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The Protective Role of a Small GTPase RhoE against UVB-induced DNA Damage in Keratinocytes*

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Sarah A. Boswell[‡1], **Pat P. Ongusaha**[‡], **Paul Nghiem**[§], **and Sam W. Lee**[‡2]

From the [‡]*Cutaneous Biology Research Center (CBRC), Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129 and the* [§]*Dermatology Division, University of Washington, Seattle, Washington 98109*

***RhoE*, a p53 target gene, was identified as a critical factor for the survival of human keratinocytes in response to UVB. The Rho family of GTPases regulates many aspects of cellular behavior through alterations to the actin cytoskeleton, acting as molecular switches cycling between the active, GTP-bound and the inactive, GDP-bound conformations. Unlike typical Rho family proteins, RhoE (also known as Rnd3) is GTPase-deficient and thus expected to be constitutively active. In this study, we investigated the response of cultured human keratinocyte cells to UVB irradiation. RhoE protein levels increase upon exposure to UVB, and ablation of RhoE induction through small interfering RNA resulted in a significant increase in apoptosis and a reduction in the levels of the pro-survival targets p21, Cox-2, and cyclin D1, as well as an increase of reactive oxygen species levels when compared with control cells. These data indicate that RhoE is a pro-survival factor acting upstream of p38, JNK, p21, and cyclin D1. HaCat cells expressing small interfering RNA to p53 indicate that RhoE functions independently of its known associates, p53 and Rho-associated kinase I (ROCK I). Targeted expression of RhoE in epidermis using skin-specific transgenic mouse model resulted in a significant reduction in the number of apoptotic cells following UVB irradiation. Thus, RhoE induction counteracts UVB-induced apoptosis and may serve as a novel target for the prevention of UVB-induced photodamage regardless of p53 status.**

GTPases are regulatory proteins that function as molecular switches, cycling between the active, GDP-bound, and inactive, GTP-bound, states via control by guanine nucleotide exchange factors, GTPase-activating proteins (GAPs),[3] and guanine dissociation inhibitors (1). Rho GTPases act on numerous effector proteins to activate various signaling cascades (2, 3) and are specifically known to be involved in regulating cytoskeleton dynamics as well as proliferation and oncogenesis (4–7). Unlike typical Rho family proteins, Rnd proteins, including RhoE/Rnd3, remain in the constitutively active, GTP-bound state without GTP hydrolytic regulation or GAP activation (1, 8, 9). RhoE is known to inhibit RhoA/ROCK (Rho-associated kinase) signaling and block actin stress fiber formation, promoting the loss of actin stress fibers and focal adhesions (8–10). RhoE was also found to have a novel function in regulating cell cycle progression, independent of its ability to inhibit ROCK I (10, 11). Recently, *RhoE* was identified as a p53 target gene in response to DNA damage that promotes cell survival through inhibition of ROCK I-mediated apoptosis (12).

Normal human skin is covered with stratified epithelium composed mostly of keratinocyte cells, which undergo a continuous process of proliferation, differentiation, and apoptosis (13). Apoptosis is critical for epidermal homeostasis and is required for epidermal turnover and removal of UV-damaged cells (14–16). It is known that p53 plays an important role in inducing apoptosis in keratinocytes that have sustained UV-induced DNA damage and that this is a key step in protecting cells from transformation (14, 16). Moreover, as the first line of defense of the human body, keratinocyte cells have acquired unique coping mechanisms to protect themselves and underlying tissue from UV radiation (17, 18). Although the effects of UV radiation have been well studied, there is still a wide range of data supporting different mechanisms of UV response, in part due to the wide variety of model cell lines used to study this complex system (19). Given the importance of p53 in the UV response of keratinocytes and the up-regulation of *RhoE* by p53 upon DNA damage (12), we investigated the role of RhoE in the UVB response of human keratinocytes. We found that RhoE is an important factor in mediating cell survival in response to UVB irradiation and functions independently of p53.

EXPERIMENTAL PROCEDURES

Cell Culture—HaCaT cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. HaCaT cells stably transfected with a pBabe-U6 plasmid were maintained in 1 μg/ml puromycin. Normal human keratinocyte (NHK) cells were isolated from adult abdominal epidermis, similarly to several published methods (20–22), in a protocol approved by the Massachusetts General Hospital Institutional Review Board (MGH IRB). Fresh specimens were washed thoroughly in phosphate-buffered saline (PBS) and disinfected with 70% ethanol. The subcutaneous fat

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[1] Supported by National Institutes of Health Training Grant AR007098-31.
[2] To whom correspondence should be addressed: CBRC, Massachusetts General Hospital, Bldg. 149, 13th St., Charlestown, MA 02129. Tel.: 617-726-6691; Fax: 617-643-2334; E-mail: sam.lee@cbrc2.mgh.harvard.edu.
[3] The abbreviations used are: GAP, GTPase-activating proteins; ROCK I, Rho-associated kinase I; NHK, normal human keratinocytes; PBS, phosphate-buffered saline; DKSFM, defined keratinocyte-SFM medium; ROS, reactive oxygen species; JNK, c-Jun NH_2-terminal kinase; HA, hemagglutinin; siRNA, small interfering RNA; shRNA, short hairpin RNA; PARP, poly(ADP-ribose) polymerase; ELISA, enzyme-linked immunosorbent assay; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter.

and dermis were manually removed from the skin, which was then cut into squares (<1 cm^2) and floated on fresh dispase solution (Hanks' balanced salt solution containing 10 mM HEPES, 0.075% sodium bicarbonate, and 50 μg/ml gentamicin mixed 1:1 with dispase II solution (Roche Applied Science)) at 4 °C for 18 h. The epidermis was then pealed off and trypsinized to isolate the keratinocytes, which were plated in defined kerationcyte-SFM medium (DKSFM, Invitrogen) on collagen I-treated plates and grown at 37 °C, 5% CO_2. Cells were grown until 60–75% confluence and then split 1:3 (passage 1), grown until 60–75%, and frozen for future use. NHK cells were used between passages one and six and were collected from three different patients: females of ages 32, 37, and 66. Experiments were repeated using cells from each patient to ensure that consistent responses were obtained from different genetic backgrounds.

