Genetic deletion of TNFα inhibits ultraviolet radiation-induced development of cutaneous squamous cell carcinomas in PKCε transgenic mice via inhibition of cell survival signals

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Abstract

Protein kinase C epsilon (PKCε), a Ca2+-independent phospholipid-dependent serine/threonine kinase, is among the six PKC isoforms (α, δ, ε, η, μ, ζ) expressed in both mouse and human skin. Epidermal PKCε level dictates the susceptibility of PKCε transgenic (TG) mice to the development of cutaneous squamous cell carcinomas (SCC) elicited either by repeated exposure to ultraviolet radiation (UVR) or by using the DMBA initiation-TPA (12-O-tetradecanoylphorbol-13-acetate) tumor promotion protocol (Wheeler, D.L. et al. (2004) Protein kinase C epsilon is an endogenous photosensitizer that enhances ultraviolet radiation-induced cutaneous damage and development of squamous cell carcinomas. Cancer Res., 64, 7756–7765). Histologically, SCC in TG mice, like human SCC, is poorly differentiated and metastatic. Our earlier studies to elucidate mechanisms of PKCε-mediated development of SCC, using either DMBA-TPA or UVR, indicated elevated release of cytokine TNFα. To determine whether TNFα is essential for the development of SCC in TG mice, we generated PKCε transgenic mice/TNFα-knockout (TG/TNFαKO) by crossbreeding TNFαKO with TG mice. We now present that deletion of TNFα in TG mice inhibited the development of SCC either by repeated UVR exposures or by the DMBA-TPA protocol. TG mice deficient in TNFα elicited both increase in SCC latency and decrease in SCC incidence. Inhibition of UVR-induced SCC development in TG/TNFαKO was accompanied by inhibition of (i) the expression levels of TNFα receptors TNFRI and TNFRII and cell proliferation marker ornithine decarboxylase and metastatic markers MMP7 and MMP9, (ii) the activation of transcription factors Stat3 and NF-kB and (iii) proliferation of hair follicle stem cells and epidermal hyperplasia. The results presented here provide the first genetic evidence that TNFα is linked to PKCε-mediated sensitivity to DMBA-TPA or UVR-induced development of cutaneous SCC.

Introduction

Chronic ultraviolet radiation (UVR) exposure is the most common etiologic factor linked to the development of cutaneous squamous cell carcinomas (SCC), a non-melanoma form of skin cancer that can metastasize (1). Protein kinase C epsilon (PKC), a family of phospholipid-dependent serine/threonine kinases, is the major intracellular receptor for the mouse skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (2–5) and is activated by a variety of stress factors including UVR (6). PKCε is among six isoforms (α, δ, ε, η, μ, ζ) of PKC expressed in both human and mouse skin (2–8). To determine the in vivo functional
The mice were housed in groups of 3–4 in plastic-bottom cages in light-,
tained by mating hemizygous transgenic mice with wild-type FVB/N mice.

Materials and Methods

Chemicals, antibodies and kits

TPA was purchased from Alexis Corp (San Diego, CA) and 7,12-
Dimethylbenzanthracene (DMBA) was from Aldrich Chemical Company
(Milwaukee, WI). Surface staining conjugated antibodies for FACS (α6-
integrin PE, CD34 FITC), isotype controls and 7-aminoactinomycin D
(7AAD) dye were purchased from BD Biosciences (San Jose, CA). The anti-
bodies to PKCε, Tumor Necrosis Factor Receptor I (TNFRI), TNFRII, Matrix
metalloproteinase-7 (MMP7), MMP9, Signal transducer and activator
of transcription 3 (Stat3), NF-κB p65, proliferating cell nuclear antigen
(PCNA) and β-actin for western blotting were obtained from Santa Cruz
Biotechnologies (Santa Cruz, CA); pStat3Ser727, pStat3Tyr705 and CD34
were from BD Biosciences (San Jose, CA). Secondary antibodies anti-
mouse, anti-goat, anti-rabbit and ECL western blotting reagents were
purchased from Thermo Scientific (Rockford, IL). ABC kit (PK-7200) for PCNA
staining was purchased from Vector Laboratories.

