PKCε Overexpression, Irrespective of Genetic Background, Sensitizes Skin to UVR-Induced Development of Squamous-Cell Carcinomas

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Chronic exposure to UVR is the major etiologic factor in the development of human skin cancers including squamous-cell carcinoma (SCC). We have previously shown that protein Kinase C epsilon (PKCε) transgenic mice on FVB/N background, which overexpress PKCε protein approximately eightfold over endogenous levels in epidermis, exhibit about threefold more sensitivity than wild-type littermates to UVR-induced development of SCC. To determine whether it is PKCε and not the mouse genetic background that determines susceptibility to UVR carcinogenesis, we cross-bred PKCε FVB/N transgenic mice with SKH-1 hairless mice to generate PKCε-overexpressing SKH-1 hairless mice. To evaluate the susceptibility of PKCε SKH-1 hairless transgenic mice to UVR carcinogenesis, the mice were exposed to UVR (1–$2\,KJ\,m^{-2}$) three times weekly from a bank of six kodacel-filtered FS40 sunlamps. As compared with the wild-type hairless mice, PKCε overexpression in SKH-1 hairless mice decreased the latency (12 weeks), whereas it increased the incidence (twofold) and multiplicity (fourfold) of SCC. The SKH hairless transgenic mice were observed to be as sensitive as FVB/N transgenic mice to UVR-induced development of SCC and expression of proliferative markers (proliferating cell nuclear antigen, signal transducers and activators of transcription 3, and extracellular signal-regulated kinase 1/2). The results indicate that PKCε level dictates susceptibility, irrespective of genetic background, to UVR carcinogenesis.

INTRODUCTION

Skin cancer accounts for half of all cancers diagnosed in the United States totaling over 1 million new cases every year (American Cancer Society, 2009). Squamous-cell carcinoma (SCC) and basal-cell carcinoma are the most common forms of nonmelaoma skin cancer. SCC is the most aggressive form of nonmelanoma skin cancer and, unlike basal-cell carcinoma, can metastasize (Goldman, 1998).

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Abbreviations: ERK1/2, extracellular signal-regulated kinase 1/2; K14, human keratin 14 promoter; MAPK, mitogen-activated protein kinase; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol-3-kinase; PKC, protein Kinase C; SCC, squamous-cell carcinoma; STAT, signal transducers and activators of transcription; TPA, 12-O-tetradecanoylphorbol-13-acetate

The most important risk factor for the development of nonmelanoma skin cancer is chronic exposure to UVR in sunlight (Cooper and Bowden, 2007; Molho-Pessach and Lotem, 2007). The UV spectrum, part of the electromagnetic spectrum, lies between visible light and X-rays and is divided conventionally into three categories: UVA (315–400 nm), UVB (280–315 nm), and UVC (190–280 nm). Because stratospheric ozone absorbs most of the radiation below 310 nm, UVA and UVB components of sunlight are the most prominent and ubiquitous carcinogenic electromagnetic wavelengths in our natural environment (Wheeler *et al.*, 2004).

UVR is a complete carcinogen, which both initiates and promotes carcinogenesis. UVB initiates photocarcinogenesis by directly damaging DNA (de Gruijl and Rebel, 2008; Marrot and Meunier, 2008; Rass and Reichrath, 2008; Timares *et al.*, 2008). UVB-induced photoproducts include cyclobutane pyrimidine dimer, pyrimidine (6-4) pyrimidone dimer ([6-4]PD), and Dewar photoisomer of the (6-4)PD (Moriwaki and Takahashi, 2008). The cyclobutane pyrimidine dimer is the predominant photoproduct, accounting for 85% of the primary DNA lesions in UV-irradiated DNA (de Gruijl *et al.*, 2001). The majority of the DNA lesions are removed by the nucleotide excision repair (de Gruijl *et al.*, 2001; Moriwaki and Takahashi, 2008). However, upon DNA

replication, some cells acquire transition mutations (C→T) and tandem double mutations (CC→TT) arising at dipyrimidine sites (Brash *et al.*, 1991; Berton *et al.*, 1997). These mutations are frequently observed in UV-induced SCC in mice and humans (de Gruijl and Rebel, 2008). Among a series of gene mutations (TP53, PITCH, and oncogenes) that are associated with UV-induced skin cancer, C→T and CC→TT point mutations in the p53 gene are most frequent (Ziegler *et al.*, 1994; Kanjilal *et al.*, 1995). UVR can induce several types of epidermal injury including sunburn cell (apoptotic cell) formation (Ziegler *et al.*, 1994; Kanjilal *et al.*, 1995). The sunburn cells can be initiated by UV-induced DNA damage and subsequent induction of p53 protein. The p53-dependent apoptosis of UV-damaged normal cells (sunburn cells) is prevented due to p53 mutation. Thus, these mutated cells can clonally expand to form SCC following subsequent UVR exposures.

