Signal Transducer and Activator of Transcription 3 Is a Key Regulator of Keratinocyte Survival and Proliferation following UV Irradiation

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Abstract
UVB irradiation of signal transducer and activator of transcription 3 (Stat3)–deficient keratinocytes resulted in a high incidence of apoptosis compared with controls. Conversely, forced expression of Stat3 desensitized keratinocytes to UVB-induced apoptosis. Upon UVB exposure, keratinocyte Stat3 was rapidly dephosphorylated, followed by decreases of both Stat3 mRNA and protein levels in a p53-independent manner. Vanadate treatment reversed the UVB-induced down-regulation of Stat3 and generation of apoptotic keratinocytes, suggesting the involvement of a tyrosine phosphatase. Furthermore, Stat3 was required for UVB-induced proliferation of follicular keratinocytes, leading to epidermal thickening. Finally, constitutive activation of Stat3 was observed in UVB-induced squamous cell carcinomas of either mice or human origin. These data suggest that Stat3 is required for survival and proliferation of keratinocytes following UVB exposure and that Stat3 is tightly regulated as part of a novel protective mechanism against UVB-induced skin cancer.

Introduction
Skin is continuously exposed to environmental insults including UV irradiation. In particular, UVB irradiation (wavelengths between 280 and 320 nm) is the major risk factor for development of skin cancer. Specific mechanisms have evolved that determine whether keratinocytes survive or undergo apoptosis in response to various conditions for skin homeostasis. It is generally accepted that UVB-induced apoptosis of keratinocytes represents a scavenging mechanism designed to remove DNA-damaged cells thereby reducing the risk of skin cancer (1–3). UVB irradiation has been shown to trigger at least two apoptotic pathways in keratinocytes, one being p53 dependent and the other p53 independent (4, 5). UVB irradiation arrests cells at the G1 phase in a p53-dependent fashion to allow repair of damaged DNA thereby reducing the DNA mutation rate (1, 6). In the presence of high levels of DNA damage, however, p53 up-regulates Bax, which results in cytochrome c release from mitochondria, subsequent activation of caspase-9 and caspase-3, and ultimately cell death (7). An alternate pathway of UVB-induced apoptosis occurs via activation of death receptors present on the cell membrane, including tumor necrosis factor receptor-1 and CD95 (Fas), which initiate activation of caspase-8, and finally caspase-3 (3). Accordingly, dysfunction of these apoptotic pathways (e.g., p53 mutation; ref. 8) or a disturbance of CD95/CD95L interactions (9) may contribute to the development of UVB-induced skin cancer. Furthermore, disturbances in the apoptotic machinery through up-regulation of other antiapoptotic molecules such as Bcl-2 family members (10, 11), or activation of signaling pathways via mitogen-activated protein kinases (12), nuclear factor-κB (13), phosphoinositide 3-kinase (PI3K)/AKT (14), or signal transducers and activators of transcription (STATs; ref. 15) may also increase the risk of UV-induced skin cancer.

STATs constitute a family of cytoplasmic proteins that play critical roles in transmitting signals from extracellular stimuli to the nucleus in normal cells (16, 17). A growing body of evidence indicates that constitutive activation of STATs is associated with the development of human cancers (18–20). In particular, Stat3 is frequently associated with oncogenesis in humans (21). In addition, activation of Stat3 was reported as a prerequisite for v-Src-mediated transformation of murine cells (22, 23). Moreover, transfection of a constitutively active Stat3 gene (Stat3C) showed that Stat3 activation was both required and sufficient to promote cellular transformation of murine fibroblasts (24). Thus, given a particular set of circumstances, Stat3 may behave as an oncogene.

We have recently found that Stat3 is required for the development of chemically induced skin tumors in mice using the two-stage carcinogenesis protocol (25). This requirement for Stat3 occurs at both the initiation and the promotion stages, through the roles it plays in the inhibition of apoptosis and in cell cycle progression, respectively. In the current study, we show a critical role for Stat3 in the initial response of keratinocytes to UVB irradiation. In addition, we also found that Stat3 was constitutively activated in UVB-induced skin cancer. These data suggest that Stat3 plays a role in both the early and later stages of UVB skin carcinogenesis and that initially, Stat3 is down-regulated as part of a novel protective mechanism against UVB-induced skin cancer.

Materials and Methods
Mice. Skin-specific Stat3 −/− mice (26) were generated as previously described. K5.Stat3C transgenic mice were generated and recently reported (27). Briefly, a Stat3C cDNA (24) was excised from vector pB/CMV/S3C and ligated into the pBluescript construct (28). pBK5.Stat3C was digested with EcoRI, and...
purified, and used to generate transgenic founders on an FVB background. Transgenic mice were identified by PCR of genomic DNA with rabbit β-globin-specific primers: 5'-TTCAAGATGGTGTGGATTAGATG-3' and 5'-CAATAGAACATTATTTTCAACGCA-3'. To produce XPC null (XPC/−) mice, a targeting vector for mXPC was constructed using a 129sv mouse genomic DNA library. A 10-kb SalI fragment, containing exons 1 to 4 of mXPC, was subcloned into the SalI site of pBluescript II KS+ (Stratagene, La Jolla, CA) followed by introduction of a NotI site downstream of exon 1 and SalI and SalI sites in exon 2. The NotI-SmaI fragment of mXPC, the SmaI-HindIII fragment of the lacZ gene, the SalI-XhoI flanked-neo gene fragment of pTnBo, the SalI fragment of mXPC containing exons 3 and 4, and the XhoI-SalI diphtheria toxin A fragment from pMC1DIPa were joined in pBluescript II KS+ to generate the targeting vector pBS-lmoD0TAXPC. The lacZ gene was fused in-frame to 41 amino acids of mXPC. The targeting vector was electroporated into 129Sv-derived embryonic stem cell line E12, and G418-resistant clones were analyzed by Southern blotting. Targeted clones were injected into host blastocysts, and heterozygous F1 progeny were mated to generate homozygous XPC null mice. The p53-null mice were purchased from GenPharm (Mountain View, CA). All experiments were carried out with strict adherence to institutional guidelines for minimizing distress in experimental animals. Mice were sacrificed under anesthesia with sodium pentobarbital.

