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# An Epinephrine-Dependent Mechanism for the Control of UV-Induced Pigmentation

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## TO THE EDITOR

Tanning results from a coordinated set of signals that induce skin hyperpigmentation in response to exposure to UV radiation (Park *et al.*, 2008). This is classically thought to occur through the actions of proopiomelanocortin-derived peptides and α-melanocyte stimulating hormone (Abe *et al.*, 1969a, b; Wakamatsu *et al.*, 1997; Thody and Graham, 1998; Slominski *et al.*, 2000, 2004; Tsatmali *et al.*, 2000; Rousseau *et al.*, 2007) on the melanocyte melanocortin-1 receptor by increasing intracellular cAMP (Im *et al.*, 1998). However, as studies in proopiomelanocortin-deficient mice have shown that these mice retain the capacity to produce eumelanin even in the absence of α-melanocyte stimulating hormone or proopiomelanocortin-related peptides (Smart and Low, 2003; Slominski *et al.*, 2005), and animals with a nonfunctional melanocortin-1 receptor are still able to produce melanin in response to forskolin (Friedmann *et al.*, 1990; D'Orazio *et al.*, 2006), it is likely that alternate cAMP-dependent pathways can induce melanogenesis.

One alternate cAMP-dependent pathway involves the adrenergic receptor. The adrenergic receptors are pharmacologically divided into two subgroups, α and β, and both receptor subfamilies have been implicated in the control of pigmentation in frog skin (McGuire, 1970; Taylor and Teague, 1976) and human uveal melanocytes (Hu, 2000; Hu *et al.*, 2000). Human epidermal melanocytes express the α1-adrenergic receptor and β2-adrenergic receptor (B2AR; Schallreuter *et al.*, 1996; Scarparo *et al.*, 2000), and activation of the B2AR was shown to increase melanin synthesis (Gillbro *et al.*, 2004) whereas activation of the α1-adrenergic receptor had no effect (Schallreuter *et al.*, 1996). Human melanocytes also increase their expression of the B2AR in response to UV irradiation (Yang *et al.*, 2006), further suggesting a role for the B2AR in UV-induced hyperpigmentation.

Catecholamines are endogenous ligands for the adrenergic receptor and epinephrine has the greatest affinity for the B2AR. Indeed, epinephrine has been shown to increase melanin synthesis in human uveal melanocytes (Hu *et al.*, 2000) and increase intracellular cAMP in human epidermal melanocytes (Gillbro *et al.*, 2004). Epidermal melanocytes can synthesize the catecholamine norepinephrine but are unable to produce epinephrine as they do not express the enzyme phenylethanolamine-*N*-methyltransferase, which is necessary for synthesis of epinephrine (Gillbro *et al.*, 2004). Norepinephrine, though synthesized by melanocytes, does not seem to alter melanogenesis (Schallreuter *et al.*, 1996). However, keratinocytes possess the capacity to synthesize epinephrine (Schallreuter *et al.*, 1992; Pullar *et al.*, 2006). Thus, we hypothesized the existence of a paracrine interaction whereby keratinocytes secrete epinephrine in response to UV irradiation, which could then stimulate neighboring β-adrenergic receptors (BARs) on melanocytes to increase melanin synthesis.

Primary human keratinocytes and melanocytes were isolated from human neonatal foreskin and cultured in keratinocyte serum-free media (Cascade Biologics, Portland, OR) and phorbol-free

*Abbreviations: B2AR, β2-adrenergic receptor; BAR, β-adrenergic receptor; PBS, phosphate buffer solution*

melanocyte growth media (Cascade Biologics), respectively. Melanocytes were grown to 70–90% confluence and incubated for 48 hours in five different treatment groups: 10 μM epinephrine (Sigma, St. Louis, MO), 10 μM epinephrine and the nonselective β-adrenergic antagonist 10 μM timolol (Sigma), 10 μM norepinephrine (Sigma), 10 μM norepinephrine and 10 μM timolol, and 10 μM timolol. The cells were then collected, resuspended in 1 M NaOH, and vortexed for 15 minutes. After centrifugation, the supernatant was collected and its absorbance at 475 nm was measured and compared against a standard curve of melanin ranging from 0 to 150 μg $ml^{-1}$. The melanin measurement was then normalized to the cell count to express the melanin content as pg per cell. Only viable cells, as determined by Trypan blue exclusion, were included in the cell count.