Tumor-induction experiments

Wild type (WT), TNFα knock out (TNFαKO), PKCε over-expressing mice line
TG224 (TG) and TG/TNFαKO mouse lines on FVB/N background were used in
this study. PKCε transgenic and PKCε transgenic-TNFα knockout mice were
generated as described previously (9,12). Transgenic mice were main-
tained by mating hemizygous transgenic mice with wild-type FVB/N mice.
The mice were housed in groups of 3–4 in plastic-bottom cages in light,
humidity- and temperature-controlled rooms; food and water were avail-
able ad libitum. The animals were kept in a normal rhythm of 12 h light
and 12 h dark periods. The transgene was detected by polymerase chain
reaction analysis using genomic DNA isolated from 0.5 mm tail clips (9,12).
All of the animal protocols were approved by the University’s Research
Animal Resource Committee in accordance with the NIH Guidelines for
the Care and Use of Laboratory Animals.

Skin carcinogenesis by repeated UVR exposures

The UVR source was Kodacel-filtered FS-40 sun lamps (approximately
60% UBV and 40% UVA). Kodacel filters were purchased from Unique
Photo (Fairfield, NJ). UV lamps from National Biologicals Corporation,
(Beachwood, OH) and UVX-radiometer from UVR. (Upland, CA). Mice were
exposed to UVR (2 kJ/m²) from a bank of six Kodacel-filtered sunlamps.
UVR dose was routinely measured using a UVR radiometer. Mice were
used for experimentation at 6–8 weeks of age. The dorsal skin of the mice
was shaved 3–4 days before experimentation. Mice were exposed to UVR
twice a week (Monday, Wednesday and Friday). If needed, mice were also
shaved during the course of the tumor-induction experiment. Tumor mul-
tiplicity was observed and recorded every other week. Carcinomas were
recorded grossly as downward-invading lesions, which were confirmed
histologically (12,16,20).

Skin carcinogenesis by DMBA-initiation and TPA-
promotion protocol

Mouse skin tumors were induced by the initiation-promotion regimen
(21). For mouse skin tumor initiation, a single dose (200 nmol) of DMBA
in 0.2 ml of acetone was applied topically on the shaved back of mice. Two
weeks after initiation, TPA (10 nmol) in 0.2 ml of acetone was applied twice
a week to the skin for entire period of the experiment.

Histology and immunofluorescence analysis

For immunofluorescence studies, mouse skins were excised promptly
after killing and immediately placed in 10% neutral-buffered formalin
for fixation, and then embedded in paraffin. Four to five μm sections were
Cut for H&E staining and immunostaining of skin stem cells. Briefly, the extra
paraffin was removed using three xylene gradient washes followed by
alcohol gradient for 10 min each. The slices were washed with Milli-Q
water and then 1XPBS. The antigen retrieval was done using antigen
unmasking solution as per protocol (vector laboratories). The blocking
process was done in normal goat and normal horse serum for 1 h at room
temperature. After blocking, primary antibody to CD34 (dilution 1:200)
was incubated to tissue section on the slides overnight. Tissue sections
were incubated with their secondary antibodies (Alexa-Fluor 594-Donkey
anti-rat IgG (H + L) (Invitrogen; 1:1000 dilution) for 1 h at room tempera-
ture. After incubation with secondary antibody, slides were washed three
times with 1XPBS, mounted with DAPI (Vector Laboratories) and observed
under the fluorescent microscope (Vectra). The fluorescent pictures of
CD34 staining were captured through TRITC channel (red color) in a
Nanouc Fluorescent microscope.

Keratinocyte isolation and flow cytometric analysis

For the skin stem cell estimation, the dorsal skin of mice was harvested
for keratinocyte isolation 24-h post UVR (2 kJ/m²) exposure (22). In each
experiment an equal size of skin was excised from the indicated four
groups of mice. Viable cell counts were determined using Trypan Blue
(0.4%). Keratinocytes were incubated for 1 h in the dark at 4°C with
PE-conjugated Rat Anti-Human α6-integrin antibody at 10 µL per 10⁶ cells
and FITC-conjugated rat anti-mouse CD34 antibody at 2 µg per 10⁶ cells.
Keratinocyte preparations of single cell suspension were sorted based on
α6-integrin and CD34 status using a FACS Aria cell sorter (BD Biosciences).
A 488 nm laser was used to detect FITC with a 530/30 filter and a 532 nm
laser for PE with a 575/25 filter. The nozzle size was 130 µm and the pres-
sure used was 14 p.s.i. The live cell population gate was estimated using
forward and side scatter positioning and confirmed with 7AAD staining.