siRNA Experiments—Oligonucleotides corresponding to the following cDNA sequences were purchased as siRNA from Dharmacon: 5′-CAGATTGGAGCAGCTAC-3′ (nucleotides 501–518 (R1)) or 5′-GTAGAGCTCTCCAATCACA-3′ (nucleotides 439–457 (R2)) for RhoE, 5′-AGACAATCGGCTGCTCTGAT-3′ for GFP control, and 5′-ATTGTATGCGATCGCAGAC-3′ as a nonspecific control (Dharmacon). Cells were transfected by nucleofection (amaxa Inc.) using the human dermal fibroblast nucleofector kit and program T-24. Cells were harvested by trypsinization and resuspended in nucleofector solution at ~2–4 × 10^6 cells/100 μl of solution, electroporated with 100 μmol of R1 siRNA, and split to the appropriate number of collagen I-coated 60-mm plates. The cells were allowed to recover for 24 h prior to any further experiments. For stable knockdown of RhoE in HaCat cells, the R2 RhoE siRNA sequence was used to make a stable silencing construct of shRNA in the pBabe-U6 plasmid at the BamHI and XhoI cloning sites. The empty pBabe-U6 vector was used as a control.

UV Irradiation—UV was delivered from a panel of four UVB bulbs (RPR-3000, Southern New England Ultraviolet), which have a peak emission at 312 nm, delivering 90% UVB and <10% UVA. A Kodacel filter (catalog number K6808, Eastman Kodak Co.) was used to eliminate any UVC light (<290 nm). For *in vitro* studies, cells were seeded 24 h prior to irradiation and washed once with PBS immediately prior to irradiation. Irradiation was performed with the cells covered with 1.5 ml of PBS, and the UV dose was monitored with a Photolight IL1400A radiometer equipped with a SEL240/UVB detector. Following irradiation, fresh DKSFM or Dulbecco's modified Eagle's medium was added to the keratinocyte or HaCat cells, respectively. Cells were assayed 16 h after UV irradiation unless otherwise noted.

Western Blot Analysis—Cells were lysed in fresh lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 2 mM sodium orthovanadate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1:100 dilution of protease inhibitor mixture (Roche Applied Science)) for 45 min on ice. Antibodies used for immunoblotting are: β-actin (AC-15, Sigma), COX-2 (BD Biosciences), cyclin D1 (M-20 Santa Cruz Biotechnology), HA (F10, Roche Applied Science), JNK and phospho-Thr-183/Tyr-185 JNK (Cell Signaling), p21 (Ab-1, Calbiochem, and Ab-6, Oncogene), p38 (C-20, Santa Cruz Biotechnology), phospho-Thr-180/Tyr-182-p38 (Cell Signaling), p53 (Ab-1/Ab-2 Calbiochem), phospho-ser15-p53 (Cell Signaling), RhoE/Rnd3 (Upstate Biotechnology), total (Calbiochem), and cleaved PARP (Cell Signaling). Bands were detecting using the ECL chemiluminescence detection method.

Northern Blot Analysis—Total RNA was extracted using an Qiagen RNeasy kit with QIAshredder according to the manufacturer's protocol. Samples were quantified and denatured, and equal amounts were electrophoresed through 1% a agarose gel by the formaldehyde denaturation method. RNA was transferred to a nylon membrane (Bio-Rad), UV-cross-linked (Stratagene), and then baked at 80 °C for 1 h. Hybridization was performed with ^{32}P-labeled probes prepared by the random prime DNA labeling method (Invitrogen).

Cell Death Assays—Cell death was measured *in vitro* using a cell death detection ELISA (Roche Applied Science) and trypan blue exclusion. UV-irradiated cells, both the adherent and the floating, were harvested by trypsinization. Cells used for the ELISA were analyzed according to the manufacturer's protocol. Trypan blue exclusion was performed on cells harvested and resuspended in DKSFM and mixed 1:1 with 0.4% trypan blue. The percentage of dead cells was determined as the number of cells that stain blue versus the total cell count.

Flow Cytometry—Cells were harvested 16 h after UV irradiation and fixed with 70% ethanol while gently vortexing. Cells were stored at 4 °C up to 1 week prior to analysis. Just prior to analysis, the cells were washed once with PBS and then incubated in PBS containing 500 μg of RNase A for 30 min at 37 °C followed by the addition of 25 μg of propidium iodide and 15 min of room temperature incubation. Cells were analyzed on a FACSCalibur (BD Biosciences), and the acquired data were analyzed using the FlowJo 6.3.4 software package (Tree Star Inc.).

Detection of Intracellular ROS—UV-irradiated cells were treated with 12 μg/ml 2–7-dichlorofluorescein diacetate (Fluka) for 30 min at 37 °C. The adherent cells were harvested by trypsinization and resuspended in PBS. Intracellular reactive oxygen species oxidize 2–7-dichlorofluorescein diacetate, resulting in an increase in fluorescence as measured by flow cytometry.

Immunofluorescence—Cells were grown on coverslips and fixed with 3.7% (v/v) formaldehyde followed by permeabilization with 0.2% Triton-X. Actin filaments were visualized by incubating the fixed cells for 1 h at room temperature with rhodamine-conjugated phalloidin (Molecular Probes) (1:500). Images were collected by a Leica TCS4D confocal microscope and processed using Adobe PhotoShop software.