For irradiation studies, the keratinocyte culture medium was exchanged for prewarmed (37 °C) phosphate buffer solution (PBS), and then the cells were irradiated with either 15, or 40 mJ $cm^{-2}$ of UVB by exposing them to a precalibrated UVB lamp. Culture dishes covered by aluminum foil served as controls. The PBS was then collected, filtered through a 0.2 μm filter, then spiked with 100 μl of 0.1 M HCl to stabilize any catecholamines and saved for epinephrine measurement. The UVB-irradiated keratinocytes were lysed and sonicated in 0.1 M HCl, and the post-centrifugation supernatant collected. The epinephrine in the PBS and the keratinocyte lysates were subsequently measured by an enzyme immunoassay method (Biosource, Camarillo, CA). Epinephrine in cell extracts was expressed as pg of epinephrine per mg of protein. In a subsequent experiment, the PBS overlying the UVB-irradiated keratinocytes was collected, sterile filtered through a 0.2 μm filter, and then used to treat melanocytes for 2 hours. Each treatment medium was split into two treatment groups, where one of the treatment groups was augmented with β-blocker, timolol (10 μM). Melanin content for each of the treated melanocyte groups were determined as described above. Two different strains of keratinocytes and melanocytes derived from different individuals were used and the data were averaged from each group.

We found that melanocytes increased their synthesis of melanin by 91% when treated with epinephrine. Interestingly, norepinephrine had no effect (Figure 1). Moreover, treatment with the BAR antagonist timolol abrogated the epinephrine-induced increase in melanin generation.

UVB irradiation of keratinocytes led to a twofold (15 mJ $cm^{-2}$ recipients) or twenty-fold (40 mJ $cm^{-2}$) increase in the levels of epinephrine over nonirradiated controls (Figure 2a). Epinephrine levels in the lysate of these cells were below the level of detection (data not shown), suggesting that UV irradiation led to an immediate release of intracellular epinephrine.

When the medium from UVB-irradiated (40 mJ $cm^{-2}$) keratinocytes was transferred onto cultured melanocytes and the cells incubated for an additional 2 hours, there was a 29% increase in their production of melanin (Figure 2b). Inclusion of the BAR antagonist timolol in the medium conditioned by the UVB-exposed cells completely abrogated this melanogenic response (Figure 2b). Therefore, these results suggest that keratinocytes release epinephrine in response to UVB irradiation, which then acts in a paracrine fashion to activate BARs on melanocytes and consequently stimulate melanogenesis.

Our findings support a BAR-mediated pathway in cutaneous melanogenesis that is responsive to epinephrine, in accordance with previous findings (Hu *et al.*, 2000; Gillbro *et al.*, 2004). We also found that keratinocytes can acutely release epinephrine in response to UVB irradiation, which is particularly interesting as epinephrine is an endogenous ligand of the B2AR, and B2AR activation is a known potent activator of melanogenesis (Gillbro *et al.*, 2004). Notably, melanocytes are unable to synthesize epinephrine (Gillbro *et al.*, 2004), which we show as the necessary catecholamine for BAR-mediated melanogenesis. It will be interesting to further pursue the interaction of stress and UV-mediated pigmentation *in vivo*, as our work and the work of others (reviewed in Costin and Hearing, 2007) suggest that these are linked.

The role of the epidermal epinephrine–BAR network continues to be defined and it serves as a pathway of paracrine communication between keratinocytes and melanocytes. We show this to be especially true for keratinocytes activated by UVB irradiation. It is possible that exogenous epinephrine may serve as a protective mechanism to induce non-UV-mediated pigmentation as has been

