

# Epidermal Growth Factor Receptor-Mediated Activation of Stat3 during Multistage Skin Carcinogenesis

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## ABSTRACT

In the present study, we have investigated the possible role of signal transducers and activators of transcription (STATs), particularly Stat3, in mouse skin tumor promotion and multistage carcinogenesis. Stat1, Stat3, and Stat5 were activated in mouse epidermis after treatment with different classes of tumor promoters, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), okadaic acid, and chrysarobin. In addition, Stat1, Stat3, and Stat5 were constitutively activated in skin tumors generated by the two-stage carcinogenesis regimen using 7,12-dimethylbenz(*a*)anthracene as initiator and TPA as promoter. Several approaches were used to examine the possible role of epidermal growth factor receptor (EGFR) in modulating Stat3 activity during tumor promotion. In primary cultures of mouse keratinocytes, addition of exogenous EGF led to activation of Stat3 as shown by an elevation in tyrosine phosphorylation and nuclear translocation. In epidermis of transgenic mice expressing transforming growth factor  $\alpha$  under control of the keratin 14 promoter, Stat3 was constitutively activated. Abrogation of EGFR function in mouse epidermis using an EGFR kinase inhibitor or by overexpressing a dominant negative form of EGFR led to a reduction in Stat3 activation in response to TPA treatment. Immunoprecipitation analyses using lysates from TPA-treated epidermis and skin papillomas showed enhanced interaction between the EGFR and Stat3. Finally, Stat3 deficiency in mouse epidermis significantly reduced the proliferative response after TPA treatment. Collectively, the current results suggest that Stat3 activation may be a critical event during mouse skin tumor promotion, possibly through regulation of keratinocyte proliferation. In addition, Stat3 activation in tumor promoter-treated epidermis and in skin papillomas may occur, at least in part, via interaction with and phosphorylation by the EGFR. Finally, constitutive activation of Stat3 in both papillomas and squamous cell carcinomas suggest a role in both the development of autonomous growth and the progression of epithelial tumors in mouse skin.

## INTRODUCTION

Signal transducers and activators of transcription (STATs) are a family of latent transcription factors that are activated by a wide spectrum of growth factors, cytokines, and hormones to modulate cell proliferation, apoptosis, differentiation, and many other important biological activities (1, 2). STATs are recruited to cell surface receptors or nonreceptor kinases via a conserved src homology 2 domain. Phosphorylation of a conserved tyrosine residue activates STATs by associating two STAT monomers via reciprocal phosphotyrosine-src homology 2 interactions to form a dimer. Activated STAT dimers can translocate to the nucleus, bind to consensus DNA sequences, and modulate expression of target genes (2). Currently, seven members of the STATs have been identified, which are encoded by seven distinct

genes: Stat1 ( $\alpha$  and  $\beta$  splice isoforms); Stat2; Stat3 ( $\alpha$  and  $\beta$  splice isoforms); Stat4; Stat5a; Stat5b; and Stat6 (1).

Constitutive activation of STATs, in particular Stat3, is found in a number of primary human epithelial tumors and cancer cell lines, including squamous cell carcinomas (SCCs) of the head and neck (3), breast (4, 5), ovary (6), prostate (7), and lung (8). Stat3 has been shown to be critical in maintaining cancer cell proliferation because inhibition of Stat3 via antisense oligonucleotides, Stat3 decoy, or expression of a dominant negative Stat3 leads to suppression of growth in several epithelial cancer cell lines (3, 6, 7, 9). However, naturally occurring mutations of Stat3 leading to its constitutive activation have not been identified. It is therefore proposed that aberrant growth factor signaling, which is a frequent event in many human epithelial cancers, may play an important role contributing to the constitutive activation of Stat3 (10). Epidermal growth factor receptor (EGFR) is a 170-kDa receptor tyrosine kinase that has been shown to play an important role in epithelial carcinogenesis (11–13). Elevated expression of EGFR and/or its ligands [*e.g.*, transforming growth factor (TGF)- $\alpha$ ] are common in many types of epithelial cancer, and such changes have been shown to be an important component for maintaining the proliferative capacity of the tumor cells. Furthermore, recent studies have implicated a role for Stat3 in maintaining EGFR-mediated epithelial cancer cell proliferation (14, 15).