Animal Work—Human RhoE cDNA was cloned into a modified K14/bluescript vector. After approval by the MGH Animal Use Committee, the linearized cDNA construct was injected into fertilized C57BL/6 × SJLF2 embryos, and three founder mice expressing K14-RhoE were selected for breeding with C57BL/6 wild-type mice to yield F2 generation mice. K14-RhoE-positive F2 mice were crossed to yield a mixture of K14-RhoE-expressing mice and control transgene null littermates. All mice used in experiments were between 6 and 12 weeks old from the F3 generation. Transgenic animals were identified by

PCR analysis of genomic DNA extracted from the tail and confirmed by Western blot. For mouse irradiation, the dorsal hair was removed by shaving with electric clippers, and then the remaining hair was removed by chemical treatment (Nair). The mice were then allowed to rest for 72 h. Avertin was used to anesthetize the mice, which were then exposed to UVB irradiation, measuring the UVB dose using a radiometer as described above. 24 h after irradiation, the mice were sacrificed, and the dorsal skin was harvested for analysis. Sections of skin were taken for frozen sections, paraffin embedding, and protein extraction. Frozen sections were fixed in 1% methanol-free formaldehyde/PBS for 1 h at room temperature and then processed through a sucrose gradient (5%, 10%, 20%) before embedding in optimal cutting temperature compound (Tissue-Tek, Sakura). Paraffin sections were fixed in 4% buffered formalin for 72 h before automated embedding with a Tissue-Tek vaccum infiltration processor. Protein extracts were prepared by homogenizing a small piece of skin in lysis buffer with a tissue grinder.

Histological analysis by hematoxylin and eosin staining on 6-μm paraffin sections was performed with a linear stainer (Hacker Instruments) and imaged by bright field microscopy (Nikon E600). TUNEL analysis of 6-μm frozen sections was performed according to the manufacturer's protocol (Roche Applied Science), including a 20-min fix in formaldehyde. Sections were imaged by confocal microscopy, and the number of TUNEL-positive cells of each slide was counted from six random fields. HA staining was performed on 6-μm frozen sections. Briefly, slides were washed in PBS, fixed with 3.7% formaldehyde/PBS, permeabilized with 0.2% Triton/PBS, blocked, and then incubated in HA antibody (1:800 in blocking buffer) overnight at 4 °C. Rhodamine-conjugated secondary antibody was used at room temperature for 1.5 h, and the nuclei were stained for 15 min with 1:2000 TO-PRO3 (Invitrogen).

RESULTS

UVB Induces RhoE in Human Keratinocytes—Under various stress conditions, including DNA damage and oxidative stress, *RhoE* is induced in a p53-dependent manner (11, 12). In response to UVB irradiation, both NHK and HaCat cells show increased expression of RhoE and p21 as well as increased p53 activation, as measured by serine 15 phosphorylation (Fig. 1*A*). Although the p53 in HaCat cells is mutated (23), it is known to still be induced (24, 25) and phosphorylated in response to UV irradiation (26, 27). Thus, the observed increases in phosphorylated p53 and p21 are consistent with the literature on NHK and HaCat cells (24, 25, 28–32). To investigate the functional role of RhoE induction in the UVB response of keratinocytes, we used siRNA-targeting RhoE or GFP (negative control) to inhibit RhoE induction in response to UVB. siRNA was electroporated into HNK cells, which were treated with UVB 24 h later. The effectiveness of the RhoE siRNA is demonstrated by Northern and Western blot analysis (Fig. 1*B*). To exclude off-target effects of the RhoE siRNA, we used an alternate sequence of RhoE shRNA expressed in the pBabe U6-shRNA vector to stably knock down RhoE in HaCat cells and obtained similar results (data not shown). Although the RhoE siRNA is effective at blocking RhoE induction in response to UVB irradiation, it

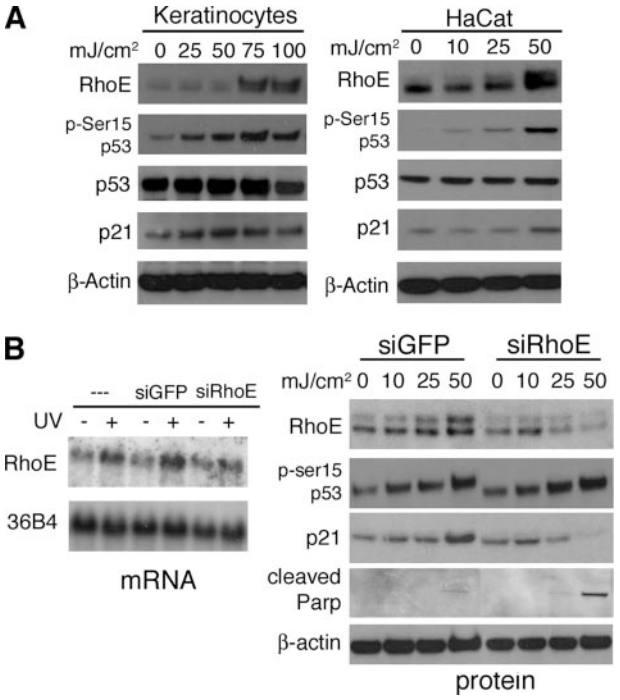

FIGURE 1. **UVB irradiation induces RhoE expression.** *A*, NHK and HaCat cells were lysed 16 h after UVB irradiation and analyzed by Western blot for RhoE, phosphorylated serine-15 p53 (*p-Ser15 p53*), p21, and *β*-actin (control). *B*, siRNA-targeting GFP (*siGFP*, control) or RhoE (*siRhoE*) was electroporated into NHK cells and treated with UVB 24 h later. Cells were harvested 16 h after irradiation for both Northern blot (*left panel*) analysis, probing for *RhoE* and *36B4* (loading control), and Western blot (*lower right panel*) analysis as described for *A*, including cleaved PARP.

did not alter the basal level of RhoE expression (Fig. 1*B*). The same blockage of RhoE induction was observed in the stable HaCat knockdown cells (data not shown). The maintenance of basal RhoE levels in siRNA-treated cells is important in maintaining normal function in the cells. This is particularly important since it has been previously observed that upon complete inhibition of RhoE in cancer cells, there is significant growth arrest and apoptosis.[4]

Although the keratinocytes treated with siRNA to GFP demonstrate a UV response very similar to control keratinocytes, treatment with siRNA-targeting RhoE greatly alters the cellular response to UVB irradiation (Fig. 1*B*). While the activation of p53 in response to UVB irradiation was not significantly affected, p21 protein levels declined rapidly, and PARP cleavage occurred at much lower doses of UVB. PARP cleavage is an indirect measurement for activated caspase-3, which is responsible for PARP cleavage upon the induction of apoptosis. Thus, the increased caspase-3-dependent PARP cleavage in UVB-irradiated cells that have reduced RhoE levels implies an increase in apoptosis.

