a causal factor in skin cancer in addition to UVB. Thus, there are believed to be at least two separate UV wavelength-dependent pathways for the induction of melanomagenesis.

Clues that UVA and UVB induce melanoma via separate pathways arose through the observation that UVB irradiation led to similar rates of melanoma in both black and albino hepatocyte growth factor (HGF) transgenic mice (engineered to express melanocytes in the epidermis and dermis). This suggested that initiation of melanoma development by UVB is pigment independent. In contrast, black HGF transgenic mice developed melanoma following UVA irradiation, whereas matched albino strains did not (Noonan et al. 2012). This is supportive of two wavelength-dependent mechanisms for UV-induced melanoma: (1) a pigment-independent pathway that is initiated by UVB and (2) a pigment-dependent pathway caused by UVA.

These authors performed several studies to better characterize these two pathways. CPD and 64PP were detectable in both black and albino transgenic HGF mice following UVB irradiation, but not UVA irradiation. UVA irradiation led to only low levels of TT-CPD lesions and no 64PP in both strains. Thus, it was thought that UVA-induced CPD formation could not explain the pigment-dependent mechanism of UVA-induced melanoma. However, one of the photooxidative products of UVA, 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), was found only in pigmented UVA-irradiated HGF transgenic mice, but not in their UVA-irradiated albino counterparts. In contrast, UVB is not effective at producing 8-oxodG. Thus, it appears that 8-oxodGuo development requires both UVA and melanin.

Additional information about UVA, melanin, and melanomagenesis came from the discovery of "dark CPDs." UVA has been traditionally considered to be inefficient at making CPDs. However, it was found that melanin-containing murine melanocytes generated CPDs for at least 3 h after UVA exposure (Premi et al. 2015). This effect was not seen in melanocytes derived from albino mice, implicating melanin as an active player in DNA damage. Dark CPDs were shown to constitute half of all CPDs. The presence of melanin itself, rather

than its synthesis following UVR, is thought to contribute to CPD formation as CPDs were particularly increased in keratinocytes at the 2-h time point. Pheomelanin is even more potent in the production of dark CPDs as both initial and dark CPDs are twice as frequent in *Mc1r*$^{e/e}$ mice compared to black mice. Thus, pheomelanin appears to be both an inferior shield and a more potent producer of dark CPDs. Ultimately, it was found that irradiating melanin-containing cells with UV induced both superoxide and nitric oxide formation and caused a spike in levels of their product and strong pro-oxidant peroxynitrite. Peroxynitrite leads to melanin degradation and the development of melanin-like granules in the nucleus. Peroxynitrite is one of the few molecules in the body that can excite electrons to a triplet state. Thus, up to several hours after the initial UV exposure, peroxynitrite also continues to excite melanin derivatives to a triplet state that has the high energy of a UV photon. These electronically excited melanin fragments can then transfer their energy to DNA, generating dark CPDs. Thus, melanin has a dual nature, in that it is both carcinogenic and protective.

Based on these results, it appears that people with fair skin are at particularly high risk of instantaneous DNA damage from UVB due to poor melanin shielding and are still vulnerable to the pigment-dependent pathway of UVA-induced melanoma.

Pigmentation and Melanomagenesis

Although the protective benefits of melanin, and eumelanin in particular, have long been lauded, its role as a protective agent has become more nuanced with the aforementioned discoveries. Despite this, there is a wealth of evidence that supports the fact that individuals with darker skin are more protected against skin cancer. Individuals with the lightest skin types are at approximately a 70-fold greater risk of developing skin cancer than individuals with the darkest skin types. It has been shown that the epidermis of the lightest skin types allows 55% of UVA and 24% of UVB to penetrate the skin, whereas the epidermis of the darkest skin types permits only 17.5% of UVA and 7.4% of UVB (Brenner and Hearing 2008). Differences in melanosome processing are also present in different skin types. Melanosomes in dark skin are resistant to degradation by lysosomal enzymes and are able to form supranuclear caps in keratinocytes. In contrast, melanosomes in light skin are degraded to "melanin dust" within the suprabasal layers, which is less effective in providing UV protection.

