HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2010 July 28.

Published in final edited form as:

Oncogene. 2010 January 28; 29(4): 492–502. doi:10.1038/onc.2009.357.

UVB induced ERK/AKT-dependent PTEN suppression promotes survival of epidermal keratinocytes

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Abstract

Ultraviolet (UV) radiation in sunlight is the major environmental cause of skin cancer. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a proven critical tumor suppressor. We report here that UVB down-regulates PTEN in primary human keratinocytes, human HaCaT keratinocytes, and mouse skin. As compared to normal skin, the PTEN levels are reduced in human actinic keratosis, a precancerous skin lesion caused by solar UV. PTEN down-regulation is mediated by two mechanisms: (1) PTEN is cleaved by active caspase in apoptotic cells in which AKT activation is reduced; and (2) PTEN transcription is suppressed in surviving cells, and this suppression is independent of caspase activation and occurs in parallel with increased ERK and AKT activation. We report here that the combination of ERK and AKT activation is crucial for PTEN suppression in surviving cells following UVB irradiation. PTEN remains suppressed in these cells. AKT activation is higher in UVB-irradiated surviving cells as compared to UVB protected control cells. ERK and AKT pathways are involved in sustaining PTEN suppression in UVB-exposed cells. Increasing PTEN expression enhances apoptosis of keratinocytes in response to UVB radiation. Our findings indicate that (1) UVB radiation suppresses PTEN expression in keratinocytes, and (2) the ERK/AKT/PTEN axis may form a positive feedback loop following UVB irradiation. Identification of PTEN as a critical molecular target of UVB will add to our understanding of the pathogenesis of skin cancer.

Keywords

PTEN; UVB; Keratinocytes; AKT

Introduction

Skin cancer is the most common cancer in the United States. Each year more than one million new cases are diagnosed, accounting for more than 40% of all cancers. The common skin cancers include basal cell carcinoma (BCC), squamous cell carcinoma (SCC), together known as non-melanoma skin cancer (NMSC), and melanoma. BCCs and SCCs account for

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approximately 80% and 16% of all skin cancers, respectively, whereas malignant melanomas account for almost 4% (Bowden, 2004).

Ultraviolet (UV) radiation in sunlight is the major environmental cause of skin cancer (Erb *et al.*, 2008; Ramos *et al.*, 2004). The rising incidence rates of BCC, SCC, and melanoma are highly associated with increased exposure to UV radiation due to factors such as sun bed tanning for cosmetic purposes, increased outdoor activities, changes in clothing style, increased longevity, and ozone depletion (Rigel, 2008). UVB (280–315 nm) induces DNA damage and perturbs signal transduction pathways that are critical for skin homeostasis.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor that negatively regulates the PI3K/AKT pathway (Maehama and Dixon, 1998). Loss of PTEN function through deletion, mutation, and/or decreased expression has been found in human sporadic cancers (Birck *et al.*, 2000; Byun *et al.*, 2003; Harima *et al.*, 2001; Kohno *et al.*, 1998; Steck *et al.*, 1997) and in hereditary cancer syndromes (Liaw *et al.*, 1997; Marsh *et al.*, 1997). Functional studies support the hypothesis that PTEN is a critical tumor suppressor of skin cancer and that loss of PTEN activity through mutation, deletion, or reduced expression may promote skin carcinogenesis. Mice with PTEN deletion are highly susceptible to tumor induction (Suzuki *et al.*, 1998). Conditional knockout of PTEN in keratinocytes leads to skin tumors (Backman *et al.*, 2004; Li *et al.*, 2002; Suzuki *et al.*, 2003), highly suggesting the pivotal role of PTEN in skin cancer development. However, it has been unclear whether PTEN is a direct target of UVB and, if so, how UVB regulates PTEN expression in keratinocytes. We hypothesize that UVB suppresses PTEN in keratinocytes and this suppression plays a critical role in cell survival. In this study, we found that UVB induces PTEN down-regulation *via* an ERK/AKT-dependent mechanism in surviving cells and a caspase-dependent mechanism in apoptotic cells. This down-regulation of PTEN by UVB irradiation leads to enhanced AKT activation and cell survival.

Results

UVB-induced down-regulation of PTEN in human keratinocytes

UVB is a complete carcinogen, inducing tumors by damaging DNA (Setlow, 1974) and activating oncogenic signaling pathways (Bowden, 2004). The PI3K/AKT oncogenic pathway is activated by UVB (Bode and Dong, 2003; Bowden, 2004). AKT activation is down-regulated by PTEN. AKT inhibition prevents UVB-induced skin damage including formation of cancer (Bowden, 2004).

We examined the effect of UVB radiation on the PTEN protein levels in human HaCaT keratinocytes to determine whether UVB is an important regulator of PTEN. When cells were exposed to different doses of UVB, PTEN was down-regulated at 6 and 24 h following exposure to 20 or 30 mJ/cm^2 UVB, but not to 10 mJ/cm^2 UVB (Figures 1A and 1B). At 72 h post-UVB, the PTEN levels were further reduced as compared to those at 6 h and 24 h. PTEN down-regulation post-UVB was correlated with AKT activation, although total AKT decreased. These data clearly indicate that UVB-induced PTEN down-regulation as well as AKT activation is both dose-dependent and time-dependent.

To determine whether UVB-induced PTEN down-regulation is specific for HaCaT cells, we evaluated the effects of UVB on PTEN levels in normal human epidermal keratinocytes (NHEK). Similar to HaCaT cells, in NHEK cells UVB irradiation suppressed the expression of PTEN and activated AKT at 72 h at 20 or 45 mJ/cm^2 in a dose-dependent manner (Figure 1C). Our data thus indicate that UVB-induced PTEN suppression is independent of the molecular differences between HaCaT and NHEK cells including p53 mutations. p53 in HaCaT cells has UV-type mutations and lost DNA-binding ability (Datto *et al.*, 1995). Although UVA radiation showed no effects at 24 and 48 h (data not shown), inhibitory effects similar to those of UVB were noted at 72 h (Figure 1D). This difference may reflect the need for much higher doses of UVA to be effective. Our results suggest that PTEN is mainly localized in the cytoplasm of human HaCaT keratinocytes (Figure 1E). PTEN levels in the cytoplasm decreased at 6 and 24 h following UVB radiation. In these studies we did not detect translocation of PTEN from cytoplasm to nucleus or *vice versa*. We could not detect PTEN in the membrane fraction in either UVB-irradiated cells or cells kept in the dark (data not shown).

PTEN is down-regulated in UVB-exposed mouse skin and human actinic keratosis and its down-regulation promotes cell survival

We have shown that PTEN down-regulation does not recover by 3 d following one exposure to UVB irradiation (Figure 1A). To determine whether this also holds true *in vivo*, we evaluated the effect of UVB irradiation on the PTEN levels in mouse epidermis. As shown in Figures 2A and 2B, in UVB-exposed mouse skin compared to that kept in the dark, the PTEN protein levels were significantly reduced in interfollicular epidermal keratinocytes ($P < 0.05$ and 0.01 for UVB at 50 and 125 mJ/cm^2, respectively).

To examine whether PTEN down-regulation detected in human keratinocytes and mouse skin is relevant to UV-induced skin carcinogenesis, we compared the PTEN levels in normal skin versus actinic keratosis (premalignant skin lesions caused by UV damage from chronic sun exposure). PTEN levels were significantly lower in actinic keratoses than in normal epidermis (EP) as well as in the adjacent hair follicle (HF) and sebaceous gland (SG) (Figure 2C, $P < 0.05$), strongly indicating that PTEN down-regulation may play a critical role during early stages of skin carcinogenesis.

