Melanocytes as Instigators and Victims of Oxidative Stress

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Epidermal melanocytes are particularly vulnerable to oxidative stress owing to the pro-oxidant state generated during melanin synthesis, and to the intrinsic antioxidant defenses that are compromised in pathologic conditions. Melanoma is thought to be oxidative stress driven, and melanocyte death in vitiligo is thought to be instigated by a highly pro-oxidant state in the epidermis. We review the current knowledge about melanin and the redox state of melanocytes, how paracrine factors help counteract oxidative stress, the role of oxidative stress in melanoma initiation and progression and in melanocyte death in vitiligo, and how this knowledge can be harnessed for melanoma and vitiligo treatment.

Journal of Investigative Dermatology (2014) **134,** 1512–1518; doi:10.1038/jid.2014.65; published online 27 February 2014

INTRODUCTION

Oxidative stress results from the overproduction of pro-oxidant species in cells, and/or reduction of cellular antioxidant capacity, and it can damage nucleic acids, lipids, and proteins, leading to mutagenesis or cell death (Sander *et al.*, 2004). Reactive oxygen species (ROS) are produced by mitochondria and peroxisomes during normal cellular metabolic processes. The ROS production may be accentuated under pathologic conditions, such as inflammation and cancer, as well as upon exposure to exogenous factors, such as UV or chemicals (Zhang *et al.*, 1997; Klaunig and Kamendulis, 2004; Sander *et al.*, 2004; Klaunig *et al.*, 2009). Skin is the largest organ that interfaces with the environment, and a major source of ROS that are induced by sun exposure. Epidermal melanocytes are particularly vulnerable to excessive ROS production owing to their specialized function: melanin synthesis, which is stimulated by sun exposure, during the process of tanning, and by inflammation that results in postinflammatory hyperpigmentation (Figure 1,2). Oxidative stress can disrupt the homeostasis of (Figure 2) melanocytes, compromising their survival or leading to their malignant transformation (Picardo *et al.*, 1996a; Schallreuter *et al.*, 1999; Govindarajan *et al.*, 2002; Casp *et al.*, 2002a; Gavalas *et al.*, 2006; Fried and Arbiser, 2008; Guan *et al.*, 2008).

MELANIN AND THE REDOX STATE OF MELANOCYTES

Melanin synthesis involves oxidation reactions and superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) generation, which subject melanocytes to oxidative stress (Figure 1) (Koga *et al.*, 1992; Simon *et al.*, 2009). Confinement of melanin synthesis to melanosomes protects other cellular components from oxidative damage. Tyrosinase, the rate-limiting enzyme for melanin synthesis, oxidizes tyrosine to dopa, and dopa to dopaquinone, a specific orthoquinone that can react with nucleophilic compounds such as thiols or amino groups. The catalytic activity of tyrosinase results in the generation of O_2^- (Tomita *et al.*, 1984; Koga *et al.*, 1992). Dopaquinone is converted into dopachrome through a redox exchange. After spontaneous decarboxylation, dopachrome either generates dihydroxyindole (5,6-DHI), which is oxidized into indole quinone, or produces dihydroxyindole carboxylic acid (5,6-DHICA) after tautomerisation by tyrosinase-related protein 2 (TRP2), and 5,6-DHICA is then converted into the corresponding quinone. Moreover, TRP2 protects against oxidative stress by increasing glutathione levels and by reducing the toxicity of quinones and DNA damage induced by free radicals (Michard *et al.*, 2008). The redox cycling from indoles to quinones generates ROS (Nappi and Vass, 1996). Polymerization of these reactive quinones finally leads to the formation of the brown/black eumelanin. The red-yellow pheomelanin differs from eumelanin in that it has a higher ratio of sulfur to quinones, and its synthesis involves the generation of cysteinyl-dopa (instead of dopa), which is converted into benzothiazine derivatives. These differences account for the higher sunlight-induced pro-oxidant property of pheomelanin compared with eumelanin.