The mouse skin carcinogenesis model has been instrumental for investigating the molecular mechanisms contributing to human epithelial cancer development. Tumor development in this model can be subdivided into three mechanistic stages: initiation; promotion; and progression (16, 17). Initiation involves an irreversible genetic change induced by a subcarcinogenic dose of a carcinogen such as 7,12-dimethylbenz(*a*)anthracene (DMBA). Initiation with DMBA leads to mutations in the *Ha-ras* gene, and typically, this stage does not involve morphological changes in skin (16, 17). The promotion stage involves repetitive application of tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to mouse skin which causes selective clonal expansion of initiated cells. The hallmarks of the tumor promotion stage include a dramatic induction of epidermal cell proliferation and hyperplasia. The end point of the promotion stage is the development of premalignant clonal outgrowths (*i.e.*, papillomas; Refs. 16, 17). Tumor progression is characterized by the accumulation of additional genetic changes in papillomas, whereby these tumors convert to SCCs (16, 17).

Our laboratory has been using the mouse skin carcinogenesis model for understanding molecular mechanism contributing to the development of human epithelial cancers. We have previously shown that skin tumor promoters up-regulate EGFR ligands (*e.g.*, TGF- $\alpha$ ), leading to activation of the EGFR (18, 19). In addition, skin tumors generated in this model system exhibit constitutively up-regulated EGFR ligands (20, 21). Transgenic mice overexpressing TGF- $\alpha$  showed a hyperproliferative phenotype and were highly susceptible to skin tumor development induced by the two-stage regimen and also developed skin tumors after treatment with either DMBA or TPA alone (22–25). In contrast, transgenic mice expressing a dominant negative EGFR were resistant to two-stage carcinogenesis (26) and EGFR null keratinocytes transduced with v-Ha-ras produced papillomas that showed both

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an impaired growth and reduced tumor volume (27). Taken together, these data suggest a critical role for the EGFR signaling pathway in mediating TPA-induced epidermal hyperproliferation and skin tumor growth *in vivo*. However, the events downstream of the EGFR that lead to critical gene activation important for hyperproliferation and skin tumorigenesis remain to be fully elucidated. In the present study, we show for the first time that STATs are activated in mouse epidermis after topical treatment with several types of skin tumor promoters and that STATs are constitutively activated in skin papillomas and SCCs generated in this model system. In addition, Stat3 activation appears to occur, at least in part, through interaction with and activation by the EGFR. Finally, data are presented showing that Stat3 signaling may play a critical role in regulating tumor promoter-induced keratinocyte proliferation and skin tumor growth *in vivo*.

## MATERIALS AND METHODS

**Chemicals.** DMBA was purchased from Eastman Kodak Co. (Rochester, NY). TPA, phorbol-12,13-didecanoate (PDD), 4- $\alpha$ -phorbol-12,13-didecanoate (4- $\alpha$ -PDD), and okadaic acid were purchased from LC Laboratories (Woburn, MA). Chrysarobin was purchased from ICN Pharmaceuticals, Inc. (K&K Laboratory Division, Plainview, NY) and purified before use as described previously (28). The EGFR kinase specific inhibitor AG1478 was purchased from CalBiochem-NovaBiochem Corporation (San Diego, CA). EGF was obtained from UBI (Lake Placid, NY). DMEM was obtained from Life Technologies, Inc.-Invitrogen Corporation (Carlsbad, CA). Thirty percent Acrylamide/Bis Solution (29:1) and polyvinylidene difluoride membrane were purchased from Bio-Rad Laboratories (Hercules, CA). 5-Bromo-2'-deoxyuridine and protease inhibitor mixture were obtained from Sigma-Aldrich Co (St. Louis, MO).

