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The Autophagy Receptor Adaptor p62 is Up-regulated by UVA Radiation in Melanocytes and in Melanoma Cells†

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Abstract

UVA (315–400 nm) is the most abundant in sunlight and is used in indoor tanning beds. However, much remains to be understood about the regulation of the UVA damage response in melanocytes and melanoma. Here we show that UVA, but not the shorter waveband UVB (280–315 nm), up-regulates adaptor protein p62 in an Nrf2- and ROS-dependent manner, suggesting a UVA-specific effect on p62 regulation. UVA-induced p62 up-regulation was inhibited by a mitochondria-targeted antioxidant or Nrf2 knockdown. In addition, p62 knockdown inhibited UVA-induced ROS production and Nrf2 up-regulation. We also report here a novel regulatory feedback loop between p62 and PTEN in melanoma cells. PTEN overexpression reduced p62 protein levels, and p62 knockdown increased PTEN protein levels. As compared with normal human skin, p62 was up-regulated in human nevus, malignant melanoma, and metastatic melanoma. Furthermore, p62 was up-regulated in melanoma cells relative to normal human epidermal melanocytes, independent of their BRAF or NRAS mutation status. Our results demonstrated that UVA up-regulates p62, and induces a p62-Nrf2 positive feedback loop to counteract oxidative stress. Additionally, p62 forms a feedback loop with PTEN in melanoma cells, suggesting p62 functions as an oncogene in UVA-associated melanoma development and progression.

Graphical abstract

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UVA up-regulates the autophagy adaptor protein p62 in epidermal melanocytes and melanoma cells. Up-regulation of p62 requires ROS and Nrf2 activation, and p62 in turn promotes ROS generation and Nrf2 activation. Expression of p62 in melanoma cells also negatively regulates PTEN expression. Expression of PTEN in melanoma is sufficient to reduce p62 expression, creating a regulatory feedback loop.

INTRODUCTION

Skin cancer is the most common form of cancer. More than 3.5 million cases of skin cancer are expected to be diagnosed in 2017 in the United States alone (1). Skin cancers are broadly classified into melanomas and non-melanoma skin cancers (NMSCs). Melanomas account for only 4% of skin cancer diagnoses, but cause nearly half of all skin cancer deaths (2,3).

Recent advances in melanoma therapy, such as targeted therapy and immunotherapy, have benefitted a growing number of melanoma patients (4–7). However, many patients fail to have a sustained response, and better therapeutic advancements for melanoma are urgently needed. A better understanding of melanoma development and progression is necessary to achieve this goal.

Ultraviolet (UV) radiation is a major risk factor for melanoma development; 60–70% of all melanoma cases are attributed to UV exposure (8). UV radiation consists of three major types: UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm). Of these, UVA is the most abundant in sunlight and indoor tanning beds (9). UVA penetrates deep into the dermis and has been linked to oxidative stress-induced skin damage (10). Unlike UVA, UVB only superficially damages the epidermis (11), but is 1000-fold more genotoxic than UVA (10,12). UVA is absorbed by cellular photosensitizers, such as melanin in melanocytes, leading to the production of reactive oxygen species (ROS) (12,13). Evidence suggests that the absorption of UVA by melanin is photoprotective, as both UVA exposure and reduced levels of melanin are associated with melanoma risk (13–15).

UVA-induced ROS production triggers an antioxidant response, led by antioxidant response factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (16,17). Nrf2 is induced in response to ROS accumulation to activate transcription of antioxidant response genes. Nrf2 is regulated by Kelch-like ECH-associated protein 1 (KEAP1), which facilitates the degradation of Nrf2 at the proteasome. The autophagy adaptor protein p62 binds to KEAP1 and sequesters it away from Nrf2, leading to Nrf2 stabilization (18,19). In addition, Nrf2 controls p62 transcription in a positive feedback loop that perpetuates antioxidant response (20).

p62 functions as an adaptor and substrate for selective autophagy, and as an oncogenic signaling adaptor (21). We and others have found that overexpression of p62 induces proliferation, invasion, and ultimately, tumor growth and metastasis (22–25). Overexpression of p62 is seen in a number of cancers, including melanoma, and is a marker of poor prognosis (22,26). Transcription of p62 is induced by UVA in skin fibroblasts (27), further implicating p62 in the UVA response.