In the skin, the balance between the pro-oxidant and antioxidant properties of melanin are mainly determined by the relative eumelanin and pheomelanin contents, the levels of melanin intermediates, and the concentrations of reactive metals within the melanosome microenvironment (Di Donato *et al.*, 2002; Liu *et al.*, 2005). There are conflicting reports about the role of melanin or melanin intermediates as pro-oxidants or antioxidants. Constitutive pigmentation is reported to correlate directly with catalase activity in cultured human melanocytes, and with the levels of thioredoxin reductase in

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Abbreviations: 8-OxodG, 8-oxodeoxyguanosine; BER, base excision repair; DHI, dihydroxyindole; DHICA, dihydroxyindole carboxylic acid; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; MC1R, melanocortin 1 receptor; NAC, N-acetylcysteine; ROS, reactive oxygen species; TRP 2, tyrosinase-related protein 2

Received 26 November 2013; revised 8 January 2014; accepted 15 January 2014; published online 27 February 2014

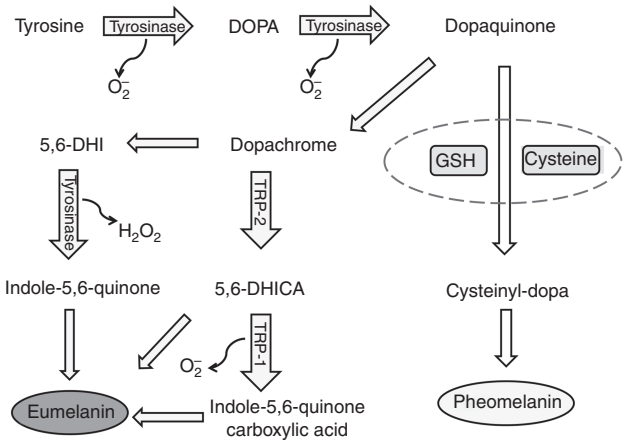

Figure 1. Generation of reactive oxygen species (ROS) by the various steps in the melanin synthetic pathway.

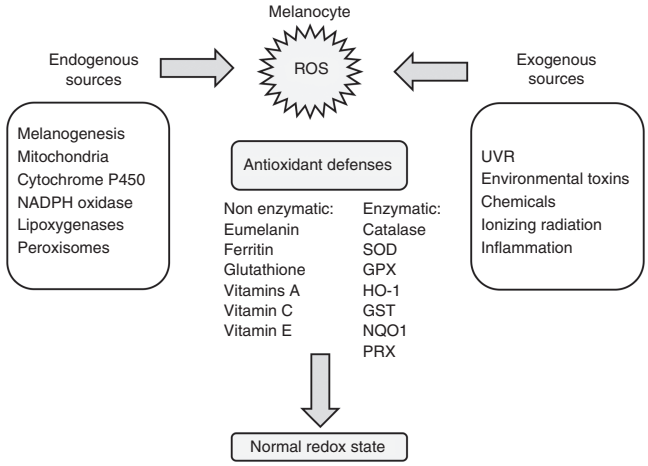

Figure 2. Induction of reactive oxygen species (ROS) by endogenous and exogenous sources and antioxidant defenses that restore normal redox state in melanocytes.

human skin (Maresca *et al.*, 2008). Generation of H_2O_2 in response to UV correlates inversely with constitutive pigmentation, suggesting an antioxidant effect of melanin (Song *et al.*, 2009). In comparison with keratinocytes, the induction of 8-hydroxydeoxyguanosine (8-OHdG), a major form of oxidative DNA damage, and expression of several base excision repair (BER) genes are higher in melanocytes (Mouret *et al.*, 2012). Paradoxically, cultured human melanocytes with high melanin content are reported to be more vulnerable to UVA-induced, but less susceptible to H_2O_2-induced, oxidative DNA damage than their counterparts with low melanin content (Hoogduijn *et al.*, 2004; Wang *et al.*, 2010). Stimulation of melanogenesis in human melanocytes or mouse melanoma cells is reported to increase UVA-induced DNA damage (Wenczl *et al.*, 1998; Kvam and Tyrrell, 1999; Marrot *et al.*, 1999). In contrast, stimulation of melanogenesis in cultured human melanocytes by α-melanocortin (α-MSH) increases the activity and protein levels of catalase, and markedly reduces UV-induced H_2O_2 generation (Maresca *et al.*, 2008; Song *et al.*, 2009). In human melanoma cells, increased pigmentation protects against UV- or H_2O_2-induced mitochondrial DNA damage (Swalwell *et al.*, 2011). The controversy about the pro-oxidant versus the antioxidant effects of melanin and its intermediates is fueled by reports using purified melanin or melanin intermediates exogenously added to cultured cells or naked DNA (Tomita *et al.*, 1984; Kipp and Young, 1999; Kovacs *et al.*, 2012). Although these data support the oxidative nature of melanin, the experimental conditions used are unlikely to be physiologically relevant, as melanin is normally confined in melanosomes.