The increased melanoma risk within individuals with red hair and light skin has historically been attributed to inadequate UV protection, but research in recent years has revealed a UVR-independent pheomelanin-dependent mechanism of melanoma development. Mitra and colleagues developed a preclinical model of melanomagenesis in redheads by engineering pheomelanin-expressing *Mc1r* loss-of-function mice possessing a *Braf*^{V600E} mutation selectively expressed within melanocytes (Mitra et al. 2012). These red-haired mice were observed to develop spontaneous melanomas in the absence of UVR at a tenfold higher rate than mice without pheomelanin. Additionally, in the absence of UV exposure, the red-haired mice were found to have significantly elevated levels of lipid peroxidation and oxidative DNA damage within the skin compared to genetically matched albino mice. This strongly suggested that pheomelanin, and in particular the oxidative stress resulting from its presence, plays an independent role as a driver of melanoma formation (Morgan et al. 2013).

Wendt and colleagues sought to study this phenomenon in humans. Specifically, they generated a large case-control study to examine the effects of *MC1R* variants on melanoma incidence among 991 melanoma patients and 800 controls (Wendt et al. 2016). To isolate UV-independent effects, the researchers adjusted the analysis for age, sex, and variables related to sun exposure (e.g., history of sunburns in childhood and adolescence and visible signs of actinic sun damage) and discovered there was a 1.5-fold (95% CI, 1.01–2.21; $P = 0.04$) to 2.63-fold (95% CI, 1.82–3.81; $P < 0.001$) melanoma risk increase. Thus, humans with *MC1R* variants also appear to suffer from the same pheomelanin-driven and UV-independent risk of melanoma development.

These findings further confirm the significant genetic contribution to melanomagenesis and highlight the need for an improved understanding of the mechanism behind pheomelanin-mediated melanoma formation. It is not clear how pheomelanin increases the amount of oxidative stress in the skin. It has been hypothesized that one of two mechanisms may occur: (1) pheomelanin generates ROS that cause oxidative DNA damage, or (2) pheomelanin synthesis consumes antioxidants that leave the cell susceptible to ROS-mediated damage. Pheomelanin has also been shown to affect the cellular redox system itself, as Panzella and colleagues demonstrated that pheomelanin significantly lowered levels of both reduced glutathione (GSH) and nicotinamide adenine dinucleotide (NADH) (Panzella et al. 2014). Although individuals with light skin and red hair are most strongly affected by the negative effects of pheomelanin, it is possible that a dose response occurs in which individuals with lower pheomelanin levels may have tempered but still significant negative effects from oxidative stress in the skin. It is unclear whether specific antioxidants might be able to combat this newly recognized UV-independent mechanism of carcinogenesis, but it is highly likely that this effect is significantly amplified by UV exposure.

The p53 Protein and Melanomagenesis

It is understood that UV exposure of the skin causes DNA damage and that the cumulative effect of repeated damage is a contributor to skin cancer development. However, the precise mechanism whereby UVR initiates melanomagenesis is poorly understood. In an effort to answer this, the role of DNA damage in the tanning pathway as well as carcinogenesis has been closely examined. Melanization after UVR is enhanced by DNA repair. Furthermore, topical application of small dipyrimidine DNA fragments, which imitate photodamaged pyrimidine dinucleotides excised during DNA repair, upregulates tyrosinase and increases pigmentation. Alternative DNA-damaging processes, such as X-ray irradiation and exposure to chemotherapeutic agents, can also elicit a tanning response likely through overlapping pathways. Studies have established evidence of the ability of UVR to generate tumor-initiating DNA mutations in melanocytes and exome sequencing studies of melanoma have clearly demonstrated a major contribution of UV signature mutations.

The tumor p53 protein (p53), which is encoded by the *TP53* gene, has been nicknamed the "guardian of the genome" because of its role in preventing genome mutation. The p53 protein has a central role in the skin's response to UVR. p53 is a transcription factor whose stability is rapidly increased following DNA damage. p53 is involved in several signaling pathways that become activated after stressors such as DNA damage, oxidative stress, and membrane compromise. Depending on the degree of damage, p53 may activate genes involved in DNA repair or may initiate apoptosis. The actions of p53 are mediated via control of cell cycle checkpoint activity and regulation of DNA repair machinery.