To determine whether PTEN down-regulation persists in HaCaT cells, we first exposed HaCaT cells once to UVB irradiation and noted that PTEN was down-regulated at 6 h and 24 h after UVB. The surviving cells were cultured for one week, but did not recover from PTEN down-regulation (Figure 2D). If these cultured cells were then exposed to a second UVB dose, PTEN levels were reduced even slightly further at 6 and 24 h. These findings demonstrate that UVB-induced PTEN down-regulation in keratinocytes is persistent. AKT activation peaked at 24 h after UVB irradiation and was reduced by one week, but still remained higher than in cells kept in the dark. Twenty-four hours after the second UVB irradiation, AKT activation was much higher than after the first UVB dose. These data indicate that UVB-induced PTEN down-regulation coincides with AKT activation in response to the first and the subsequent UVB exposures.

To determine the role of UVB-induced down-regulation of PTEN, we reconstituted PTEN in UVB-exposed HaCaT cells by infecting them with an adenoviral vector expressing wild-type PTEN (Ad-PTEN). Infection with Ad-PTEN increased the PTEN protein levels modestly and inhibited AKT phosphorylation, as compared to empty vector expressing green fluorescent protein (GFP) (Figure 2E). The increased PTEN levels significantly enhanced apoptosis of HaCaT cells upon UVB irradiation ($P < 0.01$), as determined independently by Annexin V/propidium iodide assays and sub-G_1 assays (Figures 2F and 2G). These findings strongly indicate that down-regulation of PTEN following UVB irradiation promotes cell survival.

PTEN down-regulation is both caspase-dependent and caspase-independent

Apoptosis is critical for maintaining normal homeostasis. Keratinocytes undergo apoptosis by caspase activation after exposure to excessive doses of UVB irradiation, both *in vivo* (sunburn) and *in vitro*. Activation of caspase-3 cleaves PTEN during apoptosis (Torres *et al.*, 2003). To determine whether UVB-induced caspase activation and the resulting apoptosis are critical for PTEN down-regulation, HaCaT cells were exposed to different UVB doses, and then apoptosis was assessed. After exposure to 10 mJ/cm^2 no apoptosis was detected; however, at higher doses (20, 25, 30, or 40 mJ/cm^2) UVB caused apoptosis in a dose-dependent manner (Figures 3A and 3B). Caspase-3 activation and PARP cleavage are two early apoptosis markers. UVB irradiation at 20 mJ/cm^2 caused activation of caspase-3 and cleavage of PARP within 6 to 10 h (Figure 3C). At 24 h post irradiation neither caspase activation nor PARP cleavage was detectable, since only attached surviving cells were collected and analyzed. PTEN levels remained down-regulated in cells both with and without caspase-3 activation. To examine the PTEN levels in surviving cells and apoptotic cells, cells were exposed to UVB irradiation and floating (F) and attached (A, live) cells were collected separately. As shown in Figure 3D, PTEN was undetectable in floating cells that had activated caspase-3 and cleaved PARP. In comparison, in attached cells neither caspase-3 activation nor PARP cleavage was detected; PTEN was down-regulated, although the PTEN level was higher than in floating apoptotic cells. It is noteworthy that total AKT protein levels were also undetectable in floating apoptotic cells (Figure 3D), which may explain the loss of AKT activation in these cells. These results show that UVB-induced PTEN down-regulation is caspase-dependent in apoptotic cells, but caspase-independent in surviving cells.

PTEN down-regulation in apoptotic cells is caspase-dependent

To determine the mechanisms of UVB-induced PTEN down-regulation in apoptotic cells, we stained both control and UVB-irradiated cells with PTEN antibody conjugated with Alexa488, and active caspase-3 conjugated with PE. Flow cytometry was used to determine the PTEN levels and caspase-3 activation. Figure 4A shows that UVB (20 mJ/cm^2) induced caspase-3 activation, as indicated by two populations of cells, i.e. caspase positive (Cas+) and caspase negative cells (Cas−). The number of Cas+ cells increased with both UVB and the time interval following UVB irradiation (Figure 4B). The PTEN levels in Cas+ cells was much lower than in Cas− cells, as indicated by the left shift of the PTEN peak (Figure 4C). This was also true of cells kept in the dark. Caspase-3 activation by UVB exposure caused a significant PTEN down-regulation in apoptotic cells (Figure 4D, $P < 0.05$).

To determine whether caspase activation is required for PTEN down-regulation after UVB radiation, we inhibited caspase-3 activation by incubating these cells with the pan-caspase inhibitor VAD or the caspase-3-specific inhibitor DEVD. Inhibiting caspase activity by VAD significantly reduced apoptosis as quantified by annexin V-FITC/propidium assay (Figure 4E and 4F, $P < 0.05$). Incubation with DEVD and/or VAD blocked PTEN down-regulation at 6 h post radiation (Figure 4G). VAD was more efficient than DEVD in inhibiting caspase-3 activation and PARP cleavage, and slightly more efficient in preventing PTEN down-regulation. Other execution caspases including caspase-6 and 7 may also be involved in PTEN down-regulation as in PARP cleavage. In comparison, phosphorylated PTEN levels were not affected by caspase inhibitors. In addition, we could not detect fragments of endogenous PTEN as shown previously (Torres *et al.*, 2003). When HaCaT cells were transfected with a HA-PTEN plasmid, a N-terminus fragment was detected in UVB-exposed cells and VAD prevented HA-PTEN degradation and the formation of this fragment (Figure 4H). Therefore, UVB-induced caspase activation during apoptosis caused PTEN protein degradation.

PTEN down-regulation is caspase-independent in surviving cells

We showed that PTEN levels are suppressed both in apoptotic and in surviving cells following UVB irradiation (Figure 3C). The surviving cells may be transformed and eventuate in skin cancer *in vivo*. Identification of the molecular mechanisms of PTEN suppression in these cells is critical for understanding UVB carcinogenesis and its prevention by targeting PTEN suppression. UVB-induced down-regulation of PTEN in surviving cells was detected at 24 h, but not at 6 h post irradiation (Figures 5A and 5B). This is consistent with the decrease in the PTEN levels, despite the fact that there was no detectable caspase activation at 24 h post radiation (Figure 3C). Interestingly, PTEN down-regulation in UVB-exposed surviving cells was detectable in only a subpopulation of cells, equivalent to that of Cas+ cells (Figures 5A and 5B; left peak, indicated by arrow).

Our findings show that PTEN down-regulation occurs at UVB doses high enough to cause apoptosis. It has not been previously known whether caspase activation in apoptotic cells exerts other effects on surviving cells. The pan-caspase inhibitor VAD reduced Cas+ cells dramatically, whereas it had no effect on the subpopulation of cells with PTEN down-regulation (Figure 5C). The specific caspase-3 inhibitor DEVD, VAD, or both, had no effect on lowering PTEN levels at 24 h post UVB exposure as assessed by Western blotting (Figure 5D). These results strongly indicate that UVB-induced PTEN down-regulation in live cells is caspase-independent.