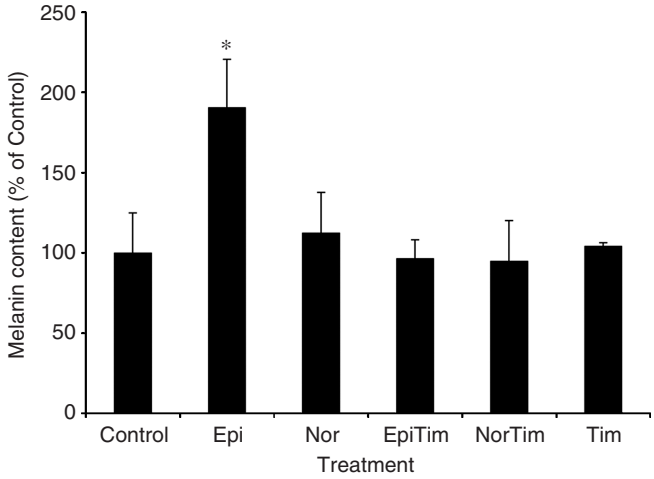


**Figure 1. Epinephrine increases melanin synthesis in melanocytes.** Melanocytes were treated with either 10 μM epinephrine (Epi), 10 μM norepinephrine (Nor), 10 μM epinephrine with 10 mM timolol (EpiTim), 10 μM norepinephrine with 10 mM timolol (NorTim), or with 10 mM timolol (Tim) for 48 hours. Melanin content was measured as outlined in the "Materials and Methods" section. Melanocytes treated with Epi produced significantly more melanin (191 ± 0.3%) than untreated controls, whereas all other treatments produced no significant changes in melanin production in comparison to control. Experiments were run in triplicate and similar results were obtained in two separate cell strains. Results are reported as mean ± s.e.m. $*P<0.05$.

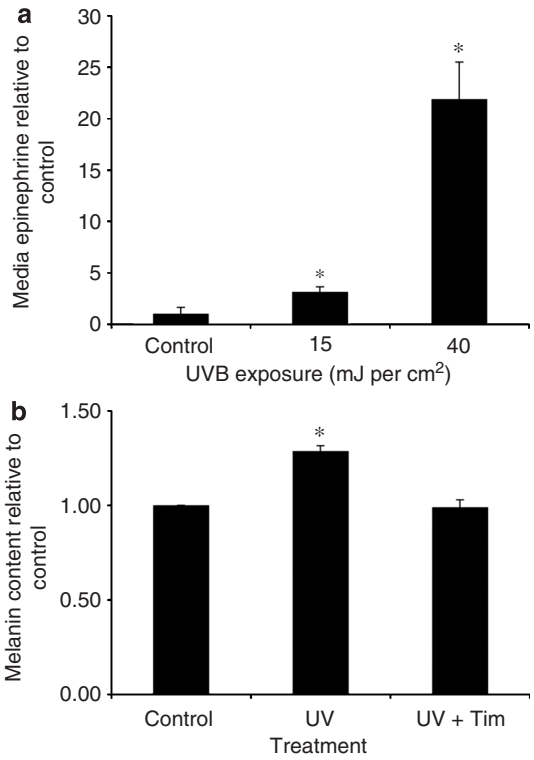


**Figure 2. UVB irradiation increases epinephrine release by keratinocytes.** (**a**) Keratinocytes were placed in PBS and were then exposed to either 15 or 40 mJ $cm^{-2}$ of UVB radiation. The PBS was subsequently collected and assayed for epinephrine as described in the ''Materials and Methods''. Control keratinocyte cultures were similarly manipulated but were shielded from the UV light during the treatment exposure. At both 15 and 40 mJ $cm^{-2}$, there was a significant increase in the epinephrine released into the medium by the irradiated keratinocytes. Epinephrine levels in the 15 mJ $cm^{-2}$ group was increased threefold over controls, and in the 40 mJ $cm^{-2}$ group the levels were 22-fold increased over controls. The data were pooled from two independent experiments with two different strains of keratinocytes and melanocytes. (**b**) Melanocytes were incubated with the conditioned medium taken from nonirradiated keratinocytes (Control), from keratinocytes that were irradiated with 40 mJ $cm^{-2}$ UVB (UV), or with medium from keratinocytes that were irradiated with 40 mJ $cm^{-2}$ UVB that was subsequently supplemented with 10 mM timolol (UV + Tim). The melanocytes were incubated in the respective media for 24 hours and then assayed for the production of melanin. Melanin production significantly increased in melanocytes that were treated with the conditioned medium from UV-irradiated keratinocytes (1.29 ± 0.03) but this increase was abrogated by the addition of 10 mM timolol (0.99 ± 0.04), a nonspecific β-adrenergic receptor antagonist. Results are reported as mean ± s.e.m. $*P<0.05$.