RhoE Promotes Cell Survival in Response to UVB Irradiation—We analyzed the effect of knocking down RhoE on cell death in NHK cells 16 h after UVB irradiation by bright field microscopy, trypan blue exclusion, DNA fragmentation ELISA, and flow cytometry analysis of cells stained with propidium iodide (Fig. 2). Bright field imaging of NHK cells demonstrates

[4] P. P. Ongusaha and S. W. Lee, unpublished results.

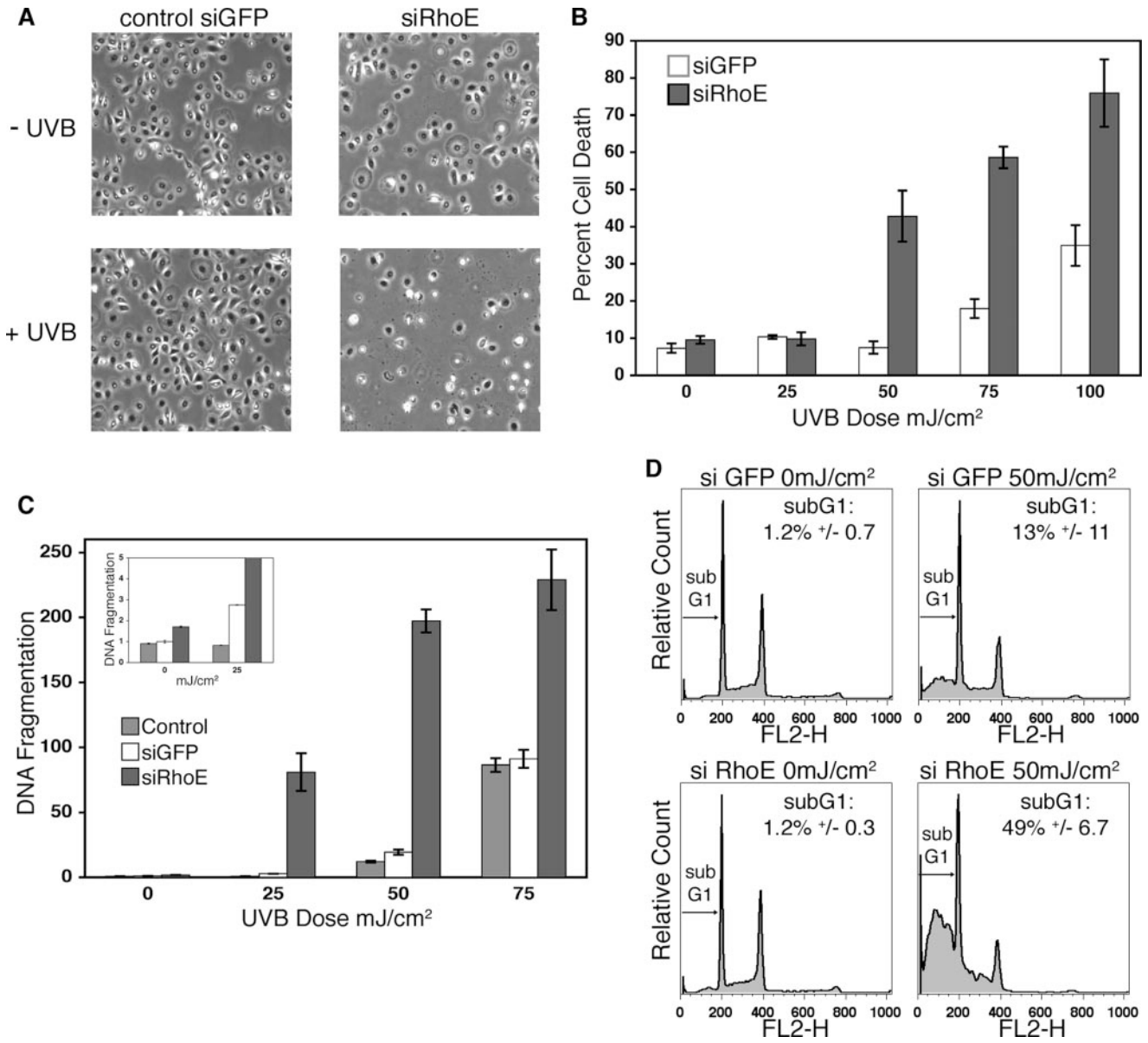

FIGURE 2. **siRNA-mediated inhibition of RhoE in UVB-irradiated keratinocytes results in increased apoptosis.** siRNA-targeting GFP (*siGFP*, control) or RhoE (*siRhoE*) was electroporated into NHK cells and treated with UVB 24 h later. Cells were assayed 16 h after irradiation as follows. *A*, bright field micrographs of keratinocytes. *B*, the percentage of cell death was quantified by a trypan blue exclusion assay as described under "Experimental Procedures." *C*, apoptosis was quantified by a cell death ELISA (Roche Applied Science) measuring DNA fragmentation as described under "Experimental Procedures." Results for *B* and *C* are shown as mean ± S.D. from three individual experiments. *D*, apoptosis was visualized by FACS analysis of propidium iodide-stained cells to quantify the number of sub-G_1 cells. A representative data set is shown. The percentage of sub-G_1 population and all *error bars* reflect the mean ± S.D. from three individual experiments.

that after electroporation, keratinocytes are healthy, and the introduction of siRNA against RhoE clearly results in a reduction of cell number after UVB irradiation (Fig. 2*A*). Cell death was then quantified by trypan blue exclusion (Fig. 2*B*). At UVB doses greater than 25 mJ/cm^2, the RhoE knockdown cells display a greater than 2-fold increase in cell death. DNA fragmentation (Fig. 2*C*) was quantified by cell death ELISA to determine whether this increase in cell death was due specifically to apoptosis. These data clearly indicate that the increase in cell death after UVB irradiation of keratinocytes treated with siRNA against RhoE is due to apoptosis. Propidium iodide staining of the UVB-irradiated cells was performed to confirm the DNA fragmentation results by quantitation of the sub-G_1 cell population by FACS analysis (Fig. 2*D*). Similar data were obtained when these experiments were performed in HaCat cells treated with siRNA or stably transfected with shRNA against RhoE (data not shown). These data demonstrate that RhoE promotes cell survival in response to UVB irradiation.