**Animals and Treatment.** Female SENCAR mice were obtained from the National Cancer Institute (Frederick, MD) and were used for experimentation at 6–8 weeks of age. SENCAR mice are an outbred line of mice that were selectively bred for sensitivity to two-stage skin carcinogenesis (16). The dorsal skin of each mouse was shaved 2 days before treatment; only those mice in the resting phase of the hair cycle were used. All solutions of DMBA, TPA, PDD, 4- $\alpha$ -PDD, okadaic acid, chrysarobin, and the EGFR kinase-specific inhibitor, AG1478, were prepared in reagent-grade acetone and were applied topically in a total volume of 0.2 ml. In the two-stage tumor experiment using DMBA as the initiator and TPA as the promoter, mice were initiated with a single topical application of DMBA at 10 nmol. Two weeks after initiation, TPA at 3.4 nmol was applied topically twice weekly. Mice were sacrificed, and skin tumors were collected 20 (papillomas) and 40 weeks (SCCs) after first TPA treatment. TPA treatment was terminated at least 1 week before harvest of the tumors. In the short-term tumor promoter experiments, mice were treated topically with single or multiple applications of tumor promoters. For multiple treatment protocols, mice were treated twice weekly for 2 weeks with TPA (3.4, 6.8, and 10 nmol), PDD (10 nmol), 4- $\alpha$ -PDD (10 nmol), and okadaic acid (10 nmol). For multiple treatments with chrysarobin (440 nmol), mice received treatment once weekly for 2 weeks. Mice were sacrificed at various time points, and epidermal lysates were collected. In the EGFR kinase-specific inhibitor experiments, mice were pretreated with AG1478 (100, 50, and 25 nmol) 15 min before and 1 h after a single treatment of TPA (3.4 nmol). Mice were sacrificed 3 h after TPA treatment, and epidermal lysates were collected. Transgenic mice overexpressing TGF- $\alpha$  under the control of a human keratin 14 promoter have been described previously (29). Skin-specific Stat3-deficient mice were generated as described previously (30). In brief, K5Cre transgenic mice were crossed with Stat3<sup>fl/fl</sup> mice to obtain K5Cre.Stat3<sup>fl/wt</sup> mice. Then, K5Cre.Stat3<sup>fl/wt</sup> mice were crossed to Stat3<sup>fl/fl</sup> mice to generate K5Cre.Stat3<sup>fl/fl</sup> mice, which are referred to as skin-specific Stat3-deficient mice. Age-matched Stat3<sup>fl/fl</sup> mice and Stat3<sup>fl/wt</sup> mice were used as control littermates.

**Cell Culture.** Epidermal basal cells from SENCAR mice were isolated and cultured in MEM-2 medium with 1% FBS, 0.04% penicillin-streptomycin solution, and a calcium concentration of 0.04 mM as described previously (20). Cells were plated at a density of 10<sup>7</sup> cells/100-mm tissue culture dish. Cells were starved for 48 h in 1% BSA/MEM-2 in the absence of all growth factors. After two washes with starvation medium, cells were stimulated with 100

ng/ml EGF and lysed with ice-cold lysis buffer 5, 15, and 20 min after EGF stimulation.

**Preparation of Epidermal Cell Lysates.** Mice were sacrificed by cervical dislocation. The dorsal skins were treated with a depilatory agent (1 min) followed by washing. The skin was excised and placed on a glass plate on ice, and the epidermis was removed with a razor blade and placed into lysis buffer. The lysis buffer contained 50 mM Tris-HCl (pH 8.6), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, and 10  $\mu$ l/ml protease inhibitor mixture. The lysates were incubated on ice for 10 min, snap frozen in liquid nitrogen, thawed, and then centrifuged at 14,000  $\times$  g for 15 min at 4°C. The supernatant was precleared with protein A-agarose beads for 10 min at 4°C and then centrifuged at 14,000  $\times$  g for 15 min at 4°C. The supernatant was used immediately for Western blot analysis, immunoprecipitation, or stored for short-term use at –80°C.