The tumor promotion component of UVR carcinogenesis, which involves clonal expansion of the initiated cells, is probably mediated by aberrant expression of genes altered during tumor initiation. UVR has been reported to alter the expression of genes regulating inflammation, cell growth and differentiation, and oncogenesis. Specific examples include upregulation of the expression of p21 (WAF1/C1P1) (Lu *et al.*, 1999), p53 (Ziegler *et al.*, 1994), AP-1 activation (Cooper and Bowden, 2007), ODC (Wheeler *et al.*, 2004), COX2 (Isoherranen *et al.*, 1999), tumor necrosis factor-α, and a wide variety of cytokines and growth factors (Wheeler *et al.*, 2004). UVR-induced initial signals linked to the development of skin cancer are not defined. We found that protein Kinase C epsilon (PKCε) overexpression in epidermal cells of FVB/N mice sensitizes the skin to UVR-induced cutaneous damage and development of SCC.

Protein Kinase C (PKC), a family of phospholipid-dependent serine/threonine kinases, is not only the major intracellular receptor for the mouse skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Mellor and Parker, 1998; Mochly-Rosen and Kauvar, 1998; Newton, 2001; Angel *et al.*, 2003; Griner and Kazanietz, 2007) but is also activated by a variety of stress factors including UVR (Mellor and Parker, 1998; Mochly-Rosen and Kauvar, 1998; Wheeler *et al.*, 2004). PKCε is among six isoforms (α, δ, ε, η, μ, ζ) expressed in the mouse skin (Mochly-Rosen and Kauvar, 1998). To determine the *in vivo* functional specificity of PKCε in mouse skin carcinogenesis, we generated PKCε transgenic mouse (FVB/N) lines 224 and 215 that overexpress approximately 8- and 18-fold, respectively, PKCε protein over endogenous levels in basal epidermal cells (Reddig *et al.*, 2000; Jansen *et al.*, 2001a, b). PKCε transgenic mice were observed to be highly sensitive to the development of SCC elicited by the DMBA (7, 12-Dimethylbenz[a]anthracene) (100 nmol) – TPA (5 nmol) tumor promotion protocol (Reddig *et al.*, 2000; Jansen *et al.*, 2001a, b). UVR exposure (1 kJ m^{-2} thrice weekly) induced irreparable skin damage in high PKCε-overexpressing mouse line 215. However, the PKCε transgenic mouse, line 224, when exposed to UVR (2 kJ m^{-2} thrice weekly), exhibited minimum cutaneous damage but increased SCC multiplicity by threefold and decreased tumor latency by 12 weeks (Wheeler *et al.*, 2004). However, it is unknown whether mouse genetic background contributes to the susceptibility of PKCε transgenic mice to UVR-induced development of SCC.

In this communication, we cross-bred PKCε FVB/N transgenic mice with SKH-1 hairless mice to generate PKCε-overexpressing SKH-1 hairless mice to determine whether PKCε not mouse genetic background, determines susceptibility to UVR carcinogenesis. We present that PKCε-overexpressing SKH-1 hairless mice are as sensitive as their FVB/N transgenic mice to UVR carcinogenesis.

RESULTS

Generation of PKCε SKH-1 transgenic hairless mice

We have previously reported that PKCε transgenic FVB/N mice, which overexpress PKCε in the basal epidermal cells and the cells of the hair follicle, are highly sensitive to UVR-induced development of SCC (Wheeler *et al.*, 2004). In this study, we generated PKCε transgenic SKH-1 hairless mice to determine whether the mouse background contributes to the sensitivity of PKCε transgenic mice to UVR-induced carcinogenesis. In this experiment (Figure 1), heterozygous (+/−) inbred FVB/N K14-T7PKCε mice were crossed for four generations to wild-type (−/−) outcrossed SKH1 hairless mice for transmission of the transgene and hairlessness. Four generations of crossing were sufficient as SKH-1 is an outcrossed line, so genetic homogeneity within the mice is not necessary. PKCε transgenic SKH-1 hairless mice were phenotypically normal (Figure 1a). Histological examination of skin of untreated transgenic mice and wild-type mice revealed no histological differences (Figure 1b). To determine epidermal PKCε expression level in transgenic and their wild-type mice, dorsal skin of untreated PKCε SKH-1 transgenic hairless mice and their wild-type littermates was excised and fixed in 10% formalin for the analysis of PKCε (Figure 1c). The dorsal skin of PKCε transgenic mice exhibited significantly more immunoreactivity throughout the epidermis compared with the wild-type dorsal skin with strong staining in the basal cells. The wild-type dorsal skin sample exhibited light immunoreactivity to the anti-PKCε antibody throughout the epidermis (Figure 1c).

The possibility was explored that PKCε may cross-talk to other PKC isoforms by modulating their levels and their associated signals. In this experiment (Figure 1d and e), we determined the levels of other PKC isoforms in the epidermis of PKCε transgenic SKH-1 hairless mice to examine whether the elevated PKCε levels in the epidermis altered the level of expression of other PKC isoforms. As compared with wild-type SKH-1 hairless mice, PKCε transgenic SKH-1 hairless mice elicited about eightfold increase in PKCε expression level. However, there was no change in other PKC isoform expression levels between wild-type and transgenic mice (Figure 1d and e).