**UVB irradiation.** Mice and cells were irradiated using two different UVB sources: Torex F20SE-30/DMR fluorescent sun lamps with a peak emission at 305 nm (Toshiba Medical Supply, Tokyo, Japan; for Figs. 1–4A-D) and a Westinghouse FS20 sun lamp bulb with a peak emission at 313 nm (for Fig. 4E-G and Figs. 5 and 6). The fluence rate was measured with either a UVR-305/365D digital radiometer (Opto-Electronic Measuring Instruments, Toshiba Medical Supply) or with an IL1400A Radiometer/Photometer coupled to a SEL240/UVB-1/TD detector (International Light, Inc., Newburyport, MA), respectively. Mice were irradiated on the dorsal skin with UVB at the indicated doses. Young (≤4 days old) or adult mice were used. Adult mice were shaved 2 days before irradiation. Twenty-four to 48 hours later, the dorsal skin was excised and fixed in 10% formalin, embedded in paraffin, and stained either with H&E or specific antibodies as indicated. Sunburn cells were counted with morphologic examination of the specimens under light microscopy (29). Three to four mice per group were used, and at least three sections of skin were evaluated for each mouse. For in vitro UVB irradiation, cells were coated with the minimum volume of PBS necessary to circumvent evaporation during irradiation. In some experiments, cells were pretreated for 4 hours with vanadate (Sigma, St. Louis, MO) at a final concentration of 100 μmol/L before UVB irradiation.

**Keratinocyte culture.** Epidermal keratinocytes were cultured from young mice (≤4 days old). Briefly, full-thickness skin was treated with 250 units/mL of dispase (Godo Shusei, Tokyo, Japan) overnight at 4 °C, after which the epidermis was separated from the dermis and trypsinized to prepare single cells. These were suspended in MCDB153 medium (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 0.1 mmol/L monoethanolamine, 0.1 mmol/L phosphorylcholine, and 0.5 mmol/L hydrocortisone at 37 °C under an atmosphere of 5% CO₂. Cells were seeded at a density of 5 x 10⁵/mL in dishes precoated with collagen type I (Iwaki Glass, Tokyo, Japan) for 5 hours and any unattached cells were removed by washing with PBS and cultured. Two to 4 days later, when cells reached ~70% confluency, they were irradiated with UVB at the indicated doses.

**Gene transfer experiments.** To introduce naked Stat3 DNA into the skin, we used a wild-type Stat3 expressing plasmid that coexpresses GFP (a generous gift from Dr. S. Akira, Osaka University, Suita, Japan). The dorsal skin of mice (≥3 days old) was stripped five times with Scotch tape and treated with acetone to disrupt the epidermal barrier. Naked plasmid DNA (20 μg, 1 μg/μL) in TE was applied and allowed to evaporate. After 24 hours, mice were irradiated with UVB. For in vitro experiments, adenoviral vectors were used for gene transfer into cultured keratinocytes. Recombinant adenoviral vectors (a generous gift from Dr. S. Akira) containing either the wild-type Stat3 (Stat3-adv) or the YF (phenylalanine substitution at 705Y) mutant Stat3 (Stat3YF-adv) in the expression cassette were constructed using the circular form of the adenoviral genome cloned in a cosmid and the Cre-loxP recombination system as previously reported (30).

**Figure 1.** Increased sensitivity to UVB-induced apoptosis in Stat3-deficient keratinocytes. A, histologic appearance of the dorsal skin 24 hours after UVB irradiation (1,000 J/m²) of wild-type (WT), and Stat3-deficient mice (Stat3/−), in which the number of sunburn cells (arrowheads) is greater than that in WT mice. Sunburn cells represent apoptotic keratinocytes (arrow in inset), characterized by eosinophilic cytoplasm and condensed nuclei. H&E staining. Bar, 100 μm. B, comparison of the number of sunburn cells (per cm slide) in skin sections from wild-type (white columns, n = 5) and Stat3/− mice (black columns, n = 5) 24 hours after UVB irradiation at the indicated doses. **P < 0.05; ***P < 0.01. C, FACS analysis of staining with propidium iodide for apoptosis defined as sub-G₀ population (%) in cultured wild-type (WT) and Stat3/− keratinocytes 24 hours after UVB irradiation (1,000 J/m²). Keratinocytes were infected with either the Stat3 adenovirus vector(s) or an adenovirus control vector during a serum starvation period for 24 hours at a multiplicity of infection (MOI) of 5.

**DNA repair assay following UVB irradiation.** Primary keratinocyte cultures at subconfluence were irradiated with UVB at 200 J/m² and harvested at 0, 1.5, 5, and 8 hours after treatment for isolation of DNA. The (6-4)phoprtoducts were determined by ELISA as previously reported (31). Global genomic DNA repair was determined by kinetic profiles of the disappearance of (6-4)phoprtoducts over time following UVB irradiation.