UVB-mediated PTEN down-regulation is mediated by suppressing PTEN transcription

To further determine the mechanisms of PTEN down-regulation by UVB, we examined the PTEN mRNA levels in control and UVB-exposed cells. UVB significantly decreased the PTEN mRNA levels as early as 6 h, and up to 48 h post irradiation (Figure 6A, $P < 0.05$). Similarly, the transcriptional activity of the PTEN promoter was reduced significantly (Figure 6B, $P < 0.01$). The inhibition of the PTEN mRNA and the transcriptional activity of PTEN promoter were reduced by > 50% as compared to controls. These results indicate that PTEN down-regulation by UVB is mediated *via* suppression of PTEN transcription.

Activation of ERK and AKT mediates PTEN down-regulation

We have demonstrated that following UVB radiation PTEN is suppressed for at least a week (Figure 2D), and even longer than 4 weeks, and will partially recover by 6 weeks (data not shown). Considering the critical role of PTEN as a tumor suppressor in skin homeostasis, recovering PTEN expression may prove beneficial in preventing carcinogenesis, e.g., following sunburn. We examined the role of the ERK and AKT pathways to further elucidate the molecular mechanisms of PTEN suppression by UVB irradiation. UVB activated the ERK and AKT pathways (Figure 7A). While ERK was activated at all the time points examined, AKT was activated only at 3 and 24 h post radiation, possibly due to loss of AKT protein in apoptotic cells between 3 and 24 h (Figures 3C and 3D). PD98059 is an inhibitor of ERK activation, and LY2940002 is an inhibitor of AKT activation. When cells were kept in the dark or exposed to UVB and then treated with PD, LY, or both, the suppression of PTEN expression at the mRNA level was completely blocked (Figure 7B, $P < 0.05$). The levels of PTEN protein were also significantly increased by PD, LY, or both ($P < 0.05$ for PD or LY and 0.01 for PD/LY) (Figures 7C and 7D). Interestingly, LY or PD increased the levels of PTEN protein for three days even when added 24 h post UVB radiation (Figure 7E). This indicates that ERK/AKT pathway is critical in maintaining PTEN suppression in UVB-exposed keratinocytes. To further determine the effect of AKT activation alone on PTEN expression, we infected HaCaT cells with constitutively active AKT adenovirus. At 48 and 72 h after infection, the PTEN protein levels decreased (Figure 7F). This AKT activation suppressed the PTEN mRNA levels significantly at 48 h post infection (Figure 7G, $P < 0.05$). These results demonstrate that AKT activation negatively regulates the PTEN levels, forming a positive feedback loop in keratinocytes. Our findings suggest that UVB-induced PTEN suppression is reversible by blocking ERK/AKT pathways.

DISCUSSION

PTEN plays an important role in skin homeostasis, and acts as a critical tumor suppressor by inhibiting PI3K/AKT pathway in both humans and mice. UV radiation is the major environmental factor causing skin carcinogenesis. In the present study, we investigated the effect of UVB on PTEN expression and its underlying molecular mechanisms. We found that UVB down-regulates PTEN in human NHEK and HaCaT cells, as well as in mouse skin. PTEN expression is significantly lower in actinic keratosis than in non-tumor sun-protected skin, suggesting that PTEN suppression is a consequence of UV damage. At the molecular level, we found that UVB down-regulates PTEN *via* two distinct mechanisms. In UVB-induced apoptotic cells PTEN down-regulation is caspase-dependent. In surviving cells PTEN suppression is caspase-independent. In surviving cells, PTEN suppression is ERK/AKT-dependent, due to reduced transcription of its gene. Thus, following UVB radiation, the activation of ERK and AKT pathways reduces PTEN levels. We showed that this suppression can persist for weeks. Following UVB radiation, PTEN suppression promotes cell survival by enhancing AKT activation.

Following UVB irradiation, PTEN is down-regulated by activated caspases during apoptosis. Biochemical analysis has clearly shown that PTEN has several target sites for

active caspase-3, located in unstructured regions within its C terminus (Torres *et al.*, 2003). The supporting evidence for PTEN down-regulation in apoptotic human HaCaT keratinocytes at 6 h after UVB requires caspase activation include: (1) Similar to caspase activation, PTEN down-regulation by UVB is both dose-and-time-dependent. (2) PTEN levels are significantly lower in caspase-3-positive cells as compared to caspase-3-negative cells. (3) Inhibiting the activity of caspase-3 or pan-caspase prevents PTEN down-regulation following UVB irradiation. Using HA-fusion PTEN, we found only one fragment in UVB-exposed cells, which would not be detectable if caspase inhibitor VAD was added. This fragment corresponds to the smaller fragments generated during PTEN cleavage by active caspases as reported previously (Torres *et al.*, 2003). However, we did not detect other cleavage fragments, because either they were rapidly degraded by other proteases, or they were not detected by commercial antibodies available to us.

In surviving keratinocytes, however, UVB-induced PTEN suppression is caspase-independent. In surviving cells, caspase-3 is inactive, and PTEN levels are lower than light-protected cells at 24 h and beyond (Figure 1A and 5A), but not at 6 h post-UVB exposure (Figure 5A). DEVD, VAD, or both failed to prevent PTEN down-regulation in surviving cells at 24 h post-UVB irradiation as shown by flow cytometry and Western blotting (Figure 5C and 5D). The decreased PTEN levels may lead to AKT activation (Figures 2E and 7A). Increased AKT activation is seen only in live cells, but not in apoptotic cells, although both populations have lower PTEN levels than control cells (Figure 3D). Interestingly, UVB-irradiated cells are able to maintain PTEN suppression for one week or longer (Figure 2D), which would result in prolonged AKT activation. A second UVB dose could further reduce PTEN expression and enhance AKT activation.

We also found that UVB-induced activation of ERK and AKT plays a critical role in both the induction and the maintenance of PTEN suppression. In surviving cells the inhibition of ERK and AKT activation prevented PTEN suppression at the mRNA and protein levels upon UVB exposure (Figure 7). One day after UVB radiation, we started inhibiting ERK, AKT daily for 3 days, and then detected increased PTEN expression. Inhibitors of ERK activation, AKT activation, or both could only partially recover down-regulation of the PTEN protein, implying that additional regulatory mechanisms are involved.

Recent studies support that activation of ERK pathway by Ras suppresses PTEN expression through c-Jun up-regulation (Hettinger *et al.*, 2007; Vasudevan *et al.*, 2007). Interestingly the AKT activation, negatively regulated by PTEN, in turn reduces PTEN expression following UVB irradiation and maintains PTEN suppression. This is different from the finding in NIH3T3 cells because AKT activation by transfecting constitutive active AKT increases the promoter activity of PTEN (Vasudevan *et al.*, 2007). However, dominant-negative AKT has no effect on PTEN transcription (Vasudevan *et al.*, 2007). Possible explanations for this discrepancy are that (1) different levels of AKT activation may be critical for its effect on PTEN expression and (2) that AKT-mediated PTEN suppression may be cell-type-dependent and UVB-specific. In fact, high AKT activation achieved by increasing adenovirus of constitutively active AKT has no effect on PTEN expression (data not shown). The modest AKT activation seen with a low concentration of adenovirus (5 MOI, multiplicity of infection) suppressed PTEN expression in human keratinocytes.