**Immunoprecipitation.** Epidermal or tumor lysates were incubated with anti-EGFR antibody (1:100 dilution; NeoMarkers, Fremont, CA) in 500  $\mu$ l of ice-cold lysis buffer for 1 h in 4°C. Thirty  $\mu$ l of protein A/G-conjugated agarose beads were added and incubated for 1 h at 4°C. The immunocomplex was precipitated by brief centrifugation and washed three times with ice-cold lysis buffer.

**Western Blot Analysis.** The protein concentration of epidermal cell and cultured cell lysates was measured with the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Epidermal cell lysates, cultured cell lysates or immunoprecipitates were separated on 7.5% SDS/PAGE according to the method of Laemmli (31), under reducing conditions. Separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes and blocked with 5% nonfat dry milk in PBS with 0.1% Tween 20 (TPBS) for 1 h at room temperature. Blots were then incubated with specific primary antibodies for Stat1, p<sup>Tyr</sup>Stat1, Stat3, p<sup>Tyr</sup>Stat3, p<sup>Tyr</sup>Stat5 (Cell Signaling Technology, Inc., Beverly, MA), p<sup>Ser</sup>Stat3 (Affinity BioReagents, Golden, CO), Stat5 (BD Transduction Laboratory, Los Angeles, CA), and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h at room temperature. Blots were washed with TPBS and subjected to corresponding horseradish peroxidase-conjugated secondary antibodies (rabbit, Amersham, Arlington Heights, IL; mouse and goat, Santa Cruz Biotechnology, Inc.). Blots were washed with TPBS and detected with the LumiGlo chemiluminescent kit (Cell Signaling Technology, Inc.).

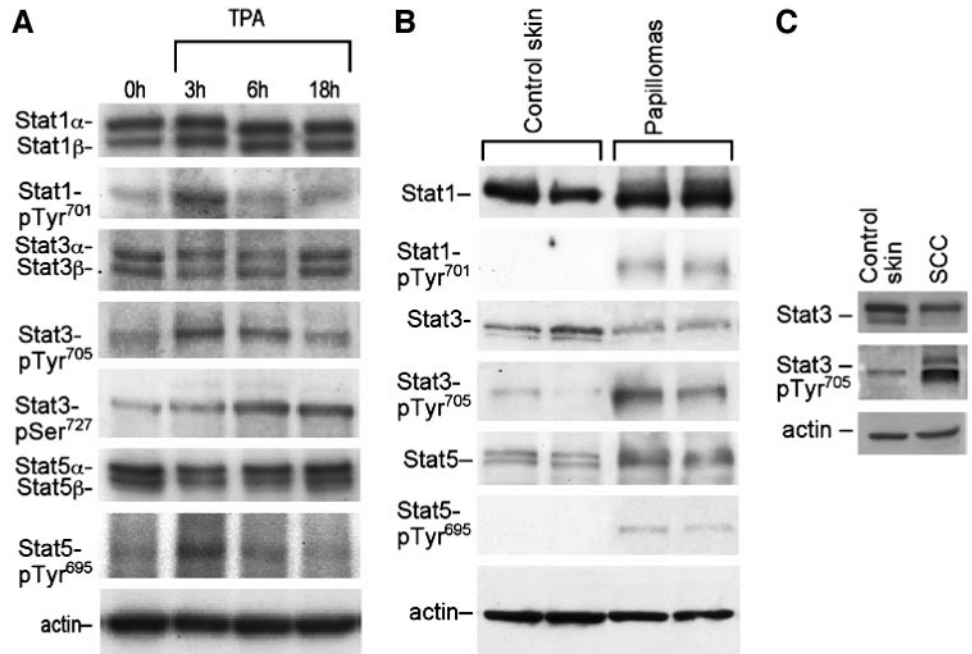
**Indirect Immunofluorescence Staining.** Primary keratinocytes were washed with PBS and fixed for 5 min in 4% paraformaldehyde, which contained 0.5% Triton X-100 for permeabilization. After washing with PBS, fixed cells were blocked in PBS containing 1% BSA. Cells were stained with a 1:500 dilution of Stat3 (C-20) antibody (Santa Cruz Biotechnology, Inc.). Cells were then incubated with a FITC-conjugated antirabbit secondary antibody (The Jackson Laboratory, Bar Harbor, ME) and washed with PBS. Images were analyzed using a fluorescence microscope.