UVR has been linked to p53 expression. Immunohistochemistry of UV-irradiated human skin demonstrates an increase in p53 within suprabasal cells peaking at 4 h after exposure, as well as in basal cells peaking 48 h after exposure (Pontén et al. 1995). Topical sunscreen and more darkly pigmented skin eliminated UV-induced expression of p53. Following p53 upregulation, p53 simultaneously stimulates expression of POMC in keratinocytes, leading to transcription of tyrosinase and TRP1 genes within underlying melanocytes (Cui et al. 2007).

Two common polymorphisms in p53 are an arginine and proline at position 72 (Arg72 and Pro72). The Arg72 form has a greater tendency toward promoting apoptosis, whereas the Pro72 form confers elevated transcriptional activity (Miller and Tsao 2010). The effects of the Pro72 polymorphism are nuanced, as it is more commonly found in individuals living closer to the equator (likely due to the improved tanning ability conferred by this polymorphism) but has also been discovered to be associated with a greater risk for melanoma and basal cell carcinoma (Han et al. 2006). In particular, the Pro72 allele may be especially detrimental in individuals with loss of function in MC1R, as damaged cells would have a lower tendency to undergo apoptosis after DNA

damage. Indeed, Nan and colleagues found the highest risk of melanoma in women with light pigmentation along with MC1R variants (Nan et al. 2008).

Sunscreen

Sunscreen is a topical product containing UV filters that absorb or reflect a fraction of solar UVR and thus protects against sunburn. Sunscreens can be divided into inorganic UV filters (those that reflect sunlight) and organic UV filters (those that absorb UV light). Inorganic filters include titanium dioxide and zinc oxide, and organic UV filters include avobenzone, oxybenzone, octinoxate, octocrylene, and padimate O.

Sunscreens are categorized according to SPF (sun protection factor), which is measured by calculating the minimal dose of UVR necessary to cause confluent erythema at 24 h after exposure on the protected skin of a certain phototype, compared to the unprotected skin. SPF, as a measure of erythema, is primarily based on UVB protection rather than UVA, because erythema is induced by UVB irradiation. "Broad-spectrum" solar protection would extend into the UVA range, but the United States currently lacks a federally regulated system for quantitatively rating UVA protection. In Europe there are several different rating systems to measure effectiveness in blocking UVA rays, such as the immediate pigment darkening assay (IPD) and the persistent pigment darkening assay (PPD).

Fewer UVA-protecting sunscreen ingredients are available in the United States compared to other countries. However, in 2006, the Food and Drug Administration (FDA) approved an efficacious UVA-protecting compound, ecamsule. Despite this increased availability of UVA-protecting UV filters, it was discovered that half of all sunscreens marketed as having broad-spectrum protection within the United States provided only low to medium protection against UVA. This may be due in part to the observation that the UVA filter avobenzone is degraded in the presence of the UVB filter octinoxate following UV exposure (Sayre et al. 2005).

Despite the widespread and continued recommended use of UV filters (including by these authors), there have been some uncertainties about the safety details of these compounds. It has been suggested that the aromatic organic compounds might penetrate through either the stratum corneum or hair follicles into the epidermis. Researchers have also voiced concerns about systemic absorption, especially after noting discernable levels of UV filters within both breast milk and urine samples following topical sunscreen application. Furthermore, both organic and inorganic UV filters have been observed to induce ROS following UVR within the epidermis.

Even though numerous epidemiologic studies have demonstrated both an increased incidence and risk of melanoma with increased ambient solar radiation and cutaneous sun sensitivity, the protective effect of sunscreen against melanoma has been surprisingly difficult to demonstrate. Mouse experiments have shown that sunscreen can delay the onset of melanomagenesis. However, findings from case-control and cohort studies on sunscreen use in humans have been largely uninformative and generally fail to achieve statistical significance. Unfortunately, many past studies were based upon antiquated sunscreen formulas, which make results difficult to extrapolate to current day usage. Studies were also often based on indirect measures of melanoma risk, such as nevi quantification. One commonly cited study performed by Gallagher and colleagues on Canadian children revealed a small reduction in new melanocytic nevi following regular sunscreen use (Gallagher et al. 2000). It is difficult to interpret how exactly these observations contribute to melanomagenesis, particularly when current thinking is that only a minority of melanomas arise from preexisting nevi.