The surviving keratinocytes post excessive UVB irradiation appear different in shape within a week from cells kept in dark. It seems that UVB alters the cytoskeleton of keratinocytes. Previous studies support that UVB causes reorganization of actin cytoskeleton in CHOAA8 cells (Grzanka *et al.*, 2006). UVB induces perinuclear cytoplasmic actin filaments into a broad perinuclear distribution due to phosphorylation of 27 kDa heat shock protein (HSP27) (Wong *et al.*, 2000). Further investigation is needed to understand the regulation and function of the actin cytoskeleton alteration by UVB and whether it is connected with PTEN suppression.

Causing apoptosis in human HaCaT keratinocytes, 30 mJ/cm^2 UVB used in our study takes about 30 min at noon time in the summer time in Boston (Webb *et al.*, 1988). The minimal erythemal dose (MED) for humans, depending on skin types, has been measured to be 34 mJ/cm^2 (14–84 mJ/cm^2) for whites in the North East of England (Diffey, 1991; Diffey and Farr, 1991). MED is the threshold UV dose that may produce sunburn, i.e. apoptosis of keratinocytes *in vivo*. Thus, the UVB doses used here are relevant to physiologically solar UV exposure.

It appears that sustained PTEN suppression by UVB may involve epigenetic modifications in keratinocytes including chromatin reorganization. It has been shown that DNA repair following UV-induced DNA damage results in the recruitment of new histone H3.1 and may compromise the recovery of chromatin organization (Polo *et al.*, 2006). The incorporation of H3.1 variants into a defined chromatin region may act as an imprint for newly repaired chromatin following the damage event. Similar to UVC (100–280 nm, a waveband that is not present in sunlight reaching the Earth's surface but that has been used extensively in DNA damage studies), UVB radiation causes DNA damage including formation of cyclobutane pyrimidine dimmer (CPD) and 6-4 photoproducts (64PP). However, it is unknown PTEN expression is inhibited because of the incorporation of new histone variants that modify the chromatin structure of the PTEN gene. If so, ERK and AKT pathway may play an important role in the imprint of newly repaired chromatin post damage; these questions need to be addressed to understand not only the mechanisms of UVB-induced PTEN suppression but also the influence of UVB on epigenetic stability in keratinocytes. Further studies are needed to elucidate how environmental UV radiation inhibits the expression of the tumor suppressor PTEN.

In summary, our results demonstrate that UVB down-regulates PTEN not only in apoptotic cells through degradation by active caspases, but also in surviving cells through caspase-independent but ERK/AKT-dependent mechanisms. Identification of PTEN as a critical molecular target of UVB irradiation will add to our understanding of the pathogenesis of skin cancer, which is critical to improve our abilities to reduce the skin cancer burden.

Materials and methods

Human normal and tumor samples and immunohistochemistry

All human specimens were studied after approval by the University of Chicago Institutional Review Board. Tissues from formalin-fixed paraffin-embedded blocks in replicates from three individuals diagnosed with actinic keratosis or without skin tumors. PTEN

immunohistochemistry (IHC) was performed by the Immunohistochemistry core facility using anti-PTEN (clone 6H2.1, Upstate). The PTEN levels were evaluated in basal keratinocytes of interfollicular epidermis. At least 50 keratinocytes were selected for analysis of PTEN levels by ImageJ (NIH).

Animal Treatments

Female outbred C57B6 mice were obtained from Jackson Laboratories. Mice were housed three animals per cage. Mice were shaved one day prior to the first UVB irradiation and as needed later. Irradiated mice were exposed to UVB dorsally three times a week over one week. Seventy-two hours after the final exposure, mice were euthanized and skin was collected followed by fixation in formalin and embedding in paraffin. The PTEN protein levels were determined by immunohistochemistry by the core facility using anti-PTEN (138G6, Cell Signaling). At least 50 interfollicular basaloid epidermal keratinocytes were selected for analysis of PTEN levels by ImageJ. PTEN levels were expressed as percentage with the protein level in the control taken to be 100%.

Cell culture, UVB treatment, transfection, and adenoviral infection

HaCaT cells were cultured in 60 mm dishes containing normal culture medium. Normal human epidermal keratinocytes (NHEK) cells were purchased from Invitrogen and cultured in EpiLife medium (Invitrogen). For UVB treatment, the Stratalinker 2400 equipped with 312-nm UVB bulbs (Stratagene, La Jolla, CA) (UVC 0%, UVB 51%, and UVA 49%) was used. Cells were exposed to UVA irradiation as described previously (He *et al.*, 2008b). The UV exposure was performed in PBS after washing the cells with PBS twice to avoid the photosensitization effect of components in culture medium on the cells. In selected experiments, cells were preincubated with inhibitors at 37 °C for 1 h prior to irradiation. Inhibitors used were as follows: PD98059 (PD), and LY294002 (LY) from Biomol (Plymouth Meeting, PA). After UVB treatment, cells were incubated at 37°C with or without the inhibitors. Caspase inhibitors DEVD and VAD or the negative control FA were added after UVB irradiation. Cells were transfected with HA-PTEN plasmid (kindly provided by William Sellers, Harvard University, Addgene plasmid 10786) as described previously (He *et al.*, 2008a; He *et al.*, 2008b). Then cells were exposed to UVB and collected for Western analysis. Adenoviral infection was conducted as described previously (He *et al.*, 2006b). The adenoviral vector expressing wild-type PTEN was kindly gifted by Dr. Christopher Kontos (Duke University Medical Center).

Cell fractionation

Cytosolic and nuclear proteins were extracted using a Nuclear Extraction Kit (Chemicon) according to the manufacturer's instructions. Briefly, cells were lysed and disrupted followed by centrifugation at 8,000 ×g at 4°C for 20 min. The supernatant was taken as the cytosolic fraction. The pellet was further disrupted followed by centrifugation at 16,000 at 4°C for 5 min and the supernatant was taken as the nuclear fraction. Membrane fraction was isolated as described previously (He *et al.*, 2006a).

Determination of the PTEN levels and the caspase-3 activation by flow cytometry

Briefly, HaCaT cells were irradiated and fixed with 4% formaldehyde. After permealization with 90% methanol, cells were double-stained with anti-PTEN Alexa Fluor 488 (Cell Signaling) and anti-active caspase-3-PE (BD Biosciences) antibodies. The PTEN levels and the activation of caspase-3 in cells were determined by flow cytometry.

Western blotting

Western blotting was performed as described previously (He *et al.*, 2008b; He *et al.*, 2006b). Antibodies used were as follows: phospho-AKT-ser473 (p-AKT) and phospho-PTEN (p-PTEN, ser380/Thr382/383) (Cell Signaling Technology); phospho-ERK1/2 (p-ERK) (Santa Cruz); PARP, PTEN, AKT, ERK, and β-actin, caspase-3 (Santa Cruz) and HA (hemagglutinin) (Sigma). The Western blot signal was exposed to film, which was then scanned into a computer using a scanner. The optical density of the scanned blot was quantified using ImageJ (NIH) relative to a standard titration curve of PTEN protein. Data were expressed as percentage, with the protein level in the control taken to be 100%.

Real-time PCR

Quantitative real time PCR assays were performed using ABI7300 (Applied Biosystems, Foster City, CA). Real-time TR-PCR fluorescence detection was performed in 96-well plates with the SYBR® Green PCR Master Mix (Applied Biosystems) as described previously (He *et al.*, 2004). Amplification primers were 5−-AGTTCCCTCAGCCGTTACCT-3− (forward) 5−-AGGTTTCCTCTGGTCCTGGT-3− (reverse) for the PTEN gene; and 5−-ACTGGAACGGTGAAGGTGACA-3− (forward) and 5−-ATGGCAAGGGACTTCCTGTAAC-3− (reverse) for β-actin. The threshold cycle number (C_T) for each sample was determined in triplicate. The C_T for values fro PTEN were normalized against β-actin as described previously (He *et al.*, 2004).