The Pro-survival Function of RhoE Is Independent of p53 and ROCK I in Keratinocytes—RhoE was recently identified as a p53 target gene up-regulated in response to DNA damage (12), and p53 is known to be stabilized in response to UVB exposure (32–35). Therefore, we investigated the p53 dependence of RhoE-mediated survival in response to UVB irradiation. Although it was expected that the pro-survival role of RhoE would be due, in part, to its role in p53-mediated survival, it

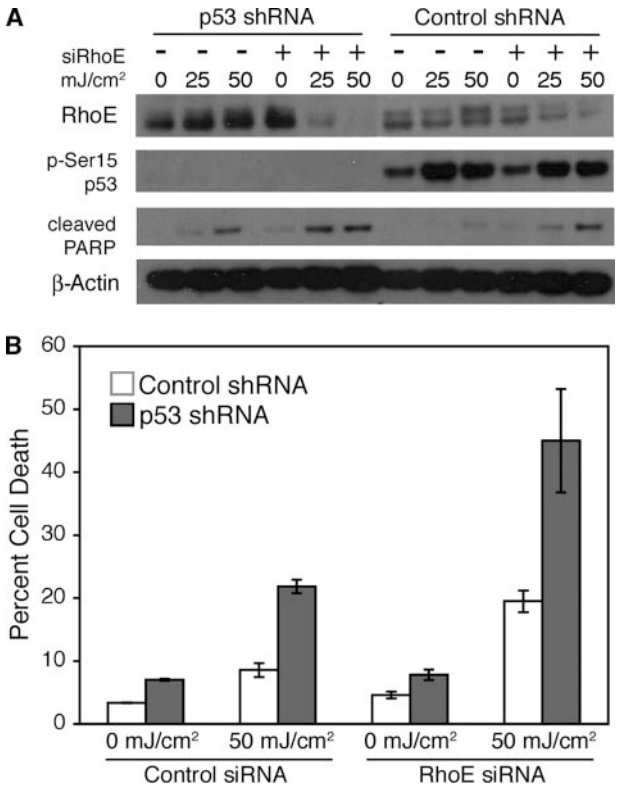

FIGURE 3. **RhoE is an upstream effector in response to UVB irradiation functioning independent of p53.** *A* and *B*, HaCat cells stably transfected with the pBabe vector expressing p53 shRNA or empty control vector were electroporated with control nonspecific siRNA or siRNA-targeting RhoE (*siRhoE*). 24 h later, cells were treated with UVB and harvested 16 h after irradiation. *A*, cell lysates were analyzed by Western blot for RhoE, phosphorylated serine-15 p53 (*p-Ser15 p53*), cleaved PARP, and β-actin (control). *B*, the percentage of cell death was quantified by a trypan blue exclusion assay as described under "Experimental Procedures." *Error bars* reflect the mean ± S.D. from two experiments.

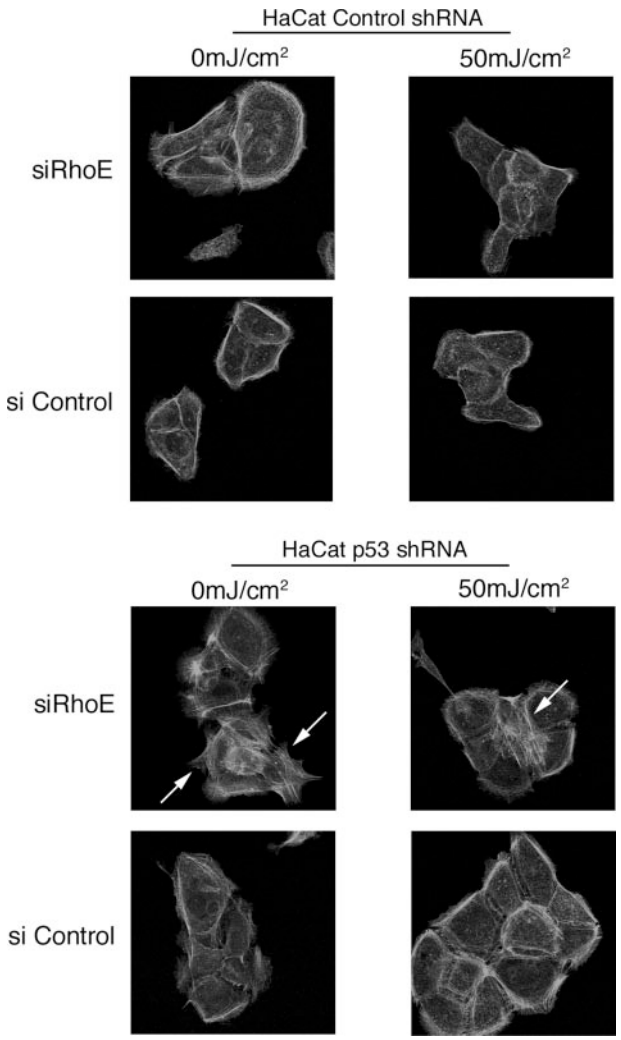

FIGURE 4. **Inhibition of RhoE does not affect the keratinocyte cytoskeleton without p53 knockdown.** HaCat cells stably transfected with the pBabe vector expressing p53 shRNA or empty control vector were electroporated with control nonspecific siRNA (*SiControl*) or siRNA-targeting RhoE (*siRhoE*) and plated on glass cover slides. 24 h later, the cells were treated with UVB, and 16 h after irradiation, cells were fixed and stained for filamentous actin with rhodamine-conjugated phalloidin. Representative images from duplicate experiments are shown. Stress fibers and filopodia are identified with *arrows*.