**Apoptosis assay.** For fluorescence-activated cell sorting (FACS) analysis of sub-G₀ DNA content, cultured cells were washed with PBS, resuspended, and 3 to 5 x 10⁶ cells were fixed with 70% ethanol for 4 hours. The cells were suspended in 100 μL phosphate-citrate buffer (mixture of 0.2 mol/L Na₂HPO₄ with 0.1 mol/L citric acid at 24:1) for 30 minutes, washed, and suspended in a final volume of 0.3 mL of PBS. Propidium iodide (3 μL of 1 mg/mL stock solution; Calbiochem, San Diego, CA) and RNase (3 μL of 1 mg/mL stock solution; Sigma) were added to the cell suspensions. Immunofluorescence with propidium iodide was measured...
with a FACSscan (Becton Dickinson, San Jose, CA). The percentage of cells below the G1 peak (sub-G1 fraction) was measured with the CellQuest software (Becton Dickinson) to determine the apoptotic cell population. In some experiments, keratinocytes in culture dishes were stained \textit{in situ} with Hoechst 33342 (Molecular Probes, Eugene, OR) at a final concentration of 1 \(\mu\)g/mL for 10 minutes, washed with PBS, and observed under a fluorescence microscope (Nikon, Melville, NY). In addition, phase-contrast microscopy was also used to determine apoptotic cells in culture based on morphologic changes (chromatin condensation and nuclear fragmentation) as previously described (28). The percentage of apoptotic cells was determined by counting a total of 300 cells per group.

\textbf{Western blotting.} Protein lysates were prepared from cultured keratinocytes that had been irradiated with UVB or were untreated. The lysis buffer contained 0.5\% NP40, 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L lytic buffer contained 0.5\% NP40, 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaVO\(_3\), and 5 \(\mu\)g/mL apro tin. Equivalent quantities of lysates (8-20 \(\mu\)g) were applied in each experiment based on protein content. Lysates were diluted with an equal volume of \(2\times\) sample buffer [4\% SDS, 20\% glycerol, 12.5 mmol/L Tris (pH 6.8), 0.004\% bromophenol blue, 10\% 2-mercaptoethanol], boiled for 5 minutes, separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blotted with antibodies against the following: Stat3 (1:1,000; Transduction Laboratories, Lexington, KY), phospho-PY-Stat3 (1:1,000, specific for phosphorytrosine at 705; Cell Signaling Technology, Beverly, MA), phospho-PS-Stat3 (1:1,000, specific for phosphoserine at 727; Cell Signaling Technology), Bcl-xL (1:500; Transduction Laboratories), Stat1 (1:1,000; Transduction Laboratories), Stat5b (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), \(\beta\)-actin (1:1,000; Sigma), and the Flag epitope (1:1,000; Sigma). Either horseradish peroxidase (HRP)--conjugated anti-mouse or anti-rabbit immunoglobulin (1:1,000, Sigma), and the Flag epitope (1:1,000; Sigma). Either horseradish peroxidase (HRP)–conjugated anti-mouse or anti-rabbit immunoglobulin (1:1,000, Amersham, Piscataway, NJ), was used as the secondary and bands were visualized using an enhanced chemiluminescence system (Amersham).

\textbf{Northern blotting.} Total RNA was extracted using TRIZol reagent (Life Technologies, Gaithersburg, MD), separated by electrophoresis in formaldehyde-containing 1\% agarose gels (15 \(\mu\)g per sample) and transferred to Hybond N+ nylon membranes (Amersham). Membranes were hybridized with DIG-labeled DNA probes and immunodetected using a DIG detection kit according to the manufacturer’s protocol (Roche Diagnostics, Pleasanton, CA). Stat3 (2.5 kb, a generous gift from Dr. T. Hirano, Osaka University, Suita, Japan) and \(\beta\)-actin (2.2 kb, Nippon Gene, Tokyo, Japan) cDNAs were used as probes.

\textbf{Reverse transcription-PCR.} Total RNA was extracted with an RNA isolation kit (Promega, Madison, WI), reverse transcribed using M-MLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) with random oligonucleotide hexomers and amplified for the indicated genes by PCR. Reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) with random oligonucleotide hexomers and amplified for the indicated genes by PCR.