Luciferase Reporter Assays

HaCaT cells were seeded in 6-well plates and grown to 50–60% confluence. The plasmid mixtures, containing 1 μg of PTEN promoter luciferase construct (PTEN-Luc in pGL3 vector, kindly provided by Ian de Belle at the Burnham Institute of Medical Research) and 0.025 μg of pRL-TK (Promega, used as a transfection efficiency control), were transfected using FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's protocol. The empty vector pGL3 was used as a vector control. At 48 h after transfection, the cells were harvested in 1x luciferase lysis buffer (Promega), and luciferase activity was measured with a TD-20/20 Luminometer (Turner BioSystems) and normalized with the values of pRL-TK luciferase activity using a dual luciferase assay kit (DLR, Promega).

Statistical Analyses

Data conducted in cells were expressed as the mean of three independent experiments (mean ± SE) and were analyzed by Student's *t*-test and ANOVA. The PTEN levels in mouse skin and human skin specimens were analyzed by Student's *t*-test and Fisher's exact test. A two-sided value of $P < 0.05$ was considered significant in all cases.

Acknowledgments

This work was supported by the Department of Medicine and the Section of Dermatology at the University of Chicago, the American Skin Association, and the University of Chicago Cancer Research Center (P30 CA014599), the Wendy Will Case Cancer Fund, Inc, and the CTSA (NIH UL1RR024999). We thank Terri Li for PTEN immunohistochemistry. We are grateful for Dr. Christopher Kontos for the adenoviral PTEN vector and Dr. William Sellers for the HA-PTEN plasmid.

Abbreviations

AKT	a serine-threonine kinase, downstream of PI3K, also called protein kinase B
DEVD	DEVD-fmk, a specific inhibitor of caspase-3 activation
ERK	extracellular signal-regulated kinase
FA	FA-fmk, a negative control for caspase inhibitors DEVD or VAD
LY	LY294002, a specific PI3K/AKT activation inhibitor
NMSC	non-melanoma skin cancer
PD	PD98059, an ERK activation inhibitor
PI3K	phosphoinositide-3 kinase
PTEN	phosphatase and tensin homologue deleted on chromosome 10
SCC	squamous cell carcinoma
UV	ultraviolet
UVB	Ultraviolet B (280–315 nm)
VAD	VAD-fmk, an inhibitor of pan-caspase

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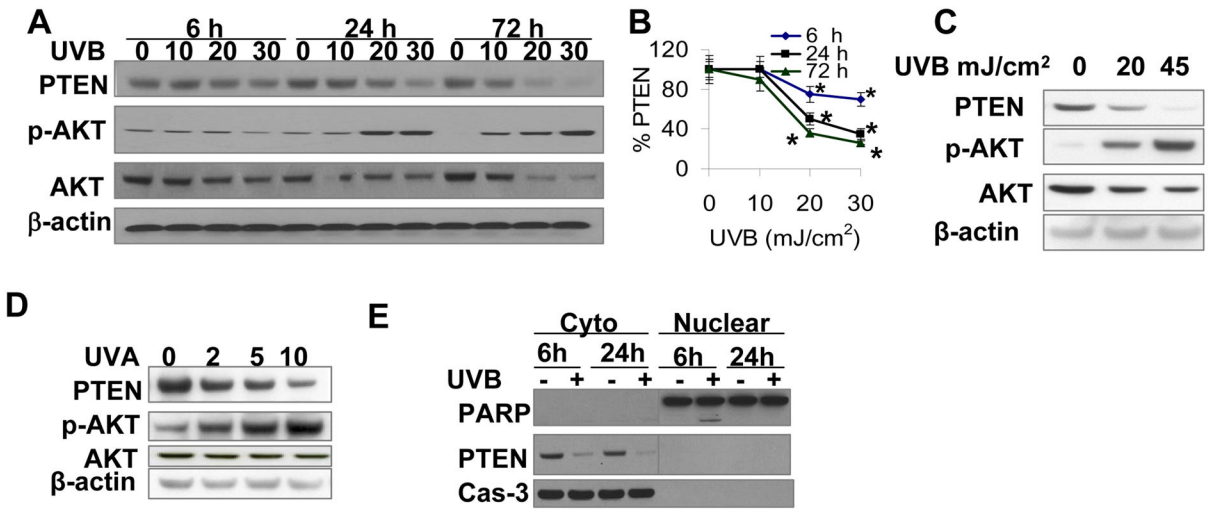

FIGURE 1. UVB effect on the PTEN levels in human HaCaT cells

Western analysis demonstrates dose- and time-dependent PTEN down-regulation following UVB irradiation. β-actin was used as an equal loading control. The data shown represent one of three separate experiments with similar results. A. HaCaT cells were kept in the dark or exposed to 10, 20, or 30 mJ/cm^2 UVB. Attached cells were collected at different time points. PTEN, p-AKT (serine 473), and total AKT were measured by Western analysis using specific antibodies. B, quantification of the PTEN levels in A. C, Normal human epidermal keratinocytes (NHEK) were exposed to UVB (0, 20, or 45 mJ/cm^2) and cells were collected at 72 h following UVB radiation. D, NHEK cells were exposed to UVA (0, 2, 5, or 10 J/cm^2) and cells were collected at 72 h following UVA radiation. E, Cells were exposed to UVB as in A and harvested at 6 and 24 h. Cytosolic and nuclear proteins were isolated. Caspase-3 (cytosolic marker) and PARP (nuclear marker) were used as isolation controls for successful cytosolic and nuclear fractionation and equal loading controls for these two fractions. *, $p < 0.05$, significant differences from cells kept in the dark.