ACTIVATION OF ANTIOXIDANT DEFENSES IN MELANOCYTES BY PARACRINE FACTORS

The homeostasis of epidermal human melanocytes is maintained primarily by a complex paracrine network consisting of growth factors and cytokines synthesized by epidermal keratinocytes and dermal fibroblasts, and modulated by UV. The keratinocyte-derived endothelin-1 is a potent mitogen and melanogenic factor that reduces H_2O_2 generation and apoptosis in UV-irradiated human melanocytes (Imokawa *et al.*, 1992; Tada *et al.*, 1998; Kadekaro *et al.*, 2005). The melanocortins α-MSH and ACTH are synthesized by keratinocytes and melanocytes, and they stimulate eumelanin synthesis and melanocyte survival and proliferation by binding and activating the melanocortin 1 receptor (MC1R). The MC1R is a G_s protein–coupled receptor expressed on the cell surface of melanocytes. Treatment of cultured human melanocytes with α-MSH results in rapid reduction in the generation of H_2O_2 in response to UV exposure, consistent with earlier findings by Haycock *et al.* (Haycock *et al.*, 2000; Kadekaro *et al.*, 2005; Song *et al.*, 2009; Kadekaro *et al.*, 2010). In addition, α-MSH increases the protein and activity levels of catalase, and counteracts the inhibitory effect of UV on this enzyme (Song *et al.*, 2009). Subsequently, treatment with α-MSH reduces the induction of 8-oxodG and enhances its repair in UV-irradiated melanocytes, and also reduces oxidative DNA damage induced by H_2O_2 (Song *et al.*, 2009; Kadekaro *et al.*, 2012). The antioxidant effects of α-MSH require binding and activation of MC1R, are absent in melanocytes expressing loss-of-function *MC1R*, and are inhibited by agouti signaling protein, the physiological MC1R antagonist (Song *et al.*, 2009). These results establish the significance of the activated MC1R in protection of melanocytes from oxidative stress.

Activation of p53 is an important mechanism by which the activated MC1R reduces oxidative stress in melanocytes. It is noteworthy that p53 regulates pigmentation by increasing the expression of tyrosinase in human melanocytes and proopiomelanocortin, the precursor for melanocortins, in mouse keratinocytes (Cui *et al.*, 2007). Activation of the MC1R by α-MSH binding augments the UV-induced accumulation of p53 in human melanocytes by increasing phosphorylation of p53 on Ser15. Treatment with α-MSH also increases the levels of the BER enzymes OGG1 and APE-1 by a p53-dependent mechanism (Kadekaro *et al.*, 2012).

In addition, activation of MC1R by α-MSH regulates intracellular redox status by upregulating the expression of

antioxidant genes, including heme oxygenase-1 (HO-1), ferritin, and peroxiredoxin-1 (Song *et al.*, 2009; Kadekaro *et al.*, 2010). α-MSH activates a number of transcription factors known to regulate the redox state of melanocytes. In normal human melanocytes and melanoma cells, the redox sensor APE-1 is a target of Mitf, the master regulator of melanocyte survival and function (Liu *et al.*, 2009). Treatment of human melanocytes with α-MSH upregulates Mitf and APE-1 (Kadekaro *et al.*, 2005, 2012). Melanocytes also express Nrf-2, an important transcription factor that upregulates the expression of genes for phase II detoxification enzymes, and its main target HO-1 (Marrot *et al.*, 2008; Kaspar *et al.*, 2009; Jain *et al.*, 2010; Jian *et al.*, 2011; Taguchi *et al.*, 2011). In addition, α-MSH increases the expression of *Nrf-2* gene and its target genes HO-1, y-glutamylcysteine-synthetase, and glutathione S-transferase Pi in cultured human melanocytes, and abrogates the inhibitory effects of UV on Nrf-2 and its targets (Kokot *et al.*, 2009). Another transcription factor that is activated by α-MSH is NFκB, which is known to be activated by TNF-α and ROS (Manna and Aggarwal, 1998; Ichiyama *et al.*, 1999; Haycock *et al.*, 2000). Treatment of melanocytes with α-MSH inhibits UV-induced apoptosis by increasing the protein levels of Bcl2, a known target of NFκB and Mitf (Bohm *et al.*, 2005; Kadekaro *et al.*, 2005).