does not appear that the UVB induction of RhoE is solely p53-dependent since HaCat cells, which have a mutant low functioning p53 (23, 36) with an increased half-life (37–40), still induce RhoE after UVB irradiation (Fig. 1*A*). To further investigate the p53 dependence of RhoE expression in keratinocytes, stable HaCat cells were constructed in which p53 was ablated completely with shRNA-targeting p53 expressed in the pBabe-U6 vector. These cells were able to induce RhoE in response to UVB irradiation similar to control pBabe (empty vector) knockdown cells (Fig. 3*A*). There actually appears to be increased RhoE induction in the p53-knockdown HaCat cells when compared with the vector control HaCat cells (Fig. 3*A*). While elevated doses of UVB result in PARP cleavage in both NHK and HaCat cells, HaCat cells, expressing mutant p53, are more sensitive to UVB irradiation and become apoptotic at lower doses of UV than NHK cells, as reported previously (18, 41, 42). Although the p53 knockdown HaCat cells are more susceptible to UVB-induced apoptosis when compared with the pBabe vector control cells, RhoE knockdown in these cells increased the sensitization in an additive manner as measured by PARP cleavage and trypan blue exclusion assay (Fig. 3, *A* and *B*). These data clearly demonstrate that RhoE induction by UVB is not solely dependent on p53 activation in keratinocyte cells.

RhoE is well known to act as a negative regulator of RhoA-mediated ROCK I activation (9–11), and RhoE overexpression results in a transient loss of actin stress fibers (11, 43). Recently, it has been shown that knocking down RhoE in cells exposed to genotoxic stress resulted in the maintenance of stress fibers and increases apoptosis through the enhancement of the ROCK I-mediated apoptosis (12). However, depletion of RhoE in HaCat cells did not significantly alter the actin cytoskeleton between 12 (data not shown) and 16 h after UVB irradiation (Fig. 4, *top panel*). The same observations were made in RhoE siRNA-treated NHK cells (data not shown). Also, little change in ROCK I activation, through caspase-3 cleavage, was observed in the RhoE knockdown cells (data not shown). When RhoE was inhibited in the p53-depleted HaCat cells, changes in the actin cytoskeleton appeared following UVB irradiation (Fig. 4, *bottom panel*) similar to those observed by Ongusaha *et al.* (12), including an increase in the number of stress fibers and filopodia (Fig. 4, *bottom panel*). Therefore, although RhoE may function in cytoskeleton regulation, these functions appear to

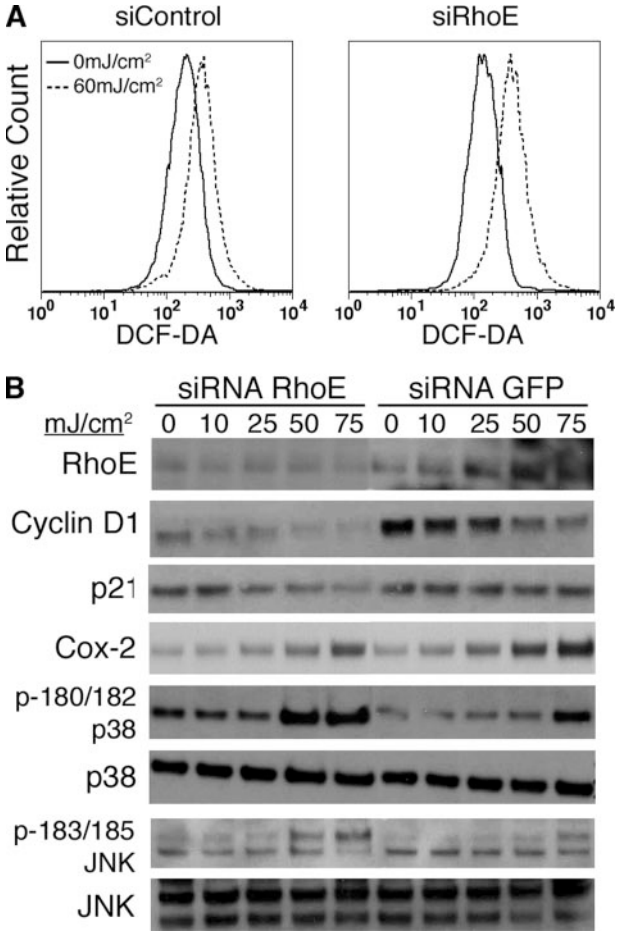

FIGURE 5. **siRNA-mediated inhibition of RhoE in UVB-irradiated cells increases ROS production and alters pro-apoptotic signaling.** *A* and *B*, siRNA-targeting RhoE (*siRhoE*) or a nonspecific control (*SiControl*) was electroporated into NHK cells and treated with UVB 24 h later. 16 h after UVB irradiation, cells were treated as follows. *A*, cells were loaded with 12 μg/ml 2′,7′-dichlorofluorescein diacetate (*DCF-DA*) and harvested for FACS analysis of 2′,7′-dichlorofluorescein oxidation by ROS. A representative data set is shown. *B*, cells were lysed and analyzed by Western blot for the following proteins: RhoE, cyclin D1, p21, Cox-2, phosphorylated Thr-180/Tyr-182-p38 (*p-p38*), p38, phosphorylated Thr-183/Tyr-185-JNK (*p-JNK*), and JNK.

be related to another p53-dependent mechanism, while the pro-survival UVB stress response of RhoE appears to act through a p53- and ROCK I-independent mechanism.

RhoE Acts as an Upstream Mediator of ROS, p38, JNK, and Cyclin D1—UV irradiation is known to induce oxidative stress via production of ROS (44, 45). To investigate the possible mechanism by which RhoE may be involved in the UVB response, we investigated whether RhoE plays any role in responding to ROS after UVB irradiation (Fig. 5*A*). NHK cells transfected with siRNA against RhoE or a control siRNA were irradiated with UVB, and the intracellular ROS was measured by monitoring the oxidation of the dye 2′,7′-dichlorofluorescein diacetate (DCF-DA) by FACS. A representative data set demonstrates that knocking down RhoE resulted in increased ROS after UVB irradiation when compared with control knockdown cells (Fig. 5*A*). The increase in ROS was reproducible with an average 1.4 ± 0.07-fold increase over the control cells in triplicate experiments. These data indicate that RhoE is important for cell survival in response to UVB irradiation, in part, by inhibiting ROS generation. We next aimed to gain further insight into the pro-survival function of RhoE by examining the expression patterns of several survival and stress response proteins in NHK cells. Depletion of RhoE resulted in a dramatic reduction in the levels of cyclin D1 and p21 (Fig. 5*B*). Cell cycle analysis confirmed this effect on cyclin D1 as demonstrated by an increase in G_1 arrest of the RhoE knockdown cells (data not shown). Additionally, the pro-survival factor Cox-2 was not induced to the same extent in the RhoE knockdown cells when compared with that of control cells in response to UVB irradiation. There is also an earlier activation of p38 and JNK kinases in the RhoE knockdown cells (Fig. 5*B*). These results were consistent in HaCat cells expressing an alternate RhoE shRNA in the pBabe-U6 vector (data not shown). These data suggest an overall upstream effect of RhoE on several UV response pathways.