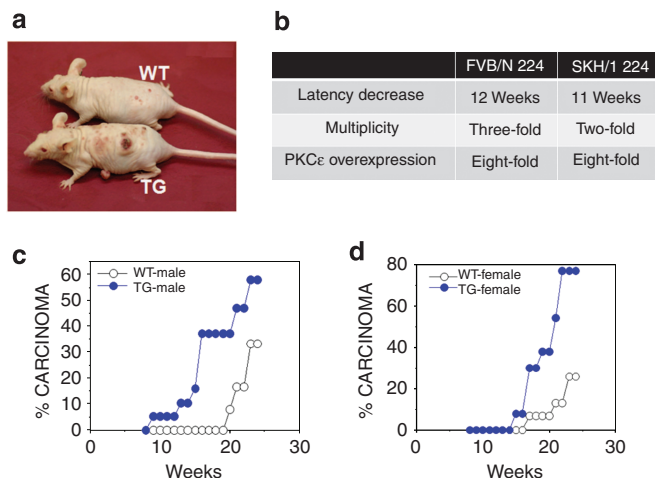### Susceptibility of SKH-1 PKCε transgenic mice to UVR-induced development of SCC

The PKCε transgenic SKH-1 hairless mice and their wild-type littermates (male and female) were treated three times
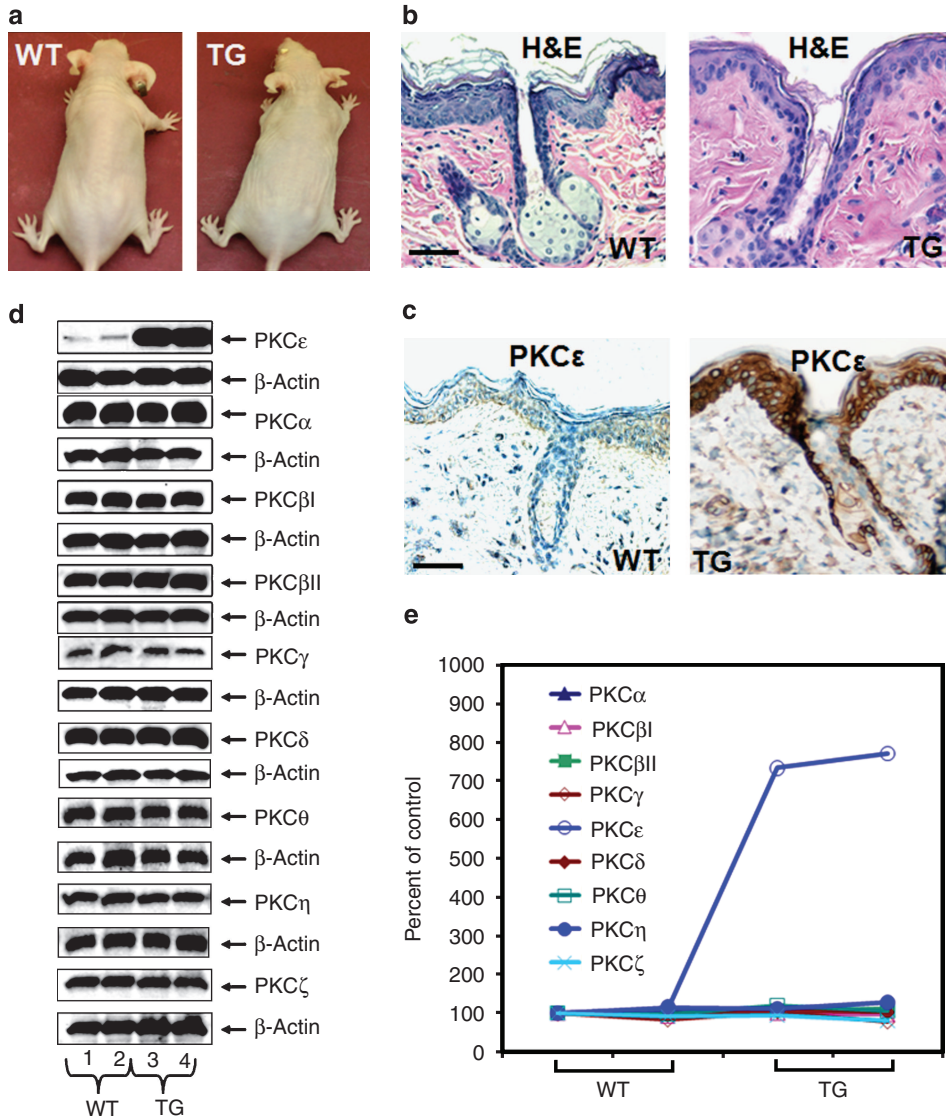
Figure 1. Generation of K14-T7PKCε transgenic mice on SKH-1 background. Heterozygous ($+/-$) FVB/N K14-T7PKCε mice were crossed for four generations to wild-type ($-/-$) outcrossed SKH-1 hairless mice for transmission of the transgene and hairlessness. (**a**) Phenotype of female SKH-1K14-T7PKCε transgenic mice. Shown are the photographs of adult SKH-1 hairless PKCε transgenic mouse and its wild-type littermate at 11 weeks of age. (**b**) Skin histology. The dorsal skin from a wild-type and PKCε SKH-1 hairless transgenic mouse was fixed in 10% neutral-buffered formalin and embedded in paraffin, and sections (4 μm) were stained with hematoxylin and eosin. (**c**) Immunohistochemical staining for PKCε expression, scale bars = 50 μm. (**d**) Epidermal PKC isoform expression profiles in PKCε SKH-1 hairless transgenic mice and their wild-type littermates; results representative of three replicates. (**e**) The quantification of proteins (normalized to β-actin) was performed by densitometry analysis using Total Lab Nonlinear Dynamics Image Analysis Software (Nonlinear USA Inc.).