PTEN is a crucial tumor suppressor and undergoes loss-of-heterozygosity (LOH) in a proportion of melanomas (28). Loss of PTEN promotes tumorigenesis by increasing cell survival (28). High levels of p62 have been found to be associated with PTEN deletion in prostate cancer, and loss of PTEN coupled with high p62 levels were associated with poor prognosis (29). In breast cancer, p62 knockdown leads to PTEN accumulation concurrent with reduced AKT activation (30). However, little is currently known regarding the function and regulation of p62 in UVA response and melanoma. Here, we examine the regulation of p62 by UVA and the functional implications for melanoma.

MATERIALS AND METHODS

Human Skin Samples

Human Skin Tumor Samples—All human specimens were studied after approval by the University of Chicago Institutional Review Board. Formalin-fixed, paraffin-embedded tissue blocks were obtained from the archives in the tissue bank (Dept. of Medicine, Section of Dermatology, University of Chicago). Non-sun-exposed normal skin, nevus, and malignant and metastatic melanoma tissues were used for immunohistochemical analysis of p62 protein levels.

Cell Lines

A375 human amelanotic melanoma cells, wild-type (WT) mouse embryonic fibroblasts (MEFs), and p62 knockout (KO) MEFs were kindly provided by Dr. Seungmin Hwang at the University of Chicago. These cells were maintained in a monolayer culture in 95% air/5% CO₂ at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). iMC23 and iMC65 melanocytes were generated and cultured as described previously (31). Normal human epidermal melanocytes (NHEM) were obtained from Invitrogen and cultured according to the manufacturer's instructions for less than 4 passages.

Inducible expression of PTEN in WM793TRPTEN cells was obtained by treatment of cultures with doxycycline (Sigma) at a final concentration of 100 ng/ml. Cells were maintained in DMEM with GlutaMAX (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 g/ml streptomycin, and 4 g/ml insulin (Sigma). Other melanoma cells were generously provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia) and cultured as described previously (32).

Chemical treatments

To study the role of oxidative stress, cells were pretreated with 10 mM NaN₃ for 1 h, 2.5 µM mito-CP for 1.5 h, 1 mM NAC overnight, or 1 mM GSH-ester overnight prior to UVA exposure.

siRNA transfection

NHEM were transfected with siNC and siNrf2 siRNA (Dharmacon) using Amaxa nucleofector according to the manufacturer's protocol.

UVA Radiation

For UVA irradiation, four parallel PUVA lamps were used, and doses were measured using a Goldilux UV meter equipped with UVA and UVB detector (Oriel). Contamination from UVB irradiation was eliminated using a 0.13 mm Mylar filter material from Cope Plastics. This filter limits UVB exposure to 0.003% of total emitted UV radiation (33,34). We have found these lamps emit no UVC radiation (100–280 nm). The UVA dosages used here are relevant to human exposure. The dose of UVA which will cause erythema is 30 J/cm² for human skin (9) and the UVA dose (20 J/cm²) used in the in vitro studies equates to about 1 hour in the midday sun during the summer at latitude 48°N in Paris, France (35). In our

laboratory, obtaining 20 J/cm² of UVA irradiation requires approximately 1 hour. Therefore, the UVA dose used here is relevant to human exposure.

ROS Assay

Cells were treated with or without UVA radiation and then collected at 24 h post-UVA or -sham irradiation and then incubated with CM-DCFH-DA (2 μ M, Invitrogen) for 30 min at 37°C. Cells were washed with PBS three times, and oxidative stress was analyzed by flow cytometry, as described previously (36).

Western Blot

Protein concentration was determined using the BCA assay (Pierce). Western blotting was performed as described previously (37). Antibodies used included GAPDH, Nrf2 (Santa Cruz), p62 (Progen), and PTEN (Cell Signaling).