SIGNIFICANCE OF OXIDATIVE STRESS IN MELANOMA

Sunlight is a major inducer of ROS formation in the skin and a major contributor to skin cancer (Sander *et al.*, 2004). Irradiation of the skin by UVA and/or UVB impairs natural antioxidant defenses and induces high levels of ROS. Acute exposure to UV is the main etiological factor for melanomagenesis. Irradiation of cultured human melanocytes with UV (75% UVB, 25% UVA) results in rapid dose-dependent generation of H_2O_2 (van der Kemp *et al.*, 2002); (van der Kemp *et al.*, 2002), and subsequent decrease in catalase activity and protein levels, and reduced HO-1 expression (Kadekaro *et al.*, 2005; Kokot *et al.*, 2009; Song *et al.*, 2009; Kadekaro *et al.*, 2010, 2012). Exposure of human OGG1 protein, an important BER enzyme, to UVB results in its inactivation (van der Kemp *et al.*, 2002).

There is increasing evidence for the significance of oxidative stress in the initiation and progression of melanoma. The role of oxidative stress in melanoma is supported by the findings that mutations in several melanoma-associated genes result from, or exacerbate, oxidative stress. The activating ^{V600E}BRAF mutation, a somatic mutation commonly expressed in nevi and melanoma, may be oxidative stress–induced (Landi *et al.*, 2006). In melanocytes, p16 is an important regulator of oxidative stress, and its depletion in cultured human melanocytes significantly increases ROS levels (Jenkins *et al.*, 2011). Melanocytes are more sensitive to p16 depletion than either keratinocytes or fibroblasts, which may impart the association of *p16* mutations with melanoma. Loss of PTEN is associated with melanoma progression, presumably owing to increased superoxide anion resulting from sustained activation of Akt (Govindarajan *et al.*, 2007). Loss-of-function alleles of the *MC1R* that are associated with increased melanoma risk cause sustained oxidative stress in human melanocytes owing to the inability to respond to α-MSH (Kadekaro *et al.*, 2010). In addition, oxidative stress can impair nucleotide excision repair, the main repair pathway for UV-induced DNA photoproducts, via lipid peroxidation products that inactivate DNA repair enzymes (Feng *et al.*, 2004, 2006). Null polymorphisms of *GSTM1* and *GSTT1* that belong to the glutathione S-transferase family of antioxidant genes have been associated with a high risk of melanoma, especially in subjects with a history of sunburns in childhood (Fortes *et al.*, 2011). One single nucleotide polymorphism in the glutathione S-transferase gene *GSTP1,* which reduces the activity of the enzyme, has been associated with melanoma susceptibility and with further increase in melanoma risk when coexpressed with *MC1R* variant alleles (Ibarrola-Villava *et al.*, 2012). These results strongly suggest that oxidative stress is a driver of melanomagenesis (Cassidy *et al.*, 2013).

There is increasing evidence for aberrant redox state in melanoma. Melanocytes derived from melanoma patients display increased sensitivity to oxidizing agents owing to endogenous antioxidant imbalance (Picardo *et al.*, 1996b; Grammatico *et al.*, 1998; Picardo *et al.*, 1999; Meyskens *et al.*, 2001). Melanoma tumor cells have higher intracellular levels of O_2^- compared with normal melanocytes, and they aberrantly activate the transcription factors NF-κB and AP-1 (Meyskens *et al.*, 2001). Moreover, melanoma tumor cells express higher levels of neuronal nitric oxide synthase, thus generating higher levels of nitric oxide than normal melanocytes, and this increase correlates with the disease stage in melanoma (Yang *et al.*, 2013). The significance of oxidative stress in melanoma is further supported by the finding that the antioxidant N-acetylcysteine inhibits tumor formation in the HGF-survivin melanoma mouse model (Cotter *et al.*, 2007), and selective inhibitors of neuronal nitric oxide synthase inhibit melanoma cell growth and metastatic potential (Yang *et al.*, 2013). Accordingly, antioxidants are being considered for the prevention, as well as for the treatment, of melanoma.