A key study published by Green et al. examined the incidence of melanoma in 1,621 patients in Australia in a randomized controlled trial of daily sunscreen application and beta-carotene supplementation over a 5-year period with a 10-year follow-up (Green et al. 2011). Participants assigned to the sunscreen intervention were asked to apply on a daily basis a broad-spectrum sunscreen containing the chemical filters 2-ethylhexyl-*p*-methoxycinnamate and 4-*tert*-butyl-4′ methoxy-4-dibenzoylmethane,

with an overall SPF of 16. Control participants were asked to continue their normal behavior of sun protection, which ranged from sporadic sunscreen use to no sunscreen use. Also of note, half of study participants were randomly assigned to supplementation with 30 mg of the antioxidant beta-carotene that has been hypothesized to counteract UV-induced oxidative DNA damage, whereas the other half was provided a placebo supplement. The results demonstrated a 50% reduction in invasive melanoma among those who used sunscreen compared to those who did not, and this trial provides the strongest evidence to date of reduction in the incidence of invasive melanoma after regular application of sunscreen in adults. Thus, the evidence suggests that sunscreen affords partial UV protection, and it is suggested that sunscreen be utilized in combination with sun avoidance strategies.

There are several potential explanations for the modest or even conflicting results from large studies analyzing melanoma prevention by sunscreen use. These include old formulations, inadequate application or reapplication of the formulations, and insufficient follow-up intervals. It is also possible that ROS produced by chemical sunscreens within the skin may antagonize the UV-protective benefits. Other confounding factors include selection bias of study participants (in which higher-risk individuals use sunscreen more often) and inadequate education of proper sunscreen use. However, there are promising alternatives that may soon be coming our way. Deng and colleagues recently devised a method of encapsulating UV filters within bioadhesive nanoparticles, which have the advantages of being adherent to the stratum corneum without penetrating deeper into the epidermis (Deng et al. 2015). Broad-spectrum protection against UVA as well as UVB is hopefully shortly on the horizon in the United States and may provide significantly enhanced protection.

The role of topical antioxidants in the prevention of melanoma is also debatable. Within this study, there was no observed effect of the beta-carotene intervention on either increasing or decreasing the risk of melanoma incidence. One of the concerns of antioxidant supplementation is that if applied to an area of the skin with islands of UV-induced mutations, the presence of antioxidants may in fact stabilize cells containing these mutations and allow their continued survival via anti-apoptotic mechanisms.

Therapeutic Pigmenting Agents

Several therapeutic pigmenting agents have been researched to harness the protective properties of eumelanization. A UV-independent agent that hyperstimulates pigment synthesis may be valuable, as pigmentary protection gained by tanning cannot otherwise be achieved without the detrimental side effects from UVR. In particular, researchers have attempted to perturb the UV signaling pathway at various points to modulate the activity of MC1R, adenylate cyclase, cAMP, and MITF.

Piperine, a compound extracted from black peppers, has been marketed as a natural supplement to aid in tanning. Experimental studies have suggested that piperine can induce melanocyte proliferation and dendrite formation in combination with UV exposure in vitro (Soumyanath et al. 2006), as well as repigmentation in a sparsely pigmented vitiligo mouse model (Faas et al. 2008). However, human studies are lacking. Additionally, the combination of UV exposure to maximize tanning is obviously not ideal as it apparently requires UV and its effects on UV carcinogenesis are unknown.

In an effort to sidestep the DNA damage pathway, a chemically modified superpotent α-MSH analogue, [Nle^4-D-Phe^7]-α-MSH, has been tested via subcutaneous injection in human subjects. Researchers demonstrated that supplementation with an α-MSH analogue led to a melanin increase of 41% in subjects with low-MED skin type and a melanin increase of 12% in those with high-MED skin type (Barnetson et al. 2006). Additionally, they observed that formation of epidermal sunburn cells and thymidine dimer formation was halved in subjects with low-MED skin type following UV exposure.