Phenotyping and estimation of the frequency of
CD34+/α6-integrin+ stem cells (HSCs)

The phenotyping assays were acquired on a BD FACSCalibur (BD
Biosciences). The BD FACSCalibur instrument was calibrated daily by
the University of Wisconsin Carbone Cancer Center Flow Cytometry
Laboratory staff using the manufacturer’s Cytometer Settings and Tracking calibration software. Data were analyzed using FlowJo software version 9.4.3 (Treestar, Ashland, OR). Positive staining and gating strategy were determined by comparison to isotype controls. Dead cells were excluded using 7AAD staining on FACS Calibur assays. Data demonstrate frequency of cells in a parent population (of live intact cells for α6-integrin and CD34 expression).

The frequency of CD34+/α6-integrin+ stem cells (Hair follicle stem cells or HSCs) represent the percent of CD34+/α6-integrin+/7AAD− cells (‘cells’ determined by FSC/SSC morphologic gate) in the total 7AAD+ population. The absolute number of HSCs in individual samples was calculated by multiplying percentage frequency of HSCs by the total number of Trypan-Blue excluding cells in the single cell keratinocyte preparation. The data represent absolute number of HSCs from the equal size of dorsal skin from all four indicated mice groups used in the study.

**Analysis of PCNA-positive cells**

At the end of the tumor induction experiment, all four groups of mice (WT, TNFαKO, TG and TG/TNFαKO) (n = 3) were used for evaluation of UVR-induced levels of PCNA and epidermal hyperplasia. Mice were killed at 24 h post last UVR treatment. Skin specimens were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Five-μM-thick sections were cut for PCNA staining. Briefly, the slides were incubated overnight at 4°C with PCNA primary antibody. Antigen retrieval pretreatment was done by using Tris-urea solution in a digital decloaking chamber. The process of blocking and secondary antibody incubation was done using R.T.U Vectastain Kit. A detailed process of PCNA staining and quantitation is described elsewhere (12).

The visualization was performed using diaminobenzidine as a substrate for the peroxidase reaction. Pictures were taken using a Nuance Bright field Microscope ( Vectra). An average percentage was calculated based on the total number of cells and the number of PCNA positive epidermal keratinocyte cells from each set of 10 fields count. Results are expressed as mean of percentages ± SEM.

**Western blot analysis**

Mice were shaved and depilated before experimentation. The mouse skin was excised and scraped to remove the subcutaneous fat. The epidermis was scrapped off on an ice-cold glass plate and homogenized in lysis buffer [50 mmol/l HEPES, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/l MgCl₂, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 200 μmol/l NaN₃, 200 μmol/l NaF and 1 mmol/l EDTA (final pH 7.5)]. The homogenate was centrifuged at 14,000 g for 30 min at 4°C. Protease and phosphatase inhibitors were procured from Sigma-Aldrich (St. Louis). Epidermal cell lysates proteins (50–100 μg) were fractionated on 10% Criterion precast SDS–polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA). The protein was transferred to a 0.45 μm Hybond-P polyvinylidene difluoride (PVDF) transfer membrane (Amersham Life Sciences, Piscataway, NJ). The membrane was then incubated with the indicated antibody followed by a horseradish peroxidase secondary antibody (Thermo Scientific), and the detection signal was developed with Amersham’s enhanced chemiluminescence reagent and using FOTO/Analyst Luminary Work Station (Fotodyne). The Western blots were quantitated (normalized to β-actin) by densitometric analysis using TotalLab Nonlinear Dynamic Image analysis software (Nonlinear USA, Durham, NC).