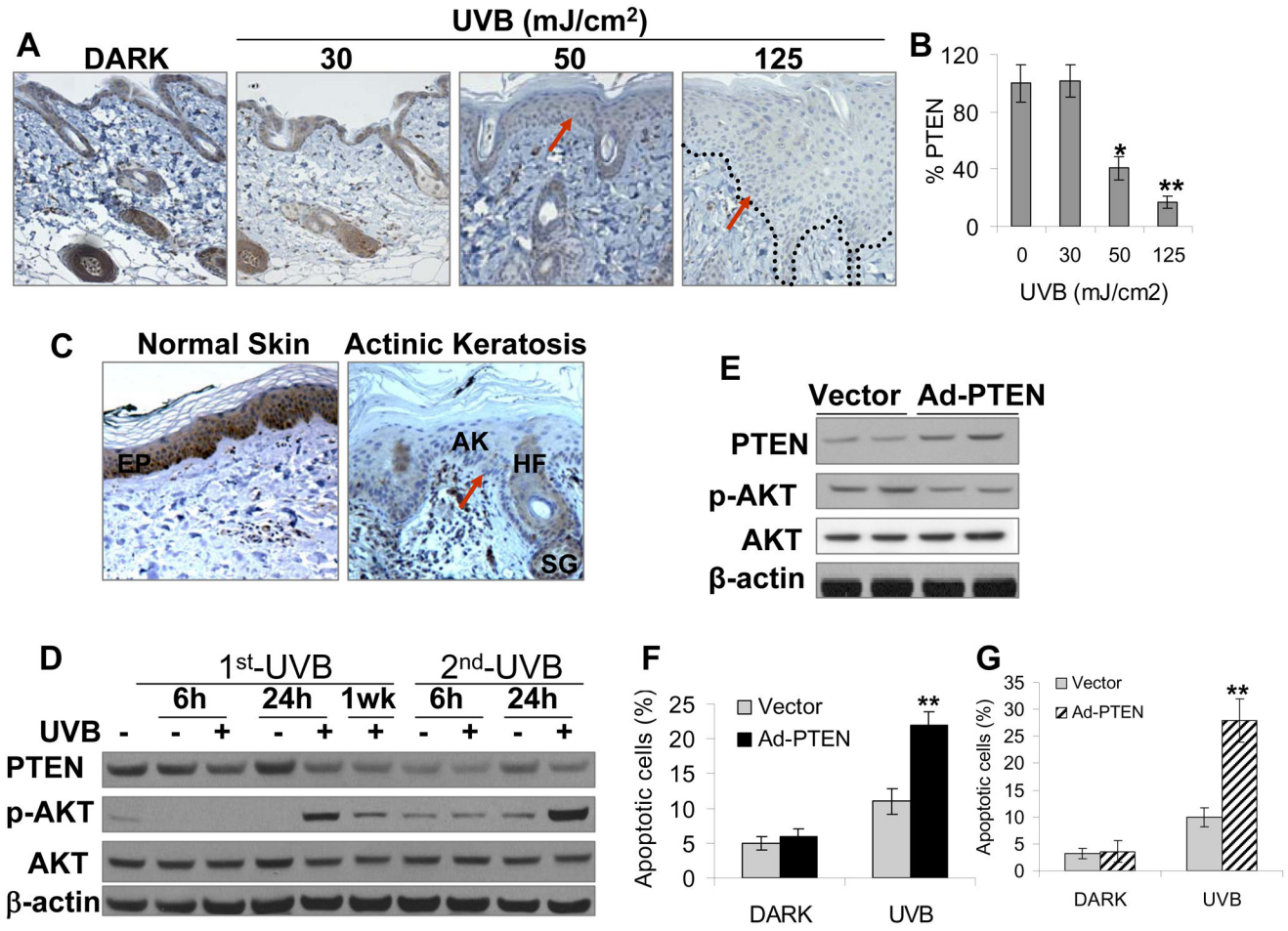

FIGURE 2. PTEN down-regulation in UVB-exposed mouse skin and human actinic keratosis specimens and maintenance of PTEN suppression following UVB radiation and its effect on AKT activation

A, C57B6 mice (n=3) were exposed to UVB (0, 30, 50, or 125 mJ/cm^2) three times over one week. Skin samples were collected at 72 h following UVB irradiation. The PTEN levels were determined by immunohistochemistry. Red arrows indicate basal keratinocytes that were quantified for PTEN expression. Dotted line indicates the boundary between epidermis and dermis. B, quantification of the PTEN levels in A. C, skin specimens from three individuals with or without actinic keratosis were analyzed for the PTEN protein levels in interfollicular epidermis by immunohistochemistry. The red arrow indicates basaloid layer of the AK tissue. AK-actinic keratosis; EP-epidermis HF-hair follicle; and SG-sebaceous gland. D, UVB-induced PTEN down-regulation is maintained in surviving cells and correlated with increased AKT activation. HaCaT cells were exposed to UVB (20 mJ/cm^2) and incubated for 6h, 24 h, or one week. A separate set of UVB-irradiated surviving cells were exposed to a second UVB exposure (20 mJ/cm^2) and attached cells were collected at 6 or 24 h. E, cells were infected with an adenoviral vector expressing wild-type PTEN or GFP as an infection control. The PTEN levels and AKT phosphorylation were determined by Western blotting. F, cells were infected as in E and then exposed to UVB (20 mJ/cm^2). Apoptosis was evaluated by Annexin-V/propidium iodide followed by flow cytometric

analysis. G, Same as in F except that apoptosis was determined by propidium iodide staining of fixed cells followed by flow cytometric analysis. *, $p < 0.05$; **, $p < 0.01$; significant differences from cells kept in the dark.

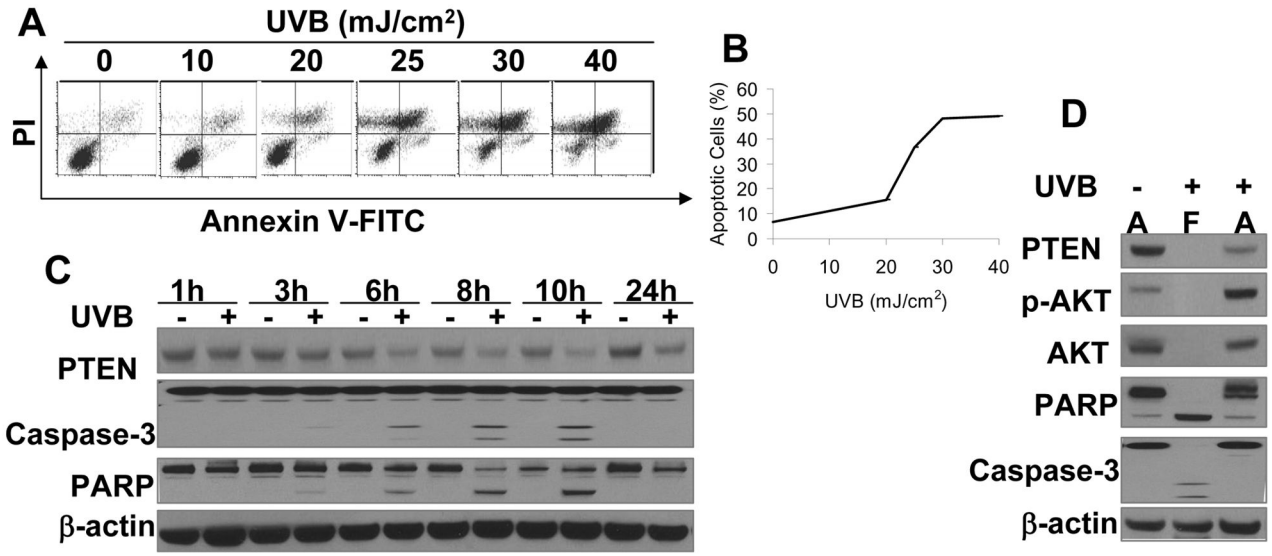

FIGURE 3. Caspase-dependent and –independent PTEN down-regulation following UVB irradiation

A, cells were exposed to different doses of UVB and collected at 14 h after UVB for Annexin-V/PI analysis. B, quantitative analysis of apoptosis induced by UVB from A. C, cells were treated as in FIGURE 1A. Caspase-3 activation and PARP cleavage were determined by Western blotting. D, HaCaT cells were exposed to UVB (30 mJ/cm^2). Twenty-four hours later attached (A) and floating (F) cells were collected.