**Histological Analysis.** For the analysis of labeling index, mice were injected i.p. with 5-bromo-2'-deoxyuridine in PBS (100  $\mu$ g/g body weight) 30 min before sacrifice. Dorsal skin samples were fixed in formalin and embedded in paraffin before sectioning. Sections of 4- $\mu$ m were cut and stained with anti-5-bromo-2'-deoxyuridine antibody (BD PharMingen, Los Angeles, CA) as described previously (32).

## RESULTS

**Activation of STATs in Mouse Epidermis by the Tumor Promoter TPA.** To elucidate a possible role for STATs in mouse skin tumor promotion, we examined the protein levels and tyrosine phosphorylation status of Stat1, Stat3, and Stat5 in epidermal lysates at various time points after topical application of the phorbol ester, TPA using Western blot analysis. As shown in Fig. 1A, a single treatment with the phorbol ester tumor promoter TPA did not significantly affect the protein levels of Stat1, Stat3, and Stat5 in mouse epidermis. However, after TPA, there was a transient elevation of tyrosine phosphorylation of all three STATs examined with a peak at 3–6 h. The tyrosine phosphorylation of Stat1 and Stat5 in TPA-treated mouse epidermis was confirmed by additional immunoprecipitation analysis (data not shown). In addition, serine phosphorylation of Stat3 was

Fig. 1. Activation of Stat1, Stat3, and Stat5 during multistage skin carcinogenesis. Groups of at least three mice received a single topical treatment of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 3.4 nmol), and the epidermis was collected at the indicated time points. Skin papillomas and squamous cell carcinomas (SCCs) were generated by using 7,12-dimethylbenz(*a*)anthracene as the initiator and TPA as the promoter and collected after 20 and 40 weeks of promotion, respectively. Tumors were harvested at least 1 week after TPA treatment. *A*, Western blot analysis of Stat1, Stat3, and Stat5 (protein and tyrosine phosphorylation level) and also Stat3 serine phosphorylation level; *B*, Western blot analysis of Stat1, Stat3, and Stat5 (protein and tyrosine phosphorylation level) in lysates from papillomas (pool of three or more/lane) compared with control skin; *C*, Western blot analysis of Stat3 and tyrosine phosphorylated Stat3 in lysates from SCCs (pool of two SCCs) compared with control skin.



examined. As shown in Fig. 1A, phosphorylation of Stat3 on Ser<sup>727</sup> was also induced by TPA treatment occurring later (6 and 18 h) than that observed for tyrosine phosphorylation.

**Constitutive Activation of STATs in Mouse Skin Tumors.** As part of the current study, we also examined the status of Stat1, Stat3, and Stat5 in mouse skin tumors that were generated by the two-stage carcinogenesis regimen using DMBA as the initiator and TPA as the promoter. Papillomas and SCCs were harvested at least 1 week after TPA treatment to avoid any effects associated with the tumor promoter. More detailed time course experiments showed that the tyrosine phosphorylation level of Stat3 returned to control levels by 24 h after TPA treatment (data not shown). Protein lysates were obtained from samples of pooled skin papillomas (at least three papillomas/sample), and the protein level and tyrosine phosphorylation status of STATs were examined by Western blot analysis. Notably, all STATs examined were constitutively activated in the skin papillomas as shown by elevated tyrosine phosphorylation (Fig. 1B). Additionally, elevated serine phosphorylation of Stat3 was observed (data not shown). There was no significant alteration in the protein levels of the three STATs that could explain the increased tyrosine phosphorylation relative to control skin samples. Furthermore, Stat3 status was also assessed in a sample of two pooled SCCs (Fig. 1C). There were two forms of Stat3 detected in the control skin samples (Fig. 1, B and C), which corresponded with the splice isoforms  $\alpha$  (92 kDa) and  $\beta$  (84 kDa). It appeared that the lower molecular weight form (Stat3 $\beta$ ), which has been proposed to have a dominant negative function (2), was down-regulated in both papillomas and SCCs. Nevertheless, as observed with lysates from papillomas, Stat3 tyrosine phosphorylation was found to be significantly elevated in lysates from SCCs.