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\caption{Restoring Stat3 reverses the UVB sensitivity of Stat3-deficient keratinocytes. A, successful introduction of Stat3 gene–encoding naked plasmid DNA into skin is shown by detection of GFP expression in hair follicles 24 hours after application (arrows) and to a lesser extent, in interfollicular epidermis (asterisks), compared with untreated skin showing only faint autofluorescence (left). e, epidermis; d, dermis. B, introduction of Stat3 gene \textit{in vivo} into skin reverses sensitivity to UVB. Sunburn cell generation (per cm slide \(\pm\) SE) in skin from Stat3-wild type and Stat3-deficient mice treated with either the Stat3 gene (black columns, \(n = 4\)) or a control (GFP only) gene (white columns, \(n = 4\)) 24 hours after UVB irradiation (2,000 J/m\(^2\)). *, \(P < 0.05; \)**, \(P < 0.01\). C, Western blot analyses of keratinocytes that are either uninfected (cont) or infected with wild-type Stat3 gene–encoding adenovirus vector (Stat3-adv), \(D\), reversal of UVB-induced (1,000 J/m\(^2\), 24 hours) apoptosis in Stat3\(^{-/-}\) keratinocytes requires tyrosine phosphorylation of Stat3. Uninfected control (top left), mock-infected control (top right), Stat3-adv-infected (bottom left), Stat3\(^{-/}\) mutant-adv-infected (bottom right). Percentages are the ratio of apoptotic cells among at least 300 cells detected by nuclear staining with Hoechst 33342. Apoptotic ratio of unirradiated cells is <5\% (data not shown).}
\end{figure}
the primers specified below under the conditions described as follows (sense, antisense primers, optimal annealing temperature, and cycles of reaction): hypoxanthine phosphoribosyltransferase (Hprt; 5′-CAGAGCTGACAGAC-CTG-3′ and 5′-GCTTGGTGAAGGACCTC-3′, 60°C, 28 cycles), Stat3 (5′-GCCGGCGATGGAGACAGAGAAA-3′ and 5′-GGCAGCAACATCCCAAGT-3′, 60°C, 30 cycles), Stat1 (5′-GAAACCGAGGCAATCC-3′ and 5′-GTTCTCTGCAACAATGGTGA-3′, 55°C, 30 cycles), and Bcl-XL (5′-AGACCCCGATGCCCTCAATG-3′ and 5′-GCCGCCGTAGTGACAGAGAA-3′, 60°C, 24 cycles).

Immunohistochemical analyses. For detection of GFP, skin specimens treated with naked DNA plasmids were fixed with formaldehyde, and embedded in paraffin. Sections were viewed using a microscope with a GFP-specific filter. To determine bromodeoxyuridine (BrdUrd) incorporation, mice were injected with BrdUrd (Sigma) in PBS (100 μg/g body weight) 30 minutes before sacrifice. Deparaffinized skin specimens were incubated in 10 mmol/L sodium citrate, microwaved, treated with H2O2 to block endogenous peroxidase activity, and washed with PBS. Slides were blocked with 5% goat serum (DakoCytomation) and incubated with anti-Flag, anti-Stat3, and anti-β-actin antibodies. D, resistance of K5.Stat3C transgenic mice to UVB-induced apoptosis. Quantitation of UVB-induced keratinocyte proliferation. Proliferation of hair follicles was determined as the percentage of hair follicles in which follicular keratinocytes (FKC) exhibited at least three contiguous cells positive for BrdUrd from 200 hair follicles per group. Epidermal thickness was measured from 50 interfollicular sites per each group.

Skin specimens from patients. Specimens of UVB-induced squamous cell carcinomas (SCC) were taken from 11 inpatients at Osaka University Hospital, ranging in age from 73 to 94 years. All of the SCCs arose on sun-exposed areas, including the cheeks, forehead, nose, eyelids, and ears. All of the human skin specimens were obtained with informed consent.

Induction of mouse skin tumors by UVB irradiation. Female SENCAR mice ranging in age from 8 to 12 weeks were used. The dorsal hair was shaved once a week for a 2 weeks before UVB irradiation using eight UV lamps (Westinghouse FS40 sunlamps) throughout the study. They were irradiated with UVB thrice a week at an initial dose of 1,200 J/m2, which was increased 25% on a weekly basis to a maximum of 5,000 J/m2. At 31 weeks of UVB treatment, a tumor incidence of 50% was reached.

Statistical analyses. For all experiments, the data was analyzed using the Student’s t test.

Results

Stat3 deficiency sensitizes keratinocytes to UVB-induced apoptosis. Stat3 activation is associated with increased survival and protection from apoptosis in many cell types (17); therefore, we examined the sensitivity of epidermal keratinocytes from K5-Cre:Stat3flox/null or K5-Cre:Stat3flox/flox (called hereafter as Stat3−/− mice) mice to UVB irradiation. Following exposure to UVB, a significant increase in the number of sunburn cells was observed in the epidermis of Stat3−/− mice (Fig. 1A, arrowheads) compared with wild-type mice (Fig. 1A, WT). Sunburn cells represent apoptotic keratinocytes of the epidermis and are characterized by eosinophilic cytoplasm and condensed nuclei as shown by H&E staining (ref. 29; Fig. 1A, arrow in inset). We found that sunburn cells were generated in a dose-dependent manner following UVB exposure, and their numbers were significantly greater in Stat3−/− mice compared with wild-type mice at all doses from 0 to 2,000 J/m2. There was a small but significant number of sunburn (apoptotic) cells in unirradiated Stat3−/− mice, whereas virtually no sunburn cells were found in unirradiated wild-type mice, suggesting that Stat3 plays a role in the constitutive survival of epidermal keratinocytes likely through activation of antiapoptotic signals such as Bcl-XL. This is supported by the findings in Bcl-xL−/− mice, where a considerable number of sunburn cells are also found even without UVB irradiation (29). At a UVB dose of 5,000 J/m2, the number of apoptotic or sunburn cells reached a maximum and no difference was seen between wild-type and Stat3−/− mice (Fig. 1B). Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining of skin sections confirmed the increased UVB sensitivity of Stat3−/− keratinocytes (data not shown). FACS analysis showed that cultured keratinocytes from Stat3−/− mice exhibited an increase in UVB-mediated apoptosis compared
with wild-type keratinocytes cultured under identical conditions (Fig. 1C). The data presented in Fig. 1 indicate that Stat3 deficiency sensitized keratinocytes to UVB-induced apoptosis and are consistent with the hypothesis that Stat3 normally plays an antiapoptotic role in keratinocytes following UVB exposure.