suggested in previous studies (D'Orazio *et al.*, 2006). Further research will help more accurately delineate how UV-induced epinephrine may participate within the cutaneous neuroendocrine system as well as the contribution of the epidermal epinephrine–BAR network to skin pigmentation.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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# A New Paradigm for the Role of Aging in the Development of Skin Cancer

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## TO THE EDITOR

Cancers of the skin are the most common cancers to afflict Americans in the United States with over 1,000,000 new cases estimated to occur in 2008 (ACS, 2008). The primary environmental factor that influences the development of skin cancer is exposure to sunlight, in the ultraviolet B (UVB) wavelengths. Notably, a dramatic increase in the incidence of skin cancers is seen with increasing age (ACS, 2008), as evidenced by the fact that a majority of skin malignancies are found in people over the age of 60 years (Kraemer, 1997; ACS, 2008). However, the mechanisms underpinning the correlation between age and skin cancer are not well understood. New ideas on the link between age and skin cancer have arisen based on age-related accumulation of stromal senescent cells that can lead to a tumor-promoting environment (Krtolica *et al.*, 2001; Krtolica and Campisi, 2002; Dilley *et al.*, 2003; Parrinello *et al.*, 2005; Collado *et al.*, 2007). Combining these recent data from others with data from our laboratory leads us to propose a new paradigm for the role of aging in the development of skin cancer involving the insulin-like growth factor-1 receptor (IGF-1R) pathway (Kuhn *et al.*, 1999; Chuang *et al.*, 2005; Heemst *et al.*, 2005; Kurosu *et al.*, 2005; Samani *et al.*, 2005; Lewis and Spandau, 2008; Lewis *et al.*, 2008).

The historical explanation for the correlation between skin cancer and aging is that UVB-induced skin damage during childhood and early adolescence initiates mutations in keratinocytes (Kraemer, 1997; Whiteman *et al.*, 2001; Krtolica and Campisi, 2002; MacKie, 2006; Feng *et al.*, 2007). Subsequently, these keratinocytes containing mutations acquire a growth advantage that over many decades generates enough genetic change to become carcinogenic. However, can we presume that time is the sole contributor to UVB-induced skin cancers? It is reasonable to consider that the physiology of aging also lends a hand to carcinogenic events. Recent data from a variety of labs have demonstrated a modification on the theory of skin cancer and aging based on changes in stromal fibroblasts of aged individuals. There are age-related increases in the number of senescent dermal fibroblasts and epidermal keratinocytes in human skin (Dimiri *et al.*, 1995). In a study involving aging primates, an age-dependent increase in markers of senescence in skin fibroblasts was observed (Herbig *et al.*, 2006; Jeyapalan *et al.*, 2007). Given this age-associated accumulation of senescent cells, it is reasonable to propose that cellular senescence may contribute to age-related cancers by altering the surrounding tissue into a neoplasia-promoting environment. The paradoxical effect of cellular senescence on an organism's well-being has been called antagonistic pleiotropy (Williams, 1957; Krtolica and Campisi, 2002). However, cellular senescence is a powerful tumor suppressor limiting cell life span and removing damaged cells from a proliferative state preventing formation of clonal tumors (Campisi, 2005; Hornsby, 2007; Rodier *et al.*, 2007). Conversely, the accumulation of senescent cells may contribute to aging and provide a tumor-promoting environment due to their altered properties such as stromal matrix reorganization and/or degradation, secretion of growth factors, and inflammatory cytokines (Krtolica and Campisi, 2002; Parinello *et al.*, 2005). Here we present our data proposing a new paradigm to explain non-melanoma skin carcinogenesis that further substantiates the importance of stromal interactions in the progression of carcinogenic events. The stromal interactions discussed demonstrate that IGF-1 and the IGF-1R are critical in the interactions between dermal fibroblast and epidermal keratinocytes and that they play an important role in aging and the response of skin to UVB irradiation.

*Abbreviations: NMSC, non-melanoma skin cancer; UVB, ultraviolet B*