Statistical Analysis

Statistical analyses were performed using Prism 6 (GraphPad software, San Diego, CA). Data were expressed as the mean of at least three independent experiments and analyzed by Student's *t*-test. Error bars indicate the standard error of the means (SE). A *P*-value of <0.05 was considered statistically significant.

RESULTS

UVA irradiation induces p62 up-regulation in melanocytes and melanoma cells

To determine the effects of UV radiation on p62 expression, we treated lightly pigmented adult normal human epidermal melanocytes (NHEM) with UVA (20 J/cm²) or UVB (20 mJ/cm²) and then collected at 6 and 24 hours post-irradiation. In these cells, p62 was induced by UVA, but not UVB irradiation (Figure 1A). Similarly, in lightly pigmented iMC23 melanocytes (31), p62 was induced by UVA in a dose-dependent manner 24 h post-irradiation (Figure 1B). In darkly pigmented and iMC65 melanocytes (31), however, p62 induction by UVA was reduced at higher doses as compared with iMC23 cells (Figure 1B). This suggests that pigmentation reduces UVA-induced p62.

In amelanotic A375 melanoma cells, treatment with 20 J/cm² UVA was found to induce p62 expression 6 h post-irradiation, while UVB exposure (20 mJ/cm²) did not (Figure 1C).

Reactive oxygen species and Nrf2 are required for UVA-induced p62 up-regulation

One known effect of UVA radiation is the generation of reactive oxygen species (ROS). To determine whether oxidative stress has a role in UVA-induced p62 up-regulation, we treated cells with sodium azide (NaN₃), a known scavenger of the ROS species singlet oxygen. Treatment with NaN₃ prior to UVA exposure inhibited UVA-induced p62 up-regulation as well as Nrf2 accumulation in iMC23 cells (Figure 2A). Pretreatment with the antioxidant mitochondria-targeted carboxy-proxyl (mito-CP) similarly inhibited UVA-induced p62 up-regulation as well as Nrf2 accumulation (Figure 2B). Knockdown of the antioxidant response factor Nrf2 in normal melanocytes impaired the up-regulation of p62 by UVA, suggesting that p62 is up-regulated in part by Nrf2 in response to UVA (Figure 2C).

However, pretreatment with the antioxidant N-acetyl cysteine (NAC) overnight enhanced UVA-induced expression of p62 and Nrf2 (Figure 2D). Glutathione ester (GSH-ester) also caused a slight increase in UVA-induced p62 expression in iMC23 melanocytes (Figure 2E). These data indicate that NAC and glutathione fail to protect the cells from UVA-induced p62 up-regulation and Nrf2 accumulation.

p62 up-regulation promotes UVA-induced production of reactive oxygen species and Nrf2 up-regulation

Given the regulation of Nrf2 by p62 (18,19), we next investigated whether p62 regulates UVA-induced oxidative stress and Nrf2. p62 deletion did not affect basal levels of ROS (Figure 3A). In WT cells, UVA radiation increased the ROS level (Figure 3A). However, ROS were not significantly induced in p62-deficient cells following UVA radiation (Figure 3A). p62 knockdown in mouse embryonic fibroblasts (MEFs) inhibited the induction of Nrf2 in response to UVA (Figure 3B), indicating that UVA-induced p62 up-regulation is required for ROS generation and Nrf2 accumulation.

p62 and PTEN form a feedback loop in melanoma cells

Given that previous work had suggested a negative association between p62 and PTEN expression (30), we next examined whether a similar feedback loop between p62 and PTEN exists in melanoma cells. In the malignant melanoma cell line WM793, doxycycline-induced PTEN expression decreased p62 expression (Figure 4A). Knockdown of p62 in A375 melanoma cells increased PTEN expression (Figure 4B), indicating that p62 and PTEN form a feedback loop in melanoma cells.

p62 is up-regulated in human melanomas and melanoma cells

Given the importance of p62 for UVA response in melanocytes and melanoma cells *in vitro*, we next examined the presence of p62 in melanoma patient samples. As compared with normal skin, p62 protein levels increased in nevus, malignant melanoma, and metastatic melanoma (Figure 5A and 5B). 60% of melanomas express a mutant form of BRAF (V600E) and 20% of melanomas express mutant NRAS (38). In human melanoma cell lines with varying BRAF and NRAS mutation states, p62 was up-regulated compared to NHEM (Figure 5C), independent of BRAF or NRAS mutation status (Figure 5C), suggesting that p62 acts as an oncogene in melanoma development.