Restoring Stat3 reverses the sensitivity of Stat3–/– keratinocytes to UVB. To confirm that the enhanced UVB sensitivity of Stat3–/– keratinocytes was directly attributed to Stat3 deficiency, we first transferred the Stat3 gene into the skin of Stat3-deficient mice by injecting a naked plasmid DNA, containing wild-type Stat3 driven by an EF1α promoter, was topically applied to the dorsal skin. Tape stripping was used to remove the stratum corneum before application of the DNA to enhance transepidermal permeability (32). Twenty-four hours after application, we were able to detect expression of the introduced Stat3 gene in the hair follicles by probing for GFP (Fig. 2A, arrows) as previously described (33) and to a lesser extent, in the interfollicular epidermis (Fig. 2A, asterisk). Twenty-four hours after subsequent UVB irradiation on the treated site, the number of sunburn cells in the Stat3 gene–treated skin of wild-type and Stat3–/– mice was significantly decreased in comparison with those animals treated with the control gene (GFP only; Fig. 2B). Infection of cultured Stat3–/– keratinocytes with the wild-type Stat3 gene–encoding adenovirus vector (Stat3WT-adv) resulted in restoration of Stat3 and phosphotyrosine (PY)-Stat3 (Fig. 2C). Nuclear staining with Hoechst 33342 revealed that UVB-induced apoptosis of Stat3–/– keratinocytes was attenuated by infection with Stat3WT-adv (Fig. 2D, bottom right) compared with the uninfected control (Fig. 2D, top left) and the mock-infected control (Fig. 2D, top right). However, infection with Stat3YF-adv, which encodes a mutant Stat3 with a phenylalanine substitution for Tyr 705, did not reverse the UVB sensitivity of Stat3–/– keratinocytes (Fig. 2D, bottom right), clearly indicating that the antiapoptotic function of Stat3 was dependent on its tyrosine phosphorylation. Collectively, these results suggest that restoring Stat3 and resulting PY-Stat3 levels in Stat3–/– keratinocytes reversed their increased sensitivity to UVB-induced apoptosis and confirmed that Stat3 deficiency was the primary cause for this effect.

Stat3 overexpression reduces UVB-induced apoptosis. As shown in Fig. 2B, Stat3 gene transfer inhibited the formation of sunburn cells in both wild-type and Stat3–/– mice. This finding prompted us to study the effect of Stat3 overexpression on the sensitivity of wild-type epidermis to UVB. Topical application of the Stat3 plasmid DNA onto the skin of wild-type mice resulted in rapid dephosphorylation of Stat3 at Tyr705 in keratinocytes following UVB irradiation (1,000 J/m²). E, effect of vanadate treatment and expression of Stat3C transgene on Stat3 status including analysis of the levels of total Stat3, PY-Stat3, and PS-Stat3 following UVB exposure (1,000 J/m²). F, vanadate treatment desensitizes keratinocytes to UVB-induced apoptosis. Control (top), vanadate treated (bottom), unirradiated (left), UVB irradiated (right, 1,000 J/m²). Percentage ± SE of apoptotic cells among at least 300 cells is shown as determined by morphologic changes observed using phase-contrast microscopy 16 hours after irradiation. G, Western blot analyses of Stat3 status in keratinocyte lysates prepared from wild-type (p53+/+) and p53–/– mice collected at the indicated times following UVB irradiation (1,000 J/m²).

Figure 4. Down-regulation of Stat3 proteins and mRNA in keratinocytes following UVB exposure. Western blotting (A), Northern blotting (B), and RT-PCR analyses (C) of the indicated molecules in wild-type keratinocytes after UVB irradiation (1,000 J/m²) at the indicated times. D, Western blot analysis reveals rapid dephosphorylation of Stat3 at Tyr705 in keratinocytes following UVB irradiation (1,000 J/m²). E, effect of vanadate treatment desensitizes keratinocytes to UVB-induced apoptosis. Control (top), vanadate treated (bottom), unirradiated (left), UVB irradiated (right, 1,000 J/m²). Percentage ± SE of apoptotic cells among at least 300 cells is shown as determined by morphologic changes observed using phase-contrast microscopy 16 hours after irradiation. G, Western blot analyses of Stat3 status in keratinocyte lysates prepared from wild-type (p53+/+) and p53–/– mice collected at the indicated times following UVB irradiation (1,000 J/m²).
Furthermore, transfection of cultured keratinocytes from wild-type mice with Stat3WT-adv resulted in Stat3 overexpression and reduced apoptosis following UVB irradiation (data not shown). Next, we examined the sensitivity of Stat3C transgenic mice in which transgene expression is targeted to keratinocytes using the bovine keratin 5 (K5) promoter (referred to hereafter as K5.Stat3C mice; ref. 27) to UVB. Stat3C is a mutant form of Stat3 in which substitution of cysteine for amino acid residues at 661 and 663 confers constitutive homodimerization of Stat3C and subsequent nuclear translocation independently of tyrosine phosphorylation (24). Western blot analysis of epidermal protein lysates from K5.Stat3C mice revealed the presence of the Flag epitope tagged to the COOH terminus of the transgene and elevated levels of total Stat3 (Fig. 3C).

The epidermis of K5.Stat3C mice showed a marked resistance to high doses of UVB irradiation as expected (Fig. 3D). These results indicate that overexpression and/or “constitutive activation” of Stat3 can desensitize keratinocytes to UVB-induced apoptosis.