RhoE Enhances Survival of UVB-irradiated Keratinocytes in Vivo—To determine whether RhoE expression can protect against UVB-induced cell death, transgenic mice expressing RhoE on the keratin 14 promoter (K14-RhoE) were generated as described under "Experimental Procedures." RhoE transgenic mice were healthy and were macroscopically indistinguishable from their wild-type littermates. UVB irradiation of control mice results in increased RhoE expression as seen in the *in vitro* studies on NHK and HaCat cells (Fig. 6*A*, *upper panel*). The transgenic K14-RhoE mice were clearly observed to express the HA-RhoE in the skin by α-HA Western blot and immunohistochemistry (Fig. 6, *A*, *lower panel*, and *C*). Although there is some background HA signal detected in the control littermates (Fig. 6*A*, *lower panel*), this is attributed to nonspecific detection since there is no positive staining in the skin (Fig. 6*C*). Overexpression of RhoE appeared to cause no defects in the growth and development of the mice as demonstrated by normal epidermal histology (Fig. 6*B*). K14-RhoE mice and control littermates were treated with 160 mJ/cm^2 of UVB and harvested 24 h after exposure. No quantifiable difference in the thickening of the epidermis or stratum corneum was observed after UVB irradiation (Fig. 6*B*). Cell death was identified and quantified by TUNEL analysis and imaged by confocal microscopy. The K14-RhoE mice demonstrated significantly fewer TUNEL-positive cells than their control littermates (Fig. 6, *D* and *E*), and the difference was determined to be statistically different by a two-tailed *t* test (Fig. 6*E*). Therefore, we found that RhoE promotes survival by protecting skin cells from UV-induced cell death *in vitro* and *in vivo*. All of these findings imply that RhoE plays an important role in maintaining healthy skin and protecting against UV damage stress.

DISCUSSION

Apoptosis of UV-damaged keratinocytes is a vital step in preventing skin cancer (14). UV is known to induce damage through three concomitant mechanisms: direct DNA damage, death receptor activation, and ROS formation. However, the details of this complex mechanism have yet to be elucidated, due in part to the wavelength dependence of UV-induced damage (44, 46). In this study, we demonstrate that RhoE is an important pro-survival factor for the normal