DISCUSSION

Here we report that UVA induces p62 in melanocytes and melanoma cells via ROS- and Nrf2-dependent mechanisms. UVA is thought to act primarily through ROS generation to promote tumorigenesis. We show that UVA induces p62 expression through ROS production, as antioxidants and ROS scavengers inhibit p62 induction by UVA. Interestingly, the antioxidant response factor Nrf2, which is known to positively regulate p62 transcription (20), is also required for p62 induction by UVA. This work suggests that UVA could induce p62 through Nrf2 as a protective mechanism against UVA damage. p62 knockdown reduced UVA-induced ROS production and Nrf2 accumulation, suggesting that p62 is crucial for UVA-induced oxidative stress. p62 knockdown also positively regulated PTEN abundance,

and PTEN overexpression decreased p62 levels, indicating a feedback loop between p62 and PTEN. Our findings demonstrate that UVA induces p62 accumulation as part of the oxidative stress response and suggest that p62 functions as an oncogene in melanoma pathogenesis.

We show here that Nrf2 is required for UVA-induced p62 up-regulation. Nrf2 is a transcription factor that can induce p62 transcription (20). In turn, p62 can bind to the Nrf2 negative regulator KEAP1 to stabilize Nrf2 (18,19). In response to UVA irradiation, p62 and Nrf2 form a positive feedback loop and are likely to enhance the Nrf2-dependent antioxidant transcription program in the face of oxidative stress. Nrf2 is known to mediate gene expression of many antioxidant genes. Perhaps the p62-Nrf2 axis serves as an oxidative stress response mechanism for the melanocytes or melanoma cells to adopt and survive. However, we cannot exclude the role of autophagy and autophagic flux in UVA-induced p62 up-regulation. p62 is known to be a selective autophagy receptor and substrate, and it can be degraded via the autophagy pathway (39,40). Future studies will elucidate the role of autophagy in UVA-induced p62 up-regulation.

It is unclear why different antioxidants had distinct effects on UVA-induced p62 accumulation. The singlet oxygen quencher sodium azide and the mitochondria-targeted antioxidant Mito-CP abolished UVA-induced p62 up-regulation, whereas NAC and GSH had no effect. Indeed, recently, using a genetically modified melanoma mouse model, Gal and colleagues have shown that NAC increases lymph node metastases of melanoma (41). *In vitro*, NAC and Trolox, the soluble vitamin E analog, increased cell migration and invasive properties (41). These findings suggest that antioxidants such as NAC at the dose tested may not be protective against melanoma progression. Future studies are essential to define the role of different types of antioxidants on melanoma initiation and progression.

In addition, we found that p62 knockdown prevented UVA-induced ROS production and oxidative stress. The reduced ROS production in p62 knockdown cells can also contribute to the reduction in UVA-induced Nrf2 accumulation. However, the underlying mechanism is unclear. It is possible that the accumulation of p62 led to p62-mediated protein aggregation in the cells in response to UVA damage. It is also possible that p62 induction disrupted mitochondrial function, which can lead to increased ROS formation. Further investigation is required to elucidate the underlying mechanism by which p62 induction regulates oxidative stress.

Consistent with previous studies (26), we found that p62 is up-regulated in human nevus, malignant melanoma, and metastatic melanoma, as compared with normal human skin melanocytes. In addition, as compared with normal human epidermal melanocytes, p62 is up-regulated in a panel of human melanoma cell lines. These findings suggest that p62 acts as an oncogene. p62 may regulate melanoma development through multiple pathways, such as Twist1, NF- κ B, or Nrf2, or a combination of these pathways (18,19,21,25,42–44). Our recent work showed that p62 stabilizes Twist1 to promote cell proliferation and migration *in vitro* and increases melanoma tumor growth *in vivo* (22). Future studies are needed to delineate the role of other p62-controlled pathways in melanoma development.