**Stat3 deficiency does not affect DNA repair following UVB irradiation.** It was possible that the sensitivity of Stat3−/− keratinocytes or resistance of Stat3-overexpressing keratinocytes to apoptosis following UVB exposure might have been due to a delay in or acceleration of DNA repair, respectively, because it has been postulated that alterations in DNA repair affect the apoptotic response following UVB exposure (1, 7). Therefore, we compared DNA repair following UVB irradiation in wild-type, Stat3−/−, and Stat3− overexpressing (Stat3WT-adv infected) keratinocytes.

![Figure 5](link)
by assessing the levels of pyrimidine (6-4)pyrimidinone dimers (6-4) photoproducts (31). XPC-null (XPC<sup>−/−</sup>) keratinocytes were used as a positive control in these experiments. ELISA revealed no differences in the kinetics of disappearance of (6-4)photoproducts among wild-type, Stat3<sup>−/−</sup>, and Stat3-overexpressing (Stat3<sup>WT-adv</sup> infected) keratinocytes (Fig. 3E). As expected, XPC<sup>−/−</sup> keratinocytes showed a marked delay in elimination of (6-4)photoproducts, because XPC is required for nucleotide excision repair (NER), particularly global genomic repair of DNA damage (34). These results suggest that Stat3 is not involved in the DNA repair process following UVB irradiation and that the increased sensitivity of Stat3<sup>−/−</sup> keratinocytes to apoptosis is not due to a failure in repair of UVB-induced DNA damage.

**UVB irradiation down-regulates Stat3.** Because Stat3 seemed to play a critical role in keratinocyte survival, we decided to determine whether there were any alterations in the expression of Stat3 in response to UVB irradiation. Notably, Western blot analyses of lysates from wild-type keratinocytes revealed that Stat3 protein levels were markedly reduced 24 hours after UVB irradiation, whereas Stat1 and Stat5 protein levels remained relatively unchanged (Fig. 4A). In addition, Bcl-xL protein levels also proved highly sensitive to UVB exposure and were considerably reduced by 24 hours after exposure (Fig. 4A). This finding is consistent with the observation that Bcl-xL is one of the downstream targets of Stat3 (35). Furthermore, Northern blot analysis revealed that Stat3 mRNA was down-regulated as rapidly as 4 hours after UVB exposure (Fig. 4B). Reverse transcription-PCR analysis confirmed that UVB irradiation induced rapid down-regulation of Stat3 mRNA as well as Bcl-xL mRNA, whereas the level of Stat1 mRNA remained relatively unchanged (Fig. 4C). We also found that UVB irradiation resulted in the rapid disappearance of tyrosine-phosphorylated Stat3 by one hour after treatment (Fig. 4D). These findings suggested that UVB directly or indirectly deactivated Stat3, resulting in down-regulation not only of Bcl-xL but also Stat3. These data are consistent with the finding of an autoregulatory loop whereby Stat3 regulates its own expression (36). Furthermore, forced expression of a constitutively active Stat3 in keratinocytes derived from K5.Stat3C mice compensated for the UVB-induced decline of Stat3 (Fig. 4E) and thereby protected keratinocytes from apoptosis (Fig. 3D).

**Involvement of a tyrosine phosphatase in UVB-induced Stat3 down-regulation.** The data in Fig. 4D indicates a rapid dephosphorylation of Stat3 at Tyr<sup>705</sup>, suggesting activation of a tyrosine phosphatase in response to UVB exposure. To further explore this observation, we pretreated cultured keratinocytes with vanadate, a potent phosphatase inhibitor. Vanadate treatment completely inhibited the down-regulation of Stat3 upon UVB irradiation not only with respect to phosphorylation status but with regard to Stat3 protein levels as well (Fig. 4E). Correspondingly, the vanadate-treated keratinocytes were resistant to UVB-induced apoptosis (Fig. 4F). This result confirmed that a tyrosine phosphatase is rapidly induced by UVB irradiation leading to dephosphorylation and inactivation of Stat3. Interestingly, there was no change in the total level of Stat3 in K5.Stat3C keratinocytes in response to UVB, but PY-Stat3 essentially disappeared (Fig. 4E). This finding suggested that Stat3C was resistant to UVB-induced inactivation consistent with its ability to spontaneously dimerize in the absence of tyrosine-phosphorylation, although endogenous phosphorylation of Stat3 at the tyrosine residue was affected. This result was also consistent with the finding that skin of K5.Stat3C mice was highly resistant to UVB-induced apoptosis (Fig. 3D). It should be noted that UVB irradiation did not significantly decrease serine-phosphorylated (PS) Stat3 levels (Fig. 4E), which seemed

**Figure 6.** Constitutively activated Stat3 in UVB-induced SCCs. A, mouse SCC after treatment with UVB irradiation for 33 weeks. B, human SCC removed from the cheek of an 84-year-old female. Clinical diagnosis was UV-related SCC. H&E staining (left) and anti-Stat3 staining (right).

proportional to total Stat3 levels. This result further indicated the involvement of a tyrosine phosphatase, not a serine/threonine phosphatase nor a dual phosphatase in UVB-induced down-regulation of Stat3. Furthermore, down-regulation of Stat3 was reproduced in p53−/− keratinocytes upon exposure to UVB (Fig. 4G), indicating that UVB-mediated dephosphorylation of Stat3 was p53 independent.