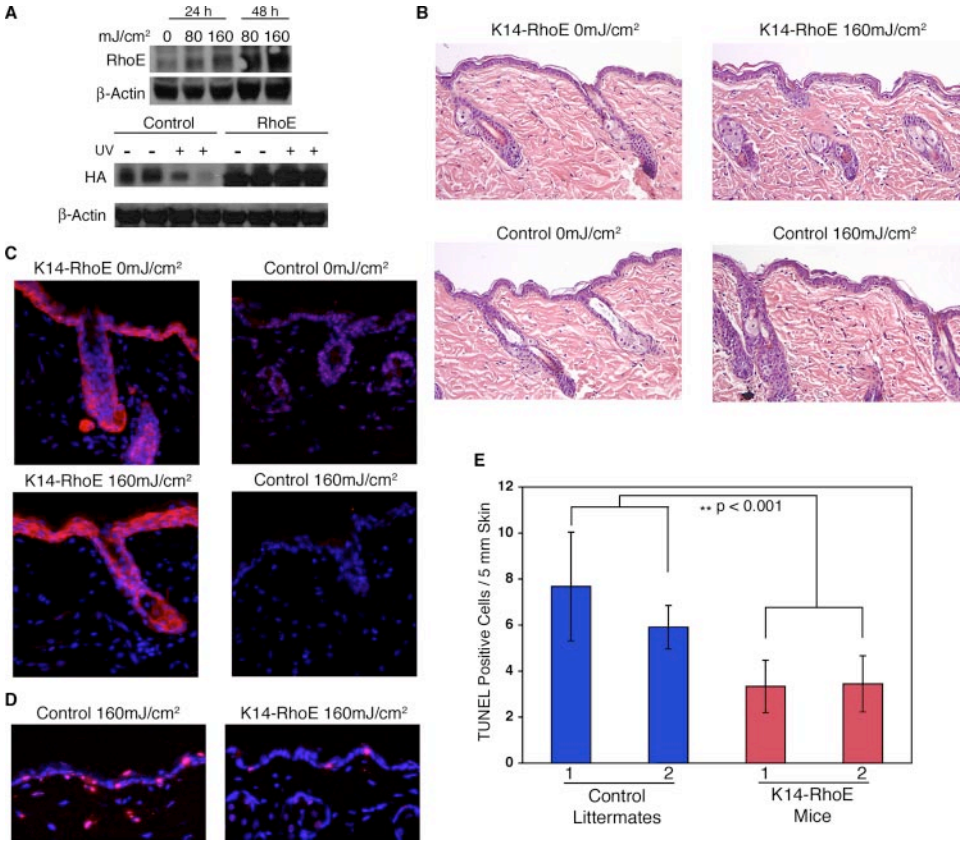

FIGURE 6. **Transgenic overexpressing RhoE mice demonstrate increased resistance to UVB-induced apoptosis.** Transgenic K14-RhoE mice, age 6–12 weeks, and control littermates were shaved, and 72 h later, irradiated with UVB. Mice were sacrificed 24 h after irradiation, unless otherwise indicated, and the dorsal skin was taken for analysis. *A*, Western blot analysis of homogenized skin samples as follows: RhoE, HA tag, and β-actin (control). Control mice were treated with UVB analyzed for RhoE expression after 24 and 48 h (*upper panel*). K14-RhoE mice and control littermates were irradiated with 160 mJ/cm^2 UVB and analyzed for HA-RhoE expression (*lower panel*). *B*, hematoxylin and eosin staining was used to observe the histology of RhoE mice and control littermates with or without UVB irradiation. *WT*, wild type. *C*, immunohistochemistry analysis of HA-RhoE expression in K14-RhoE mice and control littermates with or without UVB irradiation. HA expression is stained in *red*, and nuclear staining is *blue*. *D* and *E*, TUNEL staining was used to detect apoptotic cells in frozen sections from K14-RhoE mice and control littermates irradiated with 160 mJ/cm^2 UVB. TUNEL-positive cells are *red*, and nuclei are stained *blue*. TUNEL-positive cells were counted from at least five random fields of sections from two K14-RhoE mice and control littermates. The *error bars* represent the mean ± S.D. from three different TUNEL staining experiments. The difference between the control and K14-RhoE mice is statistically different with a p value <0.001 as determined by a Student's t test.

response of both NHK and HaCat cells to UVB irradiation and appears to act independently of both ROCK I and p53.

UVB irradiation results in RhoE up-regulation in both NHK and HaCat cells and in mouse epidermis *in vivo*. When RhoE induction is inhibited by siRNA, we find a dramatic increase in apoptosis when compared with cells treated with control siRNA.

Although *RhoE* was recently shown to be a p53 target gene (12), RhoE induction in response to UV irradiation in keratinocytes is not solely dependent on p53 (Fig. 3). Other transcription factors, including the p53 homologues p63 and p73, may aid in RhoE induction in keratinocytes. However, the increased susceptibility of the p53 shRNA HaCat cells to UVB-induced apoptosis implies that the p53 present in HaCat cells does have some residual function. Thus, p53 may still act on the RhoE promoter in keratinocytes as we observed higher levels of RhoE expression in shRNA-mediated p53 knockdown HaCat cells; however, other transcription factors or stabilization mechanisms are clearly more important for the function of RhoE in response to UV irradiation. The p53 pro-survival target *p21* has also been observed to be up-regulated in a p53-independent manner in response to UV irradiation (28, 29), as seen in this study (Fig. 1). Given that UV irradiation induces signature mutations in the *p53* gene found in carcinomas (47, 48), p53-independent up-regulation or pro-survival factors may be a general survival mechanism in keratinocyte cells. Therefore, by targeting these survival factors, we may be able to prevent growth and propagation of UV-damaged cells.

Although the mechanism of the pro-survival function of RhoE remains unclear, we propose that RhoE acts upstream of JNK, p38, and cyclin D1. JNK (49, 50) and p38 (51, 52) are known to be activated in response to UV irradiation (51, 53, 54) and play a role in apoptosis. Therefore, inhibition of RhoE induction may effect the activation of p38 and JNK after UV irradiation and cause an increase in apoptosis. Keratinocytes undergoing apoptosis, as opposed to arrest and repair, display a significant decrease in p21 protein levels (32). Keratinocytes treated with high doses of UVB and in the siRNA-mediated RhoE knockdown cells also show a reduction of p21 coincident with apoptosis (Fig. 1). In addition, although Cox-2 is known to be downstream of p38 and JNK (55, 56), it is also a pro-survival response gene in UV-irradiated keratinocytes (57), and the decrease in Cox-2 levels in RhoE knockdown cells may correlate with the loss of p21 expression and cell commitment to apoptosis. Although RhoE appears to act to inhibit p38 and JNK activation, it may also affect other upstream signaling pathways that culminate in altered Cox-2 expression and inhibition of apoptosis.

After UVB irradiation, cyclin D1 levels are known to decrease (58), as was observed in this study. However, the RhoE knockdown cells displayed a significant loss of cyclin D1 even in the absence of UV irradiation. Antisense cyclin D1 led to inhibition of squamous cell carcinoma cell growth (59) and the growth of other cancers (60, 61). Moreover, fibroblasts derived from cyclin D1$^{-/-}$ mice display increased apoptosis in response to UV irradiation (62), and cyclin D1 is overexpressed in many tumor types (63, 64). Therefore, the reduction in cyclin D1 levels in response to RhoE siRNA may play a role in the observed increase in apoptosis.

Cdc42 is a well studied member of the Rho-GTPase family and has been linked to the activation of p38 and JNK (54, 65, 66)

as well as up-regulation of cyclin D1 (7, 67–69). Also, constitutively active RhoA induces cyclin D1 (70), and Rac is known to activate JNK and p38 (71, 72). Inhibition of Rho or ROCK blocked mitogen-activated protein kinase (MAPK) activity and led to cyclin D1 induction in response to mitogenic stimuli, acting downstream of Rac (7). Interestingly, RhoA has actually been proposed to have a dual role in regulating cyclin D1, blocking early G_1 expression of cyclin D1 and promoting sustained extracellular signal-regulated kinase (ERK) activation and mid-G_1 cyclin D1 expression (73). Although RhoE is currently only known to interact with ROCK I (10) and the regulatory protein RhoGAP5 (74), it may act on other GTPases, including Cdc42. It is also possible that RhoE may act in a similar fashion to RhoA and promote cyclin D1 expression, as is observed in this study, or block cyclin D1 expression as was observed by Villalonga *et al.* (11). This correlates with the postulation by Villalonga *et al.* that RhoE acts on the cell cycle through a RhoA- and ROCK I-independent mechanism (11). Similar to other Rho GTPases, RhoE most likely acts downstream of Ras to elicit a range of effects on kinase signaling and cyclin D1 levels.

Although the exact mechanism of the RhoE-mediated prosurvival effect is under investigation, this study clearly shows the importance of RhoE in the survival of UV-irradiated keratinocytes. The pro-survival function of RhoE may be important for overall skin homeostasis. Targeting RhoE in precancerous skin cells could provide an important therapeutic mechanism for the removal of damaged skin cells.

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