(Monday, Wednesday, and Friday) weekly with $2 \, kJ \, m^{-2}$ UVR. After 7 weeks of UVR exposure, SKH-1 PKCε transgenic mice had severe cutaneous damage resulting in blistering, peeling, sloughing of the skin and the UVR dose was reduced to $1 \, kJ \, m^{-2}$ for the remainder of the study. PKCε overexpression in SKH-1 hairless mice reduced the latency for SCC (Figure 2). The first carcinoma in PKCε transgenic SKH-1 hairless male and female mice appeared after 9 and 15 weeks of UVR exposure, respectively, as compared with 20 and 17 weeks in wild-type male and female littermates, respectively. Carcinoma multiplicity in male PKCε transgenic mice was 1.18 after 23 weeks of exposure, compared with 1.0 in wild-type mice. Female PKCε transgenic mice had a carcinoma

multiplicity of 2.57 compared with 1.6 in wild-type mice. Statistical analysis for determining the difference in multiplicities from male and female mice using a Poisson model for genotype and sex found that males had a *P*-value of 0.04, whereas females had a *P*-value of 0.007. Carcinoma incidence in male PKCε SKH-1 transgenic and wild-type mice was 57.8 and 33.3%, respectively (Figure 2b). Papilloma burden in male transgenic and wild-type mice was 1.0 and 2.0, respectively. Carcinoma incidence in female PKCε transgenic and wild-type mice was 53.8 and 20.0%, respectively (Figure 2c). Papilloma burden in female transgenic and wild-type mice was 1.0 and 2.6, respectively. Carcinoma incidence was first analyzed for statistical
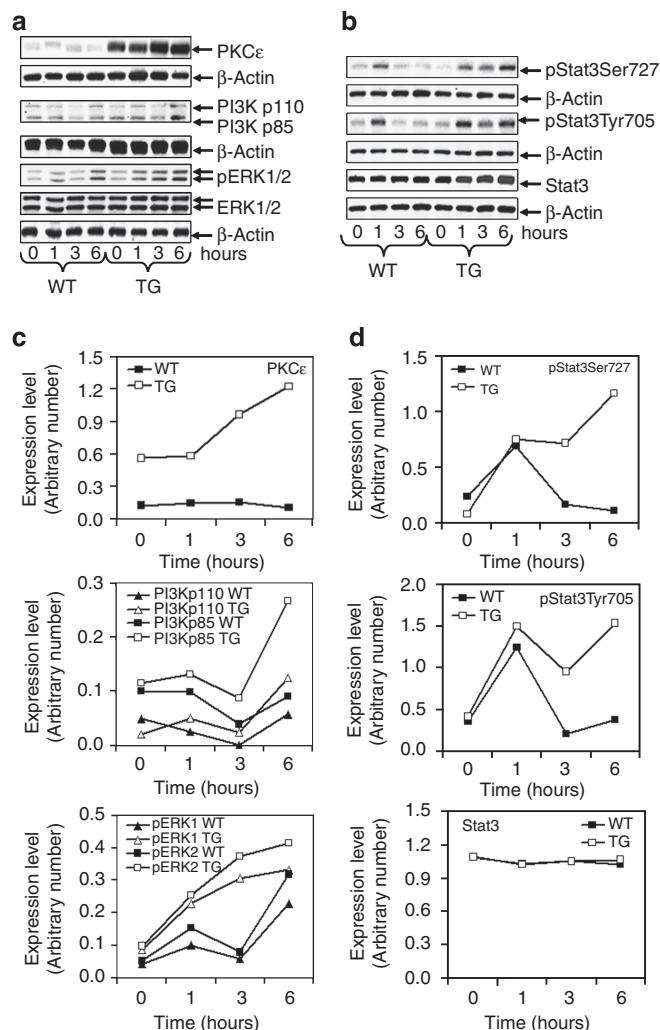
Figure 2. Susceptibility of PKCε transgenic SKH-1 hairless mice to the development of SCC by UVR. Male and female PKCε transgenic and wild-type mice at 11 weeks of age, mice were exposed to UVR ($1–2\,kJ\,m^{-2}$) three times weekly from a bank of six Kodacel-filtered FS40 sunlamps. There were 15 mice per group. Carcinomas were recorded as downward invading lesions, which were confirmed histologically. The carcinoma data are expressed as the percentage of effectual total. Carcinoma incidence was analyzed using Cox proportional hazards model. (**a**) Representative photograph of a wild-type and PKCε transgenic SKH-1 mouse after 20 weeks of UVR exposure. (**b**) Similarity between FVB/N PKCε line 224 and SKH-1 PKCε line 224, comparison of decrease in carcinoma latency, increase in carcinoma multiplicity and PKCε overexpression. (**c**) Comparison of male wild-type and PKCε transgenic mice carcinoma incidence. Carcinoma incidence in male mice was not found to be statistically different, $P=0.13$. (**d**) Comparison of female wild-type and PKCε transgenic mice carcinoma incidence. Carcinoma incidence in female mice was found to be statistically different $P=0.007$.