**Stat3 is required for UVB-induced proliferation of follicular keratinocytes and epidermal thickening.** In mouse epidermis, UVB exposure causes an initial increase in apoptotic (sunburn) cells which is followed by keratinocyte proliferation and epidermal hyperplasia (37). This latter response is likely due to a combination of mechanisms including both direct effects of UVB on signal transduction pathways leading to keratinocyte proliferation as well as compensatory mechanisms activated as a result of cell loss due to apoptosis. We recently found that Stat3−/− keratinocytes were resistant to 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal proliferation (25, 38). Therefore, we examined whether Stat3−/− mice would also be resistant to UVB-induced keratinocyte proliferation in vivo. BrdUrd pulse-chase analyses revealed that FKC (outer root sheath cells) vigorously proliferated in response to UVB irradiation in wild-type mice (Fig. 5A) and K5.Stat3C mice (Fig. 5D) but not in Stat3−/− mice (Fig. 5C). Proliferating hair follicles (defined by the presence of contiguous BrdUrd-positive FKC) were found in K5.Stat3C mice (nearly 100% of follicles) and control mice (69% of follicles) at 24 hours after irradiation (Fig. 5D). In contrast, only a small number of proliferating hair follicles (<2%) were observed in Stat3−/− mice (Fig. 5D). These results indicate that Stat3 activation is also required for UVB-induced proliferation of FKC. Forty-eight hours after UVB exposure, proliferating FKC seemed to migrate upward to the infundibulum and interfollicular epidermis in control (Fig. 5A, right) and K5.Stat3C mice (Fig. 5D, right), suggesting replenishment of the damaged epidermis with “new” basal cells. Correspondingly, control and K5.Stat3C mice showed epidermal thickening at 48 hours post-UVB (Fig. 5E).

In contrast, only marginal epidermal thickening was observed in Stat3−/− mice (Fig. 5E) and very few proliferating FKC were observed (Fig. 5C-D). In wild-type mice, immunostaining revealed that keratinocytes in hair follicles and the contiguous basal layer exhibited strong nuclear localization of Stat3 at 48 hours post-UVB (Fig. 5F, arrows), whereas cytoplasmic localization of Stat3 predominated in unirradiated control mice (Fig. 5F, left). Collectively, these results suggest that epidermal keratinocytes lost through apoptosis following UVB exposure are replenished by basal cells in which Stat3 is activated.

**Stat3 is activated in UVB-induced squamous cell carcinoma.** SCCs of the skin arise in both mice and humans through repeated UVB irradiation. Immunohistochemical staining of Stat3 revealed constitutively active Stat3, as defined by its nuclear localization, in SCC obtained from mice that received repeated UVB exposure for 8 months (Fig. 6A, right). Likewise, human SCC that had developed on areas of the face that typically receive frequent sun exposure also had predominant nuclear staining of Stat3 protein (Fig. 6B, right). The nuclear localization of Stat3 in keratinocytes from UVB-induced skin cancers (both mouse and human) suggests persistent activation of Stat3.

**Discussion**

In this study, we found that Stat3-deficient keratinocytes were highly sensitive to UVB-induced apoptosis, whereas overexpression of Stat3 or expression of a Stat3C transgene protected keratinocytes from UVB-induced apoptosis. It is known that Stat3 has a prosurvival role in a variety of cells and accomplishes this presumably through up-regulation of antiapoptotic molecules, such as Bcl-2, Bcl-xl (19, 35), and survivin (15). Furthermore, a number of studies have shown that constitutive activation of Stat3 is associated with oncogenic transformation (e.g., v-Src-transformed murine cells) and with a variety of human cancers, including hematopoietic, brain, prostate, breast, lung, and head and neck cancers (18–21). Introduction of antisense Stat3, Stat3 decoy DNA, or dominant-negative Stat3 into human tumor cells with constitutively activated Stat3 leads to apoptosis (39, 40). Very recently, we have found that Stat3-deficient mice are completely resistant to the two-stage carcinogenesis regimen using 7,12-dimethylbenz(a)anthracene (DMBA) as the initiator and TPA as the promoter, indicating that Stat3 is essential for development of chemically induced skin tumors in mice (25). Stat3-deficient keratinocytes were found more sensitive to DMBA-induced apoptosis compared with control keratinocytes and also to be less sensitive to TPA-induced epidermal hyperproliferation (25, 38). Thus, based on our previous and current data, Stat3 deficiency sensitizes keratinocytes to both chemical (DMBA) and physical (UVB) DNA-damaging agents.

UV-mediated apoptosis is a highly complex process in which different molecular and biochemical pathways are involved. DNA damage and the resulting stabilization of p53 are critical in mediating UV-induced apoptosis (1, 6). Mice that carry mutated p53 exhibit a significantly increased susceptibility to UVB-mediated skin cancer induction compared with wild-type mice (8, 41). UVB irradiation induces DNA damage by formation of cyclobutane pyrimidine dimers and (6-4)photoproducts which can be eliminated by NER (34). DNA repair mechanisms including NER protect against apoptosis following UV exposure (1, 7, 34). In the current study, no differences were found in the efficacy of NER between wild-type keratinocytes and Stat3-deficient or Stat3-proficient keratinocytes. In contrast, XPC-deficient keratinocytes were significantly impaired in NER and therefore highly sensitive to UVB-mediated apoptosis (data not shown). This result suggests that the antiapoptotic function of Stat3 is not associated with NER following UVB irradiation.