**Statistical analysis**

Number of SCCs and papillomas (where applicable) were recorded weekly starting at week 10 for the DMBA challenged arms and week 14 for the UVR challenged arms. Time to first SCC was plotted using Kaplan–Meier curves, and differences in risk between treatment arms for time to first SCC were tested using Cox proportional hazard regression models. Differences in quantitated staining levels and stem cell analysis were examined using Student’s t-test. Computations were performed with SAS and R software; figures were created with R software (23,24).
Results

TNFα deletion in PKCε transgenic mice and their wild-type littermates inhibits development of SCC elicited either by repeated UVR exposures or by the DMBA-TPA protocol

UVR-induced SCC

In this experiment (Figure 1), four groups of mice (WT, TNFα KO, TG, TG/TNFα KO) were exposed to UVR (2 kJ/m²) thrice weekly for 45 weeks. The results are shown in Figure 1A–C. As compared to wild-type littermates, PKCε over-expressing transgenic mice exhibit decrease in tumor latency and increases in SCC incidence, the results are in accord with our previous finding (12,25). PKCε overexpressing TNFα-deficient (TG/TNFα KO) mice exhibited a reduction in risk of SCC compared to their TG mice (HR: 0.14; 95% CI: 0.05–0.37, P = 0.001). Also, TG/TNFα KO mice elicited both an increase in SCC latency (21 weeks) and a decrease in SCC incidence (55%). Similarly, wild-type mice deficient in TNFα exhibited a reduction in risk of SCC compared to their WT littermates (HR: 0.21; CI: 0.06–0.74, P = 0.015) (Figure 1A and B). Also, TNFα KO mice elicited both increase in SCC latency (4 weeks) and a decrease in SCC incidence (45%) (Figure 1A and B).

DMBA-TPA-induced SCC

We have previously reported that epidermal PKCε transgenic mice are sensitive to the development of SCC elicited not only by repeated exposure to UVR but also by the DMBA-TPA tumor promotion protocol (9,12,16,21). Also, PKCε transgenic mice are more sensitive than their wild-type littermates to both UVR- and TPA-induced increase in TNFα levels in serum (13). These findings prompted us to determine the role of TNFα in the development of SCC elicited by the DMBA-TPA protocol in PKCε transgenic mice. In this experiment (Figure 2), FVB/N mice (WT, TNFα KO, TG, TG/TNFα KO) were topically treated with a single dose of DMBA (100 nmol) followed a week later by repeated treatment of TPA (10 nmol) twice a week for 37 weeks. Carcinoma and papilloma were recorded weekly. PKCε overexpressing mice deficient in TNFα (TG/TNFα KO) exhibited a reduction in risk of SCC compared to TG mice (HR: 0.07; 95% CI: 0.01–0.30, P < 0.001) (Figure 2A and B). Moreover, TG/TNFα KO mice elicited both an increase in SCC latency (14 weeks) and a decrease in SCC incidence (8%) (Figure 2A and B). Similarly, the wild-type mice deficient in TNFα (TNFα KO) exhibited a non-significant reduction in risk of SCC compared to WT mice (HR: 0.69; CI: 0.14–2.81, P = 0.54) (Figure 2). Also, TNFα KO elicited both an increase in SCC latency (8 weeks) and a decrease in SCC incidence (45%) (Figure 2). TNFα deletion significantly (P = 0.0014) inhibited papilloma multiplicity elicited by the DMBA-TPA protocol in wild-type mice (Figure 2B and C). It is notable that papilloma multiplicity is shown only in WT mice and not in TG mice (Figure 2C). TG mice develop only SCC and no papilloma (12).