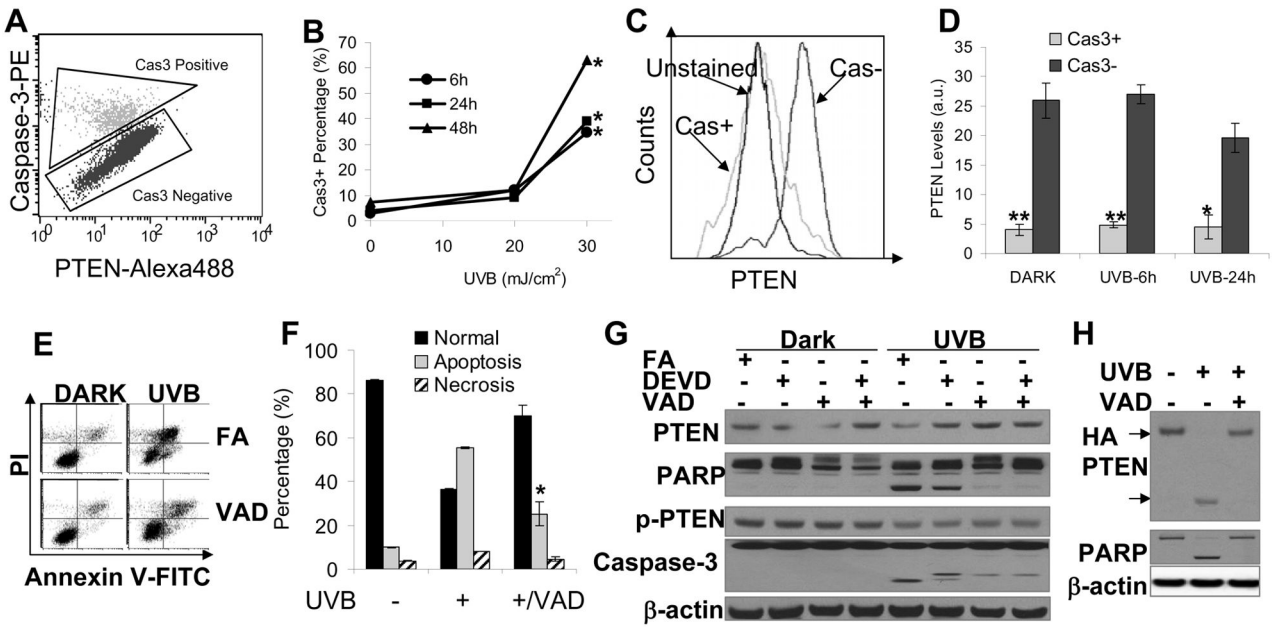

FIGURE 4. PTEN down-regulation in apoptotic cells

PTEN is cleaved by activated caspases in apoptotic cells following UVB irradiation. A, a representative plot of UVB-exposed HaCaT cells stained with PTEN-Alexa488 and active caspase-3-PE. B, quantitative analysis of percentage cells that are positive for active caspase-3 upon different doses of UVB irradiation at different times. *, $p < 0.05$; significant differences from cells kept in the dark. C, histogram shown PTEN peak in active caspase-3-negative (Cas3-), -positive (Cas3+), or unstained cells. D, quantitative analysis of the PTEN levels in Cas3− and Cas3+ cells at different times following UVB (20 mJ/cm^2). *, $p < 0.05$; **, $p < 0.01$; significant differences from UVB-exposed cells treated with FA. E, cells were exposed to UVB and incubated with or without the pan-caspase inhibitor VAD (20 μM). Cells were collected at 14 h after UVB and apoptosis were analyzed as in FIGURE 3A. F, quantitative analysis of apoptosis from E. *, $p < 0.05$, significant differences from UVB-exposed cells treated with FA. G, cells were exposed to UVB (20 mJ/cm^2) and then incubated with or without the specific caspase-3 inhibitor DEVD, VAD, or both. Cells were incubated with FA as a negative control for DEVD and VAD. Cells were collect at 6 h after UVB for Western analysis. H, cells were transfected with HA-PTEN and then exposed to UVB (30 mJ/cm^2). Cells were treated with FA (−) or VAD (+) post radiation. Cells were collected for Western blot analysis using specific anti-HA, and anti-PARP antibodies. Upper arrow indicates the full-length HA-PTEN and the lower one indicates the N-terminus fragment.

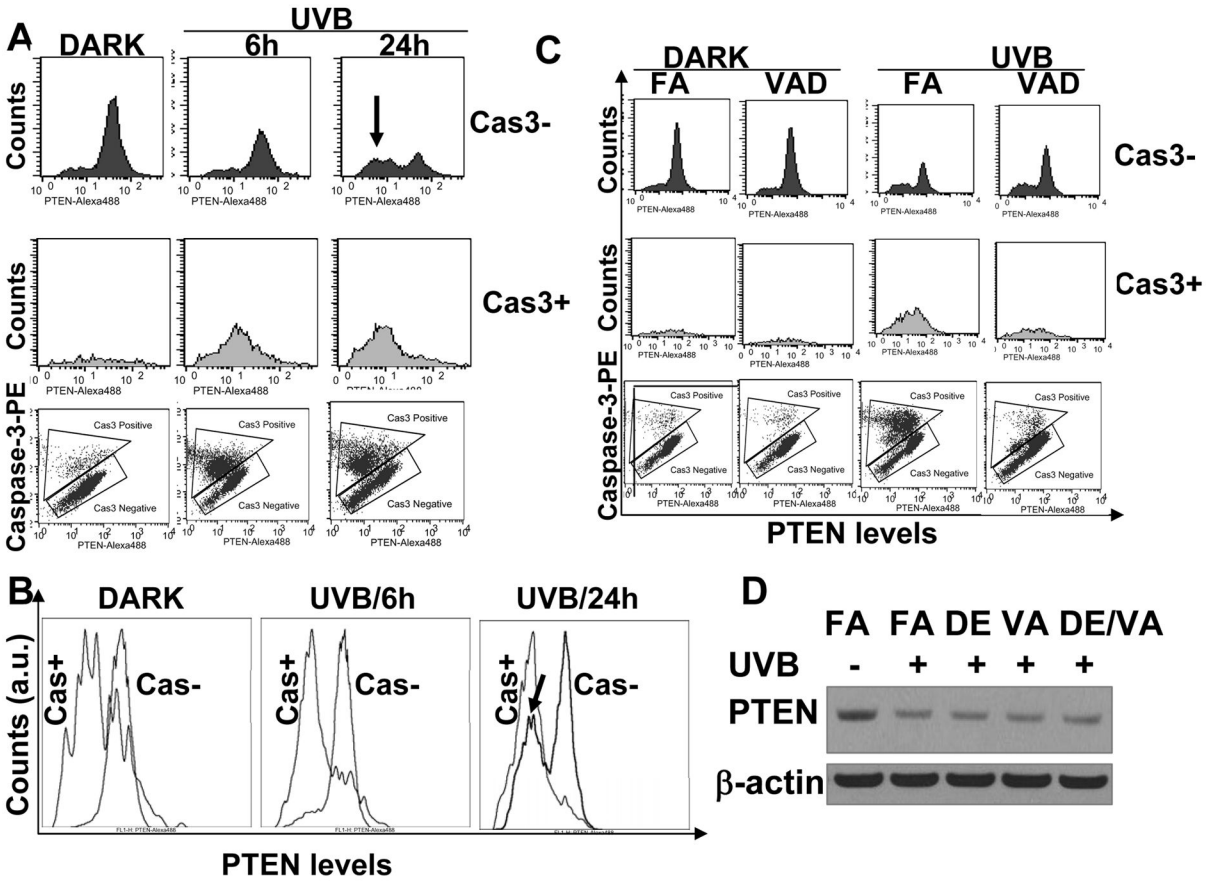

FIGURE 5. PTEN down-regulation in surviving cells following UVB irradiation

UVB-induced PTEN suppression in surviving cells is independent of caspase activation. A, cells were exposed to UVB (30 mJ/cm^2), collected at 6 or 24 h after UVB, and stained with PTEN-Alexa488 and active caspase-3-PE as in FIGURE 4. B, PTEN histogram overlap of active caspase-3-negative (Cas3−), -positive (Cas3+) cells from A. C, Similar to A except that cells were exposed to UVB and then incubated with FA or VAD for 24 h. PTEN levels in Cas3− and Cas3+ cells were determined. D, Cells were exposed to UVB (20 mJ/cm^2) and then incubated with FA, DEVD, VAD, or the combination of DEVD and VAD. Attached cells were collected for Western analysis at 6 h after UVB.