The association of aberrant melanin synthesis with oxidative stress and melanoma has been investigated by several research teams. Dysplastic nevi that are precursors for melanoma have increased ROS, and high pheomelanin, sulfur, iron, and calcium levels, and DNA damage (Salopek *et al.*, 1991; Pavel *et al.*, 2004; Smit *et al.*, 2008). Noonan *et al.*, 2012 reported that the frequency of UVA-induced melanoma tumors in HGF mice increases with skin pigmentation via an oxidative process involving melanin photoreactivity (Noonan *et al.*, 2012). Conversely, tumor formation in HGF mice is inhibited by the antioxidant N-acetylcysteine (Cotter *et al.*, 2007). In human skin, UVA-induced pigmentation was found to lack photoprotective properties (Miyamura *et al.*, 2011), indicating that exposure to UVA (e.g., in tanning beds) is not a safe practice. Recently, Mitra *et al.*, 2012 observed that recessive yellow mice with loss-of-function mc1r and coexpressing activating BRAF^{V600E} mutation develop more invasive melanoma tumors than their albino counterparts, and that pheomelanin results in oxidative DNA damage (Mitra *et al.*, 2012). They concluded that oxidative DNA damage resulting from pheomelanin synthesis is causal for melanoma,

independently of UV exposure. How these findings apply to human pigmentation and melanoma deserves to be investigated, as human melanocytes synthesize both eumelanin and pheomelanin unlike recessive yellow mouse melanocytes that only synthesize pheomelanin. The ratio of these pigments should determine the overall effects on the redox state of melanocytes particularly upon UV exposure. In addition, eumelanin and pheomelanin, as well as their intermediates, might differ chemically in human vs. mouse melanocytes, which might affect their pro-oxidant or antioxidant properties. Given that eumelanin is a scavenger of ROS (Meredith and Sarna, 2006), it can be concluded that reduction of eumelanin, as in individuals with fair skin, or the absence of eumelanin, as in recessive yellow mice, potentiates melanoma risk by increasing the vulnerability of melanocytes to oxidative stress.

OXIDATIVE STRESS AND LOSS OF MELANOCYTES IN VITILIGO

Vitiligo is a depigmentary disease that occurs in ~0.5% of the world population, and it is characterized by the loss of melanocytes in the epidermis by an autoimmune mechanism (Taieb and Picardo, 2007; Spritz, 2013). However, there is strong evidence for the role of oxidative stress as a key factor in the onset and progression of the disease. Increased sensitivity of melanocytes from vitiligo patients to UVB-induced cell death as compared with normal melanocytes was attributed to their compromised capacity to cope with increased oxidative stress (Jimbow *et al.*, 2001). Further evidence supports the exaggerated sensitivity of melanocytes from nonlesional vitiligo skin to chemical or physical oxidative stress (Maresca *et al.*, 1997; Boissy and Manga, 2004). Vitiligo patients are known to have very high levels of H_2O_2 (1 mM) and peroxynitrite in their epidermis, concomitant with reduced levels and activity of catalase (Schallreuter *et al.*, 1991; Maresca *et al.*, 1997; Schallreuter *et al.*, 1999, 2012). High levels of H_2O_2 inactivate and reduce the levels of methionine sulfoxide reductase A and B, and thioredoxin/thioredoxin reductase, thus contributing to oxidative stress and melanocyte death in vitiligo (Schallreuter *et al.*, 2008; Zhou *et al.*, 2009). In addition, high levels of H_2O_2 in the epidermis are found to oxidize proopiomelanocortin-derived bioactive peptides ACTH and α-MSH, both of which have antioxidant and survival effects on human melanocytes, and this effect can be mitigated by treatment with pseudocatalase (Kadekaro *et al.*, 2005; Spencer *et al.*, 2007; Kadekaro *et al.*, 2010). These findings suggest that the pro-oxidant state of vitiligo skin is causal for melanocyte death.

The transcription factor Nrf-2 is implicated in the pathogenesis of vitiligo. An allelic variant of the Nrf-2 gene, A^{-650}, is thought to be a risk factor for vitiligo (Guan *et al.*, 2008). More recently, Natarajan *et al.*, 2010 reported increased transcript levels of Nrf-2, as well as its targets NQO-1, y-glutamyl cysteine ligase catalytic, and modulatory subunits (GCLC and GCLM, respectively) in vitiligo lesional epidermis, as compared with nonlesional skin (Natarajan *et al.*, 2010). However, induction of Nrf-2, and its target genes HO-1, NQO-1, GCLC, and GCLM, by the electrophilic compounds curcumin and santalol is evident in nonlesional skin, but not in lesional vitiligo skin, further confirming the disruption of redox homeostasis in vitiligo (Natarajan *et al.*, 2010). Treatment of vitiligo patients with PUVA increases the expression of the Nrf-2 target HO-1 in the skin (Elassiuty *et al.*, 2011). Comparison of cultured nonlesional vitiligo melanocytes with their normal counterpart shows that the former exhibit greater induction of HO-1 than the latter in response to exposure to UVA or the phenolic compound 4-Tertiary butylphenol, demonstrating increased sensitivity of vitiligo-derived melanocytes to oxidative stress.