Figure 3. UVR-induced phosphorylation of PI3K, ERK1/2, and Stat3 in PKCε SKH-1 hairless transgenic mice and their wild-type littermates. Female PKCε transgenic SKH-1 hairless mice (TG) and their wild-type littermates (WT) (three mice per group) were exposed to a single UVR dose ($1\,kJ\,m^{-2}$). The mice were killed at 1, 3, and 6 hours post-UVR exposure. The mouse epidermis was scraped off and total lysate was prepared. Total epidermal lysate from three mice was pooled for the western blot analysis. (**a** and **b**) The epidermal lysate (25 μg protein) was subjected to SDS-PAGE followed by immunoblot analysis using indicated antibodies. Equal loading was confirmed by stripping and reprobing the blots with β-actin antibody. (**c** and **d**) The quantification of proteins (normalized to β-actin) was performed by densitometry analysis using Total Lab Nonlinear Dynamics Image Analysis Software (Nonlinear USA Inc.).

significance using the Cox proportional hazards model with *P*-values for male mice being $P=0.13$ and female mice $P=0.007$. There was no significant gender difference with either the Poisson model ($P=0.86$) or Cox proportional hazards model ($P=0.46$). Transgenic mice are clearly more sensitive than wild-type littermates to UVR-induced carcinogenesis. This sensitivity led to a massive decrease (~ 3 months) in latency and a significant increase in multiplicity.

Overexpression of PKCε in mouse epidermis increases sensitivity of skin to UVR-mediated phosphorylation of Stat3, ERK1/2, and PI3K

The effects of epidermal PKCε overexpression in SKH-1 hairless mice on UVR-induced activation of cell survival pathways (phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and signal transducers and activators of transcription 3 (Stat3)) were determined. In this experiment (Figure 3), female PKCε transgenic SKH-1 hairless mice and their wild-type littermates were exposed to a single UVR ($2\,kJ\,m^{-2}$) dose. Mice were killed at 1, 3, and 6 hours post-UVR treatment. In wild-type mice, UVR treatment elicited rapid and transient increase in the expression level of PI3K (p110), PI3K (p85), phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (Figure 3a and c), and phosphorylated Stat3 at both Tyr705 and Ser727 (Figure 3b

and d). However, UVR treatment in PKCε transgenic SKH-1 hairless mice led to persistent activation of Stat3 phosphorylation at both Tyr705 and Ser727 (Figure 3b and d), and phosphorylated ERK1/2 and PI3K (Figure 3a and c).

SCC developed in PKCε transgenic SKH-1 hairless mice elicit elevated nuclear localization of PCNA and Stat3