Forskolin, a cAMP activator, is a small molecule that has been able to activate the pigmentation pathway downstream of Mc1r, thus achieving pigmentation even in nonfunctional

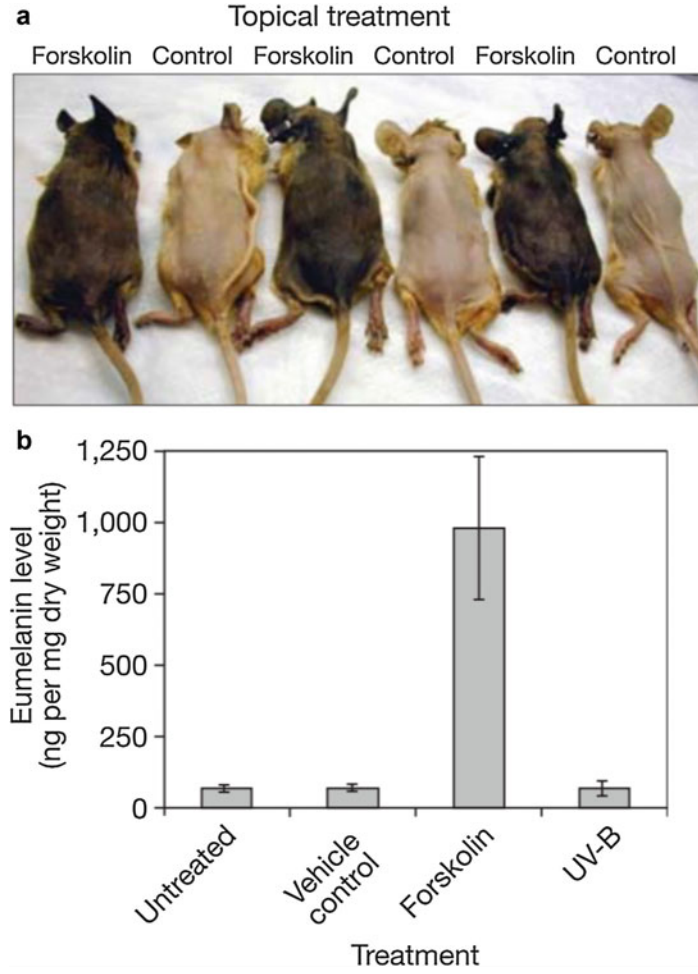

Fig. 9 Pigmentation "rescue" by topical forskolin in "redhaired/fairskinned" mice (D'Orazio et al 2006). The adenylate cyclase agonist forskolin was topically applied to the skin of redhaired (Mc1r(e/e) mice containing the K14-SCF transgene that causes retention of epidermal melanocytes. (a) Forskolin treatment induced significant skin darkening, as compared to the control vehicle treatments. (b) Quantification of eumelanin pigment demonstrated significant induction of eumelanin synthesis by forskolin. In contrast, UV-B treatment did not induce eumelanin synthesis, consistent with the observation that redhaired individuals do not tan after UV

Mc1r mutant mice (see Fig. 9). Regular topical forskolin application in mouse models leads to dark pigmentation, protection against both UVR-mediated damage and carcinogenesis, and decreased levels of both CPDs and 64PP in keratinocytes (D'Orazio et al. 2006). Unfortunately, forskolin does not have good topical penetration in the human skin.

Therapeutic pigmenting agents may not be without risk. It has been reported that use of these agents can result in atypical nevi and melanoma. Specifically, case reports exist of melanoma developing in an extremely short time period after heavy Melanotan 2 (an α-MSH analogue) usage. However, a recent controlled clinical study did not reveal evidence of increased melanoma risk (Langendonk et al. 2015).

Conclusion

Pigmentation is a multistep process. It requires adequate melanocyte development, proper homing to epidermal and follicular locations, the formation of appropriate dendritic connections with recipient epithelial cells, melanin production by melanosomes, and precise transfer of this pigment to the surrounding epithelial network. These pathways are further controlled precisely at the molecular level by enzymes, structural proteins, transcriptional regulators, transporters, receptors, and growth factors. Alterations in any of these pathways or factors can cause variations or defects in pigmentation. In addition to its social significance, pigmentation plays an important role in the

development of skin cancers including melanoma. Over the past years, there has been an explosion of knowledge about the interplay between melanin, UVR, and melanoma progression. Additionally, attempts have been made to increase pigmentation therapeutically as well as target mediators of pigmentation in melanoma. Although pigmentation is extremely complex, improved knowledge of the intricate pathways involved in the engineering of pigmentation will allow us to both better understand evolutionary conserved processes and improve health outcomes in a vast array of dermatological disorders and diseases.

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