In vitiligo, oxidative stress–induced death of melanocytes is exacerbated by abnormal levels and/or activities of other antioxidant and BER enzymes. Catalase allelic variants have been associated with vitiligo, and the levels of several antioxidant enzymes, such as catalase, glutathione peroxidase, and glutathione reductase, have been found to be altered in vitiligo, which account for sustained high levels of H_2O_2 in the epidermis (Casp *et al.*, 2002b; Gavalas *et al.*, 2006; Park *et al.*, 2006). Salem *et al.* 2009 showed that in both lesional and nonlesional vitiligo skin the levels of the BER enzymes OGG1, APE-1, and DNA polymerase β are increased (Salem *et al.*, 2009). In addition to high levels of H_2O_2, high levels of inducible nitric oxide synthase (iNos) in lesional and nonlesional skin, and increased 8-oxoG in the skin and plasma of vitiligo patients, can be detected, further indicating generalized oxidative stress in vitiligo.

TARGETING OXIDATIVE STRESS PATHWAYS FOR TREATMENT OF MELANOMA AND VITILIGO

A major benefit to understanding redox-related mechanisms occurring in healthy and diseased melanocytes is the capacity to harness these pathways for effective, targeted therapies and prevention measures. Repigmentation of depigmented skin of vitiligo, characterized by high levels of epidermal H_2O_2 and peroxynitrite, is achieved by reducing H_2O_2, such as with application of narrow-band UVB-activated pseudocatalase (Schallreuter *et al.*, 2013). For treatment of melanoma, characterized by aberrant redox state, two different strategies were proposed (Fruehauf and Meyskens, 2007). The first strategy is to use agents that increase ROS scavenging to reduce melanoma tumor growth by inhibiting H_2O_2 signaling, which mediates the proliferative effects of growth factors and inhibits the activity of protein tyrosine phosphatases, such as PTEN. Overexpression of superoxide dismutase, glutathione peroxidase, or catalase reduces tumor cell growth (Venkataraman *et al.*, 2005; Finch *et al.*, 2006; Liu *et al.*, 2006). There is increasing evidence for the efficacy of antioxidants as chemopreventative agents that inhibit melanoma onset and progression. Administration of the antioxidant NAC or selenium delays the onset of UV-induced melanoma tumors (Cotter *et al.*, 2007; Cassidy *et al.*, 2013). Honikiol, a potent scavenger of superoxide and peroxyl radicals, inhibits melanoma cell growth *in vitro* (Dikalov *et al.*, 2008; Mannal *et al.*, 2011). Selective inhibitors of nitric oxide reduce melanoma cell growth and metastasis (Yang *et al.*, 2013). Selenium, which increases glutathione peroxidase activity and the levels of glutathione, also

decreases the size of human melanoma xenografts *in vivo*, and inhibits the growth of human melanoma cells *in vitro* (Cassidy *et al.*, 2013). Treatment of human melanoma cell lines with cAMP inducers, such as α-MSH, inhibits their proliferation, partly owing to the inhibition of oxidative stress (Lyons *et al.*, 2013). The second strategy is to treat melanoma tumors with agents that trigger apoptosis by compromising ROS scavenging. Such agents include butathionine sulfoximine, which depletes GSH, and disulfiram, which inhibits copper, zinc superoxide dismutase, both of which inhibit melanoma cell proliferation *in vitro* (Fruehauf *et al.*, 1998; Cen *et al.*, 2004). In addition, quercetin, motexafin gadolinium, melphalan, and cisplatin, which inhibit thioredoxin, are effective in killing cancer cells (Witte *et al.*, 2005; Hashemy *et al.*, 2006; Lu *et al.*, 2006). Resveratrol, known to inhibit APE-1/Ref-1 endonuclease activity, sensitizes melanoma cells to DNA alkylating agents (Yang *et al.*, 2005). Combined therapy using some of the above agents can synergistically inhibit melanoma tumor growth. Understanding the complexity of oxidative stress pathways in pigmentation production, melanocyte proliferation, and malignant transformation has enormous potential to expand our armamentarium of clinically effective compounds and offer enormous promise for patients suffering from pigmentary disorders and melanoma.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported in part by R01 ES009110 (for ZAAM) and R01 ES017561 (for ZAAM and SAL).

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