In this experiment (Figure 4), SCC from PKCε transgenic SKH-1 hairless mice and their wild-type littermates (Figure 2) were excised and fixed in 10% neutral buffered formalin. Sections of SCC from PKCε transgenic SKH-1 hairless mice
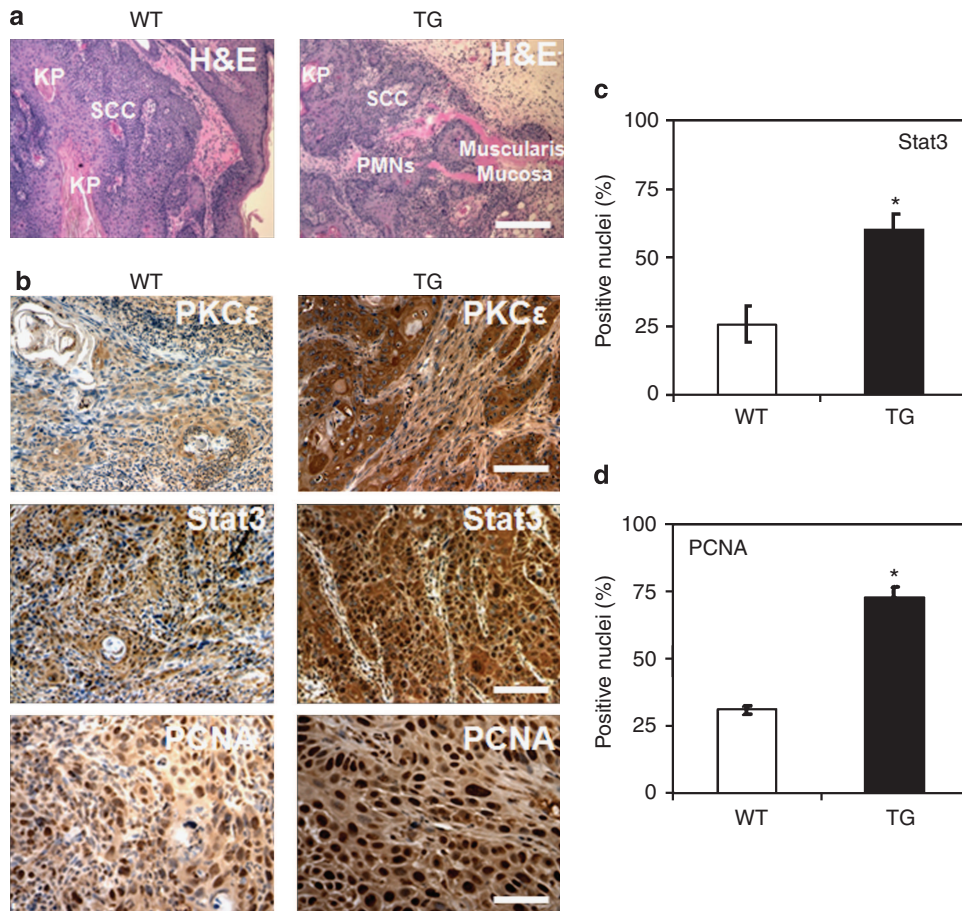
Figure 4. Immunohistochemistry of PKCε, Stat3, and PCNA in SCC. PKCε transgenic hairless mice (TG) and their wild-type littermates (WT) were exposed to UVR, as described in the legend to Figure 2 until the development of SCC. SCC was excised promptly after euthanasia, placed immediately in 10% neutral-buffered formalin, fixed for 1 hour in formalin, then transferred to phosphate-buffered saline (pH 7.4) and embedded in paraffin. Section of 4 μm thickness was cut for hematoxylin and eosin staining and immunohistochemical study. Each value is the mean ± SE from an average of 10 fields from three different mice was used to calculate Student's *t*-test. (**a**) Hematoxylin and eosin staining (scale bar = 50 μm) (**b**) Immunoreactivity of PKCε (scale bar = 50 μm), Stat3 (scale bar = 50 μm), and PCNA (scale bar = 25 μm). (**c** and **d**) Quantification of Stat3 and PCNA nuclear staining. Student's *t*-test were performed to analyze nuclear staining differences (*$P < 0.05$).

and their wild-type littermates were processed for immuno-histochemistry of PKCε, Stat3, and proliferating cell nuclear antigen (PCNA) (Figure 4b). SCC from PKCε SKH-1 transgenic mice showed a significant increase in PKCε, nuclear PCNA (Figure 4c) and nuclear Stat3 (Figure 4d) staining compared with wild-type mice (*$P < 0.05$).

DISCUSSION

The PKCε transgenic FVB/N mice, which overexpress PKCε protein eightfold over endogenous levels in basal epidermal cells and cells of the hair follicle, are threefold more sensitive than wildtype to the development of SCC by repeated exposures to UVR (Wheeler *et al.*, 2004). To determine whether it is PKCε and not the mouse genetic background that determines susceptibility to UVR carcinogenesis, we cross-bred PKCε FVB/N transgenic mice with SKH-1 hairless mice to generate PKCε-overexpressing SKH-1 hairless mice. We now present that PKCε-overexpressing SKH-1 hairless mice are as sensitive as FVB/N PKCε transgenic mice to UVR-induced development of SCC (Figure 2b).

Genetic differences in susceptibility to two-stage skin carcinogenesis have been shown to be at the level of tumor promotion by TPA (Angel *et al.*, 2003). PKCε is among the major intracellular receptor for the mouse skin tumor promoter TPA (Mellor and Parker, 1998; Griner and Kazanietz, 2007) and is activated by a variety of stress factors including UVR (Wheeler *et al.*, 2004). Furthermore, genetic mouse background has been shown to be a major determinant of conversion of benign papillomas to malignant SCC (Woodworth *et al.*, 2004). FVB/N mice are moderately susceptible to papilloma formation but are highly prone to undergo malignant conversion (Reddig *et al.*, 2000). PKCε overexpression in SKH-1 hairless mice, similar to FVB/N mice, suppressed papilloma formation but enhanced SCC development. Although, confirmation should await further experimentation involving crosses with inbred mouse strains (BALB/c, C57BL/6), the results presented imply that PKCε appears to be a highly penetrant susceptible gene.