In the current study, we found that UVB irradiation resulted in rapid dephosphorylation of Stat3 at Tyr^{705} followed by decreases of Stat3 mRNA and protein levels in wild-type keratinocytes. This finding may be of physiologic relevance and suggests a novel role for Stat3 similar to that of p53 in response to UVB. In this regard, rapid inactivation of Stat3 following UVB irradiation may mediate a protective mechanism by which severely damaged cells are eliminated to reduce future cancerous potential. UVB-induced deactivation of Stat3 was immediately followed by a decrease in mRNA levels of Bcl-xL. Reduced Bcl-xL expression would also lead to reduced keratinocyte survival as shown by studies of Bcl-xL-deficient mice (29). We have recently found that Stat3 plays a crucial role in G1-S phase cell cycle progression in keratinocytes following treatment with tumor promoters such as TPA (25, 38). Therefore, UVB-modulated Stat3 signaling (i.e., down-regulation) may also result in cell cycle arrest at the G_{1} phase. In light of these findings, Stat3 seems to share similarity with p53 when DNA repair is needed. However, Stat3 down-regulation by UVB exposure seems p53 independent, because this phenomenon was observed in p53-deficient keratinocytes. Further studies in progress are examining the
role of initial down-regulation of Stat3 by UVB exposure in protection against skin cancer. UVB irradiation rapidly decreased PY-Stat3 but did not significantly affect PS-Stat3. Furthermore, we showed that vanadate treatment prevented the UVB-induced down-regulation of Stat3 and resulting apoptosis. Collectively, these results suggest the involvement of a tyrosine phosphatase in the rapid dephosphorylation of PY-Stat3. Previous reports have shown that UVB exposure altered normal signaling pathways through modulation of tyrosine phosphatases (42–44), several of which could target activated STATs (45–47). Because Stat1 and Stat5 were relatively unaffected, the tyrosine phosphatase activated in keratinocytes following UVB exposure may be specific for Stat3. Our data seem inconsistent with a previous report showing that UVB down-regulated IFN-γ-activated Stat1 but not IL-6-activated Stat3 (42). This discrepancy may be due to the different experimental settings, as we analyzed the UVB sensitivities of STATs in cells without further stimulation with extracellular ligands. In contrast to endogenous Stat3, UVB did not affect transgenic Stat3C whose activation and nuclear translocation are independent of tyrosine phosphorylation (24). Although our current data suggest the involvement of a tyrosine phosphatase, we cannot exclude the possibility that other molecules are involved in down-regulation of Stat3 following UVB irradiation, such as SOCS (48) or PIAS (49). Further work is necessary to fully appreciate the mechanism underlying the down-regulation of Stat3 following UVB exposure in keratinocytes.

BrdUrd pulse-chase labeling of skin sections from wild-type and K5.Stat3C mice showed that following UVB irradiation, outer root sheath cells vigorously proliferated and seemed to migrate upwards through the infundibulum to the interfollicular epidermis leading to epidermal thickening. In contrast, Stat3-deficient mice showed no proliferation of follicular keratinocytes or subsequent epidermal thickening. This result is consistent with reports that the bulge stem cells contribute to regeneration of the epidermis (50). Following UVB irradiation, the majority of the apoptotic cells are found in the interfollicular epidermis. However, UVB irradiation facilitates follicular stem cells to generate transient amplifying cells (TAC). This response of follicular stem cells may be mediated by diffusible factors such as proinflammatory cytokines or other growth regulatory molecules released from keratinocytes and/or infiltrating cells. It is interesting to note that keratinocyte stem cells reside at a specific anatomic location in the hair follicles (i.e., the bulge region). Due to this location, keratinocyte stem cells receive lower doses of UVB and thus are less likely to undergo apoptosis compared with those in the interfollicular epidermis. Importantly, Stat3 seems a critical target for this response, because Stat3-deficient mice were completely resistant to UVB-induced proliferation of TAC. Thus, Stat3 is required for regeneration of epidermis after UVB-induced damage, which is similar to its postulated role in the wound healing process (26). UVB is a complete skin carcinogen that possesses both initiating and promoting activities (6). Based on our current data and a recent report in which we found that Stat3 is required for both the initiation and promotion stages of skin carcinogenesis (25), we predict that Stat3-deficient mice will be resistant to UVB carciogenesis due to both increased loss of initiated cells through apoptosis and decreased proliferation with repeated UVB exposure.

In conclusion, our data allow us to propose a novel hypothesis whereby Stat3 represents a critical regulator of keratinocytes in response to UVB irradiation. Following UVB irradiation, Stat3 is rapidly down-regulated in keratinocytes which leads to decreased cell cycle progression and increased sensitivity to UVB-induced apoptosis. In many ways, this is physiologically analogous to p53 function; levels increase following UVB irradiation leading to decreased cell cycle progression and increased apoptosis. Keratinocyte stem cells that reside in lower regions of the skin receive lower doses of UVB and are less likely to undergo apoptosis. These cells require Stat3 for proliferation and for regeneration of the epidermis following exposure to UVB. Initiated keratinocyte stem cells that have escaped normal control mechanisms can then undergo clonal expansion into a tumor. A role for functional Stat3 for survival and further proliferation of skin cancer cells is supported by our finding that UVB-induced skin cancers possessed constitutively activated Stat3 (Fig. 6) and our previous work (25). Therefore, Stat3 may be a potential target for both prevention as well as treatment of UVB-induced skin cancer.

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References

Role of Epidermal Stat3 Following UV Irradiation


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