TNFα deletion in PKCε transgenic mice and their wild-type littermates inhibits UVR-induced epidermal hyperplasia and epidermal proliferative cell nuclear antigen

We explored the possibility whether TNFα is linked to UVR-induced epidermal cell proliferation and hyperplasia. In this experiment (Figure 3), FVB/N Mice (WT, TNFα KO, TG, TG/TNFα KO) were exposed to UVR (2.0 kJ/m²) three times weekly (Monday, Wednesday and Friday) for 45 weeks, and were killed

Figure 2. Deletion of TNFα in PKCε transgenic mice inhibited the development of SCC elicited by the DMBA-initiation and TPA-promotion protocol. Four groups of 6-8-week-old FVB/N mice (WT n = 15), TNFα KO (n = 16), TG (n = 12) and TG/TNFα KO (n = 11) were initiated with one topical treatment of 200 nmol of DMBA in 0.2 ml acetone and after 1 week of DMBA treatment, mice were treated with TPA (10 nmol in 0.2 ml acetone) twice a week for the indicated weeks. Number of both papillomas and carcinomas were recorded weekly. Shown are the Kaplan-Meier survival curves (A), SCC multiplicity data (SCC/mouse) (B), number of papillomas per mouse (C) and representative photographs of indicated mice at the end of the experiment (D).
at 24 h after the last UVR exposure. For histochemistry, uninvolved skin specimens (n = 3 each) were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Five μm skin sections were used for hematoxylin and eosin and PCNA staining. Uninvolved skin of TNFα deficient mice, as compared to TG and WT mice, elicited inhibition in UVR-induced hyperplasia (Figure 3A). As reported before (12), PKCε overexpression in WT mice resulted in UVR-induced increase in number of PCNA stained epidermal keratinocyte cells (P = 0.04). TNFα deletion significantly inhibited UVR-induced increase in number of PCNA stained epidermal keratinocyte cells in both WT (P = 0.002) and TG (P = 0.001) mice (Figure 3B). Similarly, we observed the significant (P = 0.002) increase in PCNA positive epidermal keratinocytes in TG/TNFα KO mice compared to TNFα KO (Figure 3B).

TNFα deletion in PKCε transgenic mice and their wild-type littermates inhibits UVR-stimulated putative HSCs proliferation

The epidermal keratinocytes positive for both cell surface marker CD34+ and α6-integrin+ mark mouse hair follicle bulge stem cell populations (Figure 4A) which have stem cell properties such as quiescence and multipotency. We have previously reported that both single and chronic UVR treatments (1.8 kJ/m², Monday, Wednesday and Friday) result in an increase in the frequency of double positive HSCs in TG mice as compared to their WT littermates (22). We explored the possibility that the mechanism of resistance of TNFα-deficient mice to UVR-induced development of SCC may involve the role of HSCs. In this experiment (Figure 4), we compared TNFα-deficient mice and their wild-type littermates for the effects of single UVR exposure on frequencies and absolute number of HSCs. The percentages of double-positive HSCs (CD34+ and α6-integrin+) in the isolated keratinocytes were determined by flow cytometric analysis after staining with α6-integrin and CD34 antibodies conjugated with fluorochrome, the cell surface markers of mouse hair follicle bulge cells. For quantitative analysis of HSCs among all four groups we have calculated the frequencies (Figure 4B) and total absolute number of CD34+/α6-integrin+ cells (Figure 4C) from dorsal skin. We observed that UVR-induced HSCs proliferation is less in TNFα KO and TG/TNFα KO mice compared to their respective wild-type littermates (Figure 4B and C). UVR exposure resulted in significant

Figure 3. Deletion of TNFα in PKCε transgenic mice inhibited UVR-induced proliferative marker PCNA and epidermal hyperplasia. At the end of the tumor induction experiment (Figure 1), the same groups of mice (WT, TNFαKO, TG and TG/TNFαKO) (n = 3) were used for evaluation of UVR-induced levels of PCNA and epidermal hyperplasia. The mice were killed at 24 h post last UVR exposure. For histochemistry, uninvolved tumor free mouse skin specimens (n = 3) were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin for sectioning (~5 μM), and stained with H&E. PCNA staining for epidermal proliferation was done as described in Materials and Methods. Pictures were taken in a Nuance bright field microscope at 20X magnification for HE and PCNA staining. Upper panel and lower panel are showing the representative pictures of H&E stained sections and nuclear PCNA staining, respectively (A). (B) Showing the quantitation data of PCNA count from all four indicated mouse groups (n = 3) in the uninvolved epidermis. Each bar value is the percent mean ±SEM of PCNA positive nuclei counted from 10 random areas from each mouse. Black arrows are showing PCNA positive proliferative nuclei (Abbreviation: HF, hair follicle; SG, sebaceous gland). (C) Two and three star denotes P < 0.05 and ≤0.001, respectively.
HSCs increase in both WT (P = 0.01) and TG (P = 0.001) mice. We also observed that UVR-induced HSCs proliferation is significantly less (P = 0.001) in TG/TNFαKO mice compared to TG (Figure 4C).