We have previously reported generation and sensitivity of PKCε transgenic FVB/N mouse lines to UVR (Wheeler *et al.*,

2004). PKCε transgenic line 215, which overexpresses about 18-fold PKCε protein over endogenous levels, elicited severe cutaneous damage after exposure to UVR (Wheeler *et al.*, 2004). UVR-induced skin damage in PKCε transgenic line 215 after exposure to UVR (either 1 or $2 \,kJ\,m^{-2}$) was extensive and irreparable, and the experiment could not be continued until the appearance of carcinomas. However, the lower PKCε-overexpressing mice (line 224) tolerated three times weekly UVR exposures ($2 \,kJ\,m^{-2}$) for 38 weeks and, had threefold increased SCC multiplicity as well as decreased tumor latency by 12 weeks when compared with wildtype. PKCε overexpression in SKH-1 yielded similar results upon repeated UVR exposure.

The UVR-induced activated PKCε mediates two potential signals, which lead to inhibition of apoptosis and increased proliferation of preneoplastic cells. In this context, it is noteworthy that UVR-induced percentage of sunburn cells in PKCε transgenic mice was significantly lower than wild-type littermates (Wheeler *et al.*, 2004). PKCε transgenic mice were more sensitive than their wild-type littermates to UVR-induced epidermal proliferation markers PCNA, or-nithine decarboxylase, and constitutively activated Stat3 (Jansen *et al.*, 2001a, b; Wheeler *et al.*, 2004; Aziz *et al.*, 2007). Consistent with our previous report, PKCε SKH-1 transgenic mice elicited constitutive activation of Stat3 in both response to acute UVR exposure and in SCC developed during chronic UVR exposure. SCC from PKCε transgenic SKH-1 mice also had increased expression of nuclear PCNA (Figure 4).

The PI3K/AKT and MAPK pathways are involved in the regulation of survival pathways in tumor cells. Both PI3K/AKT and MAPK are activated during UVR carcinogenesis (Einspahr *et al.*, 2008). However, the mechanism by which UVR treatment mediates activation of survival pathways (PI3K/AKT and MAPK) is unclear. PKCε overexpression in SKH-1 hairless mice accompanied increased expression of phosphorylated PI3K and MAPK (ERK1/2) implying that PKCε may impart sensitivity to UVR carcinogenesis through cross-talk to PI3K and MAPK pathways.

The UVR treatment resulted in increased expression of PKCε (Figure 3b). This UVR-increased PKCε expression may be the result of the effect of UVR treatment on PKCε synthesis. It is noteworthy that PKCε levels are constant during chronic UVR treatment (data not shown) and in SCC developed by UVR treatment (Figure 4). These results (Figure 4) are in contrast to our previous findings with SCC developed in PKCε mice by DMBA-TPA protocol (Reddig *et al.*, 2000). A possible explanation for this difference in PKCε expression level in SCC may be explained by a difference in antigen retrieval protocol for immunhistochem-ical localization.

In summary, PKCε-overexpressing SKH-1 hairless mice, similar to FVB/N, are more sensitive than their wild-type littermates to induction of SCC by UVR treatment. UVR-induced activated PKCε cross-talks with survival pathways (PI3K, MAPK) and regulates constitutive activa-tion of Stat3, critical factor involved in UVR carcino-genesis (Kim *et al.*, 2009). PKCε transgenic SKH-1 hairless mice may provide a useful model to investigate UVR carcinogenesis.

MATERIALS AND METHODS

Antibodies
The antibodies and sources of the antibodies used in this study were as follows: PKCε, Stat3, phosphorylated Stat3Tyr705 (pStat3Tyr705), PI3K (p85), PI3K (p110), β-actin from Santa Cruz Biotechnology (Santa Cruz, CA); and phosphorylated Stat3Ser727 (pStat3Ser727; BD Biosciences, San Jose, CA). Blocking peptides for PKCε and Stat3 were obtained from Santa Cruz Biotechnology.

The generation of K14-T7PKCε transgenic mice on SKH-1 background
Transgenic mice-overexpressing T7 epitope-tagged PKC-ε (T7-PKCε), under the control of the human keratin 14 (K14) promoter, using the inbred FVB/N background were created as described previously (Reddig *et al.*, 2000). Heterozygous (+/−) inbred FVB/N K14-T7PKCε mice were crossed for four generations to wild-type (−/−) outcrossed SKH1 hairless mice from Charles River Laboratories (Wilmington, MA) for transmission of the transgene and hairlessness. Four generations of crossing were sufficient as SKH-1 is an outcrossed line, so genetic homogeneity within the mice is not necessary. SKH-1 mice were maintained as an outcrossed line. Thus, the difference between two "pure" SKH-1 mice is as much as the difference between a "pure" SKH-1 and a SKH-1//FVB/N cross. All animal experiments were performed in accordance with approval from IACUC at the University of Wisconsin-Madison.