Our work also establishes a feedback loop between p62 and PTEN in melanoma, in which forced expression of PTEN suppresses p62 expression and knockdown of p62 increases PTEN. We and others have shown that p62 is upregulated in melanoma and correlates with poor prognosis (22,26). The existence of a p62-PTEN regulatory loop suggests an important role for p62 in melanoma progression. Loss of PTEN in melanoma patients, therefore, may compound the up-regulation of p62 throughout disease progression. Collectively, this work implicates p62 up-regulation as an important factor in UVA response in melanocytes and in melanoma pathogenesis.

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Figure 1.

UVA induces p62 in melanocytes. (A) Immunoblot analysis of p62 and GAPDH in normal human epidermal melanocytes (NHEM) with light pigmentation treated with sham, UVA (20 J/cm²) or UVB (20 mJ/cm²) irradiation and then harvested at 6 h or 24 h post-irradiation. (B) Immunoblot analysis of p62 and GAPDH in iMC23 and iMC65 melanocytes treated with UVA radiation and collected at 6 h post-irradiation. (C) Immunoblot analysis of p62 and GAPDH in A375 melanoma cells treated with sham, UVA, or UVB irradiation and collected at 6 h post-irradiation.

Figure 2.

Oxidative stress regulates p62. (A) Immunoblot analysis of p62, Nrf2, and GAPDH in iMC23 melanocytes treated with 10 mM sodium azide (NaN₃) overnight prior to UVA irradiation (20 J/cm²). (B) Immunoblot analysis of p62, Nrf2, and GAPDH in iMC23 melanocytes treated with 2.5 μM mito-CP for 1.5 h prior to UVA exposure. (C) Immunoblot analysis of p62, Nrf2, and GAPDH in NHEM cells transfected with siRNA targeting negative control (siCon) or Nrf2 (siNrf2) for 48 h and then irradiated with UVA exposure. (D) Immunoblot analysis of p62, Nrf2, and GAPDH in iMC65 melanocytes treated with 1 mM NAC overnight and then irradiated with UVA exposure. (E) Immunoblot analysis of p62, Nrf2, and GAPDH in iMC23 cells treated with 1 mM GSH-ester overnight and then irradiated with UVA exposure. NS, non-specific band.

Figure 3. p62 regulates UVA-induced ROS and antioxidant response. (A) Flow cytometric analysis of reactive oxygen species (ROS) levels in WT and p62 KO MEFs at 0 h post-sham or -UVA (20 J/cm²) using DCFH-DA assay. (B) Immunoblot analysis of p62, Nrf2, and GAPDH in WT and p62 KO MEFs at 6 h post-sham or -UVA (20 J/cm²). NS, non-specific band.

Figure 4. PTEN and p62 feedback loop in melanocytes. (A) Immunoblot analysis of Nrf2, p62, PTEN, and GAPDH in WM793 melanoma cells treated with doxycycline for 48 h to induce PTEN overexpression. (B) Immunoblot analysis of PTEN, p-AKT, AKT, p62, and GAPDH in A375 melanoma cells stably infected with a lentiviral vector expressing shRNA targeting negative control (shNC) or p62 (shp62).

Figure 5.

p62 is upregulated in melanomas. (A) Immunohistochemical analysis of p62 protein levels in normal human skin, nevus, malignant melanoma, and metastatic melanoma. Scale bar: 25 μ M. Arrow indicates a melanocyte. (B) Percentage of tumors (in stacked column format) for each score of p62 expression. (C) Immunoblot analysis of p62, Nrf2, PTEN and GAPDH in NHEM and human melanoma cell lines with BRAF mutation, NRAS mutation, BRAF +NRAS mutations, or wild-type (WT) BRAF/NRAS.