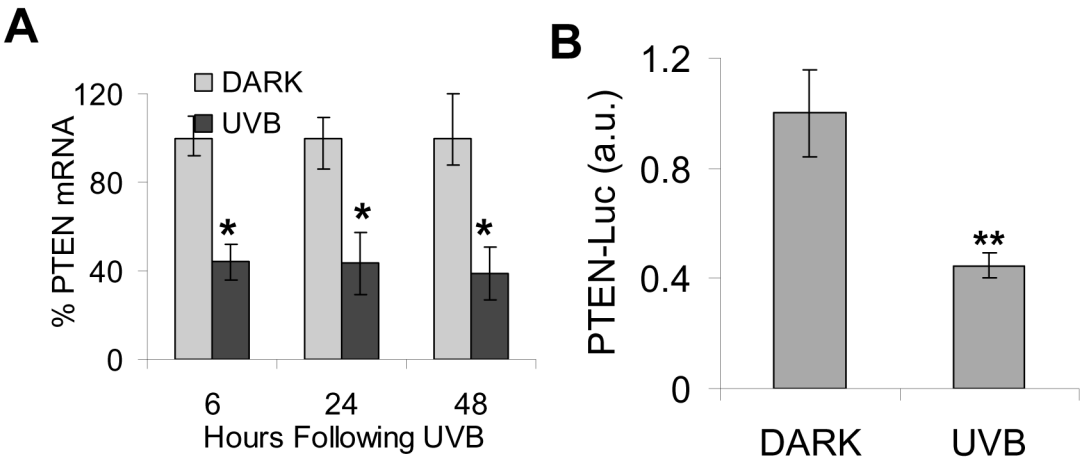

FIGURE 6. Involvement of PTEN transcription and proteasomal degradation in PTEN down-regulation following UVB irradiation

A, HaCaT cells were exposed to UVB (20 mJ/cm^2) and then collected at 6, 24, or 48 h following UVB irradiation. The PTEN mRNA levels were determined by real-time PCR. B, HaCaT cells were exposed to UVB as in A. Cells were then transfected with pGL3-Luc (vector) or pGL3-PTEN-Luc (PTEN-Luc) and pRL-tk as transfection efficiency control. Luciferase activity was analyzed by DLR assay. *, $p < 0.05$; **, $p < 0.01$; significant differences from cells kept in the dark.

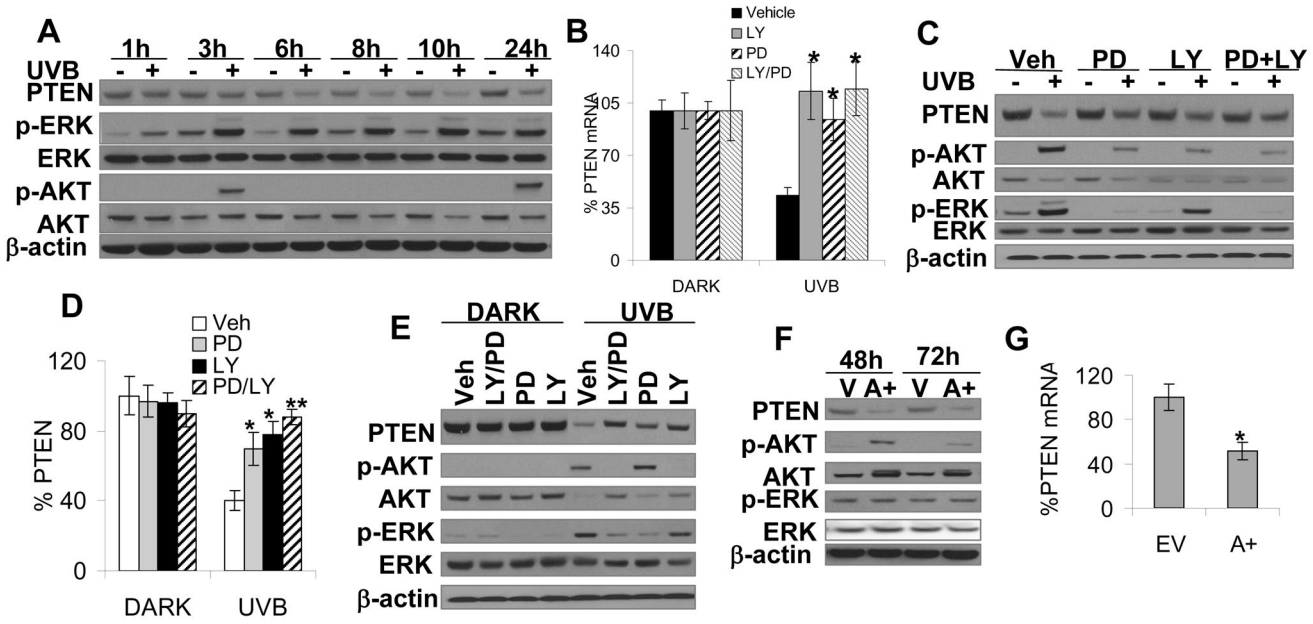

FIGURE 7. Role of ERK and AKT pathways in PTEN down-regulation in surviving cells following UVB radiation

Both ERK and AKT pathways are critical for PTEN suppression induced by UVB in surviving cells. A, cells were treated as in FIGURE 1A and ERK phosphorylation (p-ERK) was analyzed by Western blotting. B, cells were pretreated with the specific ERK activation inhibitor PD98059 (PD, 20 μM), the specific AKT activation inhibitor LY294002 (LY) (10 μM), or the combination of both, for 1 h, and then exposed to UVB (20 mJ/cm^2). Cells were then treated with PD, LY, or both and collected at 24 h. The PTEN mRNA levels were determined by real-time PCR. *, $p < 0.05$, significant differences from UVB-exposed cells treated vehicle alone. C, same as in B except that the PTEN protein levels were determined by Western blotting. D, quantification of the PTEN levels in C. *, $p < 0.05$; **, $p < 0.01$; significant differences from UVB-exposed cells treated vehicle alone. E, same as in B except that cells were treated with both PD (20 μM) and LY (10 μM) at 24 h after UVB radiation for three days and the PTEN protein levels were determined by Western blotting. F, HaCaT cells were infected with constitutive active AKT adenovirus (A+) or adenovirus expressing GFP only as a control. Cells were collected at 48 or 72 h after infection. G, same as in F except that cells were collected at 48 h post infection for real-time PCR analysis of the PTEN mRNA levels.