Genetic deletion of TNFα in mice inhibits UVR-induced expression of TNFα receptors and associated signals to epidermal cell survival

The TNFα signal transduction pathway, which may be linked to UVR-induced development of SCC, was explored. The biological effects of pro-inflammatory cytokine TNFα are mediated through their receptors, namely TNFRI and TNFRII. Although TNFα can signal through these two receptors, the majority of TNF-mediated biological events are mediated through TNFRI signaling (26–30). Both TNFα and TNFRII have been linked to UVR carcinogenesis (17–19). TNFα mediates the activation of two transcriptional factors, AP-1 and NF-kB, linked to the expression of TNFα-induced genes involved in immunity and inflammatory responses and control of cellular proliferation, differentiation and apoptosis (31,32). As shown in Figure 5, deletion of TNFα in mice suppressed the expression levels of TNFα receptors TNFRI and TNFRII, inhibited the activation of transcription factors AP-1, Stat3 and NF-kB-p65, and decreased expression levels of cell proliferation marker ornithine decarboxylase (ODC) and metastatic markers MMF7 and MMP9 (Figure 5).

**Discussion**

Skin cancer is the most common malignancy encountered in the United States with an expected diagnosis of 1.3 million new cases of non-melanoma skin cancer each year (1). SCC and basal cell carcinoma (BCC) are the most common non-melanoma forms of human skin cancer (33,34). BCC is rarely life threatening because it is slow-growing and is mostly localized. SCC, unlike BCC, invades the nearby tissues (1). The most important risk factor for non-melanoma skin cancer is chronic exposure to UVR.
to UVR in sunlight (1). We found that PKCε is an important component of UVR-induced signal transduction pathways to the development of SCC (9–16). Our FVB/N transgenic mice, which overexpress PKCε in the epidermis, develop papilloma-independent SCC elicited either by UVR (12,16) or by the DMBA-TPA protocol (9–11,21). PKCε transgenic mice provide a unique model to investigate SCC (10). We have previously reported that epidermal PKCε level in PKCε transgenic mice dictates UVR- or TPA-induced TNFα release (13,16). We now present genetic evidence that TNFα is linked to UVR- or TPA-induced development of SCC.

Both PKCε transgenic mice and their wild-type littermates develop mostly carcinomas in response to repeated UVR exposures (Figure 1). Both PKCε transgenic mice and their wild-type littermates deficient in TNFα elicited both an increase in SCC latency and a decrease in SCC incidence (Figure 1). Skin PKCε level dictates the levels of TNFα release and the susceptibility of mice to UVR-induced development of SCC (13,16). A significant inhibition of SCC incidence in TG/TNFα KO mice clearly indicates that TNFα is the downstream component of PKCε signal transduction pathways to UVR-induced development of SCC (Figures 1 and 2).

The mechanism by which PKCε-induced TNFα mediates UVR-induced cutaneous SCC is not clearly understood. TNFα has the ability to regulate a vast array of cellular responses including proapoptotic, anti-apoptotic, proliferation and inflammation (35). We have previously presented evidence that UVR-induced increased expression of TNFα is proapoptotic or a proliferating signal in UVR carcinogenesis. In this context, it is noteworthy that FADD is the key component of both Fas- and TNF-mediated apoptosis (36–39). The UVR-induced loss of FADD expression in PKCε transgenic mice lends support to the conclusion that UVR-induced level of TNFα is not proapoptotic, but rather may contribute to an increased cell proliferation of preneoplastic cells (39). We also reported that UVR-induced severe cutaneous damage (ulceration, hyperplasia and infiltration of inflammatory cells) in PKCε transgenic mice was partially prevented in bigenic PKCε transgenic TNFα knockout mice (12).