UVR treatment of mice
Mice were housed in groups of two to three in plastic bottom cages in light-, humidity-, and temperature-controlled rooms; food and water were available *ad libitum*. The animals were kept in a normal rhythm of 12-hours-light and 12-hours-dark periods. The UVR source was Kodacel-filtered FS-40 sunlamps (approximately 60% UVB and 40% UVA). UVR dose was measured weekly using UVX radiometer. Mice were used for experimentation at 11–14 weeks of age. Mice were exposed to UVR ($1–2 \,kJ\,m^{-2}$) three times weekly (Monday, Wednesday, and Friday). Tumor multiplicity was observed weekly and mouse weight was determined every other week. Carcinomas were recorded grossly as downward-invading lesions, which were confirmed histologically.

Histologic analysis
Mouse skin was excised promptly after euthanasia and immediately placed in 10% neutral-buffered formalin, fixed for 24 hours in formalin, transferred to phosphate-buffered saline (pH 7.4) and then embedded in paraffin. Four-μm sections were cut for hematoxyli-n–eosin staining and immunohistochemical study.

Immunohistochemistry of PKCε, Stat3, and PCNA
The PKCε transgenic mice and their wild-type littermates were exposed to UVR ($1–2 \,kJ\,m^{-2}$) three times weekly (Monday, Wednes-day, and Friday), for 26 weeks. SCC specimens were fixed in 10% neutral-buffered formalin for 24 hours and embedded in paraffin. Four-μm-thick sections were cut for staining. Briefly, slides were first treated for antigen retrieval by incubating first in 1 M citric acid (pH 6.0) at 95°C for 20 minutes and in 0.1 M Tris-EDTA (pH 8.0) for

20 minutes. The slides were washed with phosphate-buffered saline with 0.5% Tween and incubated overnight at 4°C with the primary antibody. Subsequent incubation steps were performed in a moist chamber at room temperature. After intermediate washing steps in Tris-buffered saline (pH 7.4), the sections were incubated with biotin-labeled rabbit antimouse IgG for 15 minutes at room temperature and then with streptavidin-peroxidase complexes for 15 minutes at room temperature. Visualization was performed using diaminobenzidine as a substrate for the peroxidase reaction. Slides were transferred into tap water and counterstained with hematoxylin for 4 minutes. Every experiment included a control that contained no primary antibody but preimmune rabbit serum. Antibody specificity was observed using blocking peptides. No immunoreactivity was observed using these controls. Specimens were analyzed using an Olympus BX 51 microscope (Lake Success, NY).

For quantitation of PCNA and Stat3-positive staining cells, 10 random fields were selected for each mouse. The number of cells demonstrating positive labeling and the total number of cells counted were recorded. An average percentage was then calculated based on the total number of cells and the number of positive staining cells from each set of 10 fields counted. Results are expressed as mean of percentages ± SE.

Western blot analysis

Mouse skin was excised and scraped to remove subcutaneous fat. The epidermis was scraped off on a ice-cold glass plate, homogenized in the lysis buffer (50 mmol l^{-1} HEPES, 150 mmol l^{-1} NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol l^{-1} MgCl$_2$, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mmol l^{-1} phenylmethylsulfonyl fluoride, 200 μmol l^{-1} Na$_3$VO$_4$, 200 μmol l^{-1} NaF, and 1 mmol l^{-1} EGTA (final pH 7.5)). The homogenate was centrifuged at $14,000 \times g$ for 30 minutes at 4°C. Twenty micrograms of cell lysate were fractionated on 10% Criterion precast SDS-PAGE (Bio-Rad Laboratories, Hercules, CA). The protein was transferred to 0.45 μm Hybond-P polyvinylidene difluoride transfer membrane (Amersham Life Sciences, Piscataway, NJ). The membrane was then incubated with the indicated antibody followed by a horseradish peroxidase secondary antibody (Santa Cruz Biotechnology), and the detection signal was developed with Amersham's enhanced chemiluminescence reagent and autoradiography using BioMax film (Kodak, Memphis, TN). The western blots were quantitated by densitometric analysis using Totallab Nonlinear Dynamic Image analysis software (Nonlinear USA Inc., Durham, NC).

Statistical methods

The primary end point of interest was carcinoma. Those mice that did not develop carcinoma were censored at the last point at which data were available for them. Differences in time to event, by gender and genotype and the interaction of gender and genotype, were examined visually using Kaplan–Meier method and tested with Cox proportional hazards models. Also of interest was tumor multiplicity. Differences in tumor multiplicity by gender, genotype, and interaction were tested with Poisson regression models. Computations were performed with R software. (R Development Core Team, 2007). Student's *t*-test was also performed for the comparison of wildtype and transgenic nuclear localization of Stat3 and PCNA.

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