Moreover, TNFα-deletion resulted decrease in both the frequencies and total number of HSCs (CD34+/6-integrin+) in WT and TG transgenic mice (Figure 4). These results imply that TNFα is linked to UVR-induced HSCs proliferation. These skin HSCs are known to be the putative precursor cells for SCC (40–43). These HSCs reside in the bulge region of hair follicles and are characterized by their attributes such as slow cycling, clonogenic, label retaining and role in induction of skin papillomas and carcinomas (44,45). It has been observed that the mice deficient in TNFα converting enzyme (TACE) show abnormal hair follicles and decreased number of HSCs (CD34+/6-integrin+) (46). We have previously reported an association of PKCε with HSCs (22). PKCε overexpression in mice increased the clonogenicity of isolated keratinocytes, a property commonly ascribed to stem cells. Both single and chronic UVR treatments resulted in an increase in the frequency of double positive HSCs in PKCε TG mice as compared to their WT littermates. In TG mice HSCs cycle at a faster rate as compared to wild-type mice. A comparison of gene expression profiles of FACS sorted double positive keratinocytes isolated from UVR treated WT and TG mice indicated increased expression in TG mice of genes (Pes1, Rad21, Tfdp1 and Cks1b) linked to cell transformation, invasion and metastasis of cancer cells (22). The current results (Figure 4) lend support to the conclusion that PKCε-induced TNFα mediates induction of SCC through direct effects on stem cells in the mouse hair follicle.

UVR-induced TNFα in PKCε transgenic mice mediates proliferative signals is further evidenced by the findings that TNFα-deficiency in PKCε transgenic mice resulted in suppression of UVR-induced activation of transcription factors Stat3, c-Jun/AP-1 and NF-κB and expression of cell proliferative markers such as PCNA and ODC (Figure 5). The constitutive expression of phosphorylated Stat3 at both pStat3Tyr705 and Stat3Ser727 residues are linked with UVR-induced skin carcinogenesis (47,48). The
phosphorylation of both of these residues is also crucial for maximum activation of Stat3, a downstream signaling component of PKCε. Inhibition of the phosphorylation of these Stat3 residues and other transcriptional factors also reveals the role of these signaling molecules in the inhibition of UVR-induced SCC in TG//TNFα KO mice. We also found inhibition of UVR-induced increased expression of TNFα receptors (TNFRI, TNFRII) in TG//TNFα KO mice (Figure 5). Notably, it is known that the biological effects of TNFα are mediated through its receptors, namely TNFRI and TNFRII. These receptors are also required for the optimal development of tumor in mice (49). The inhibition of these TNFα receptors may be one of the mechanisms of abrogation of TNFα signaling in TG//TNFα KO mice. Also, protein level of MMP7 and MMP9 are also inhibited in TG//TNFα KO mice (Figure 5), which is linked to the development, progression and metastasis of skin cancer (49–53). WT mice hardly elicited detectable changes in the indicated proteins (Figure 5). Consequently, it is difficult to conclude whether TNFα deletion has any effect on the expression of these proteins.

In summary, UVR has been shown to induce the release of pro-inflammatory (IL-1, TNFα), chemotactic (IL-6, IL-7, IL-15, GM-CSF, TNFα) and immune-regulatory cytokines (IL-10, IL-12, IL-18) in epidermal keratinocytes (54–57). Additionally, UVR-induced mRNA and protein level of epidermal TNFα correlates with the expression level of PKCε in PKCε overexpressing mouse lines (13). Our finding indicates that the inhibition of UVR-induced SCC development in TG//TNFα KO mice is accompanied by inhibition of (1) TNFα receptors (TNFRI and TNFRII), cell proliferation marker ODC, metastatic markers MMP7 and MMP9 (2), the activation of transcription factors Stat3, c-Jun/AP-1 and NF-xB, and (3) proliferation of HSCs and epidermal hyperplasia. We conclude that the genetic deletion of TNFα in PKCε transgenic mice (TG//TNFα KO) and their littermates inhibit UVR-induced SCC via abrogation of TNFα signaling and activation of cell survival pathway as evidenced by hyperplasia, HSCs proliferation and western blot analysis.

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