**Activation of Stat3 in Mouse Epidermis by Different Classes of Tumor Promoters.** To establish the generality of STATs activation in skin tumor promotion, we focused on Stat3 and examined its status in mouse epidermis treated topically with the nonphorbol ester tumor promoters, okadaic acid (33), and chrysarobin (28). As shown in Fig. 2A, the level of tyrosine phosphorylation of Stat3 was elevated in mouse epidermis treated with okadaic acid and chrysarobin at all time points examined. There also appeared to be an increase in the level of Stat3 protein at 18 h after treatment with okadaic acid and 18 and 24 h

after treatment with chrysarobin. Nevertheless, after normalization to Stat3 protein levels, Stat3 tyrosine phosphorylation levels were still significantly elevated (~1.4–2.4-fold at the time points examined). Quantitation of Stat3 tyrosine phosphorylation relative to protein level by densitometry is presented as numeric values below each lane in the Western blot shown in Fig. 2A.

**Stat3 Activation Correlates with the Tumor-Promoting Activity of Phorbol Esters.** Next, we examined the relationship between promoting activity and Stat3 activation after topical application of

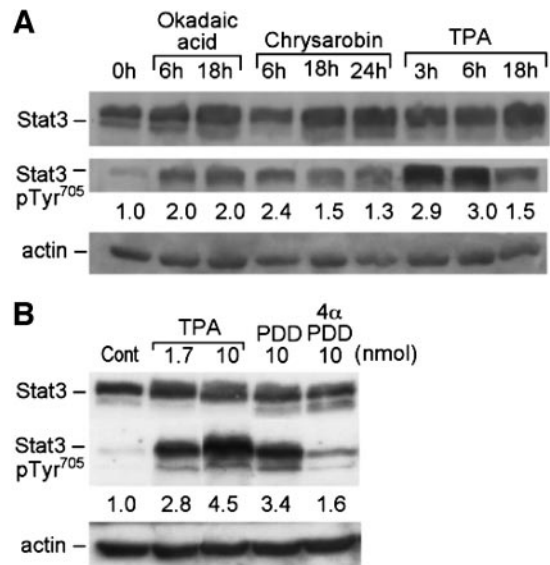


Fig. 2. Activation of epidermal Stat3 by diverse skin tumor promoters. Groups of at least three mice received a single topical treatment of different tumor promoters or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) analogue as follow: 10 nmol okadaic acid; 220 nmol chrysarobin; 1.7 and 10 nmol TPA; and 10 nmol TPA analogues. Epidermal lysates were prepared at the indicated time points. *A*, Western blot analysis of Stat3 protein and tyrosine phosphorylation level, with quantitation of the relative level (number below each lane) of phosphorylated Stat3 by densitometry (normalized to Stat3 protein levels); *B*, level of tyrosine phosphorylated Stat3 after treatment with several phorbol esters [TPA (1.7 and 10 nmol), phorbol-12,13-didecanoate (PDD; 10 nmol), 4- $\alpha$ -PDD (10 nmol)], with quantitation of the relative tyrosine-phosphorylated level (number below each lane) of Stat3 by densitometry (normalized to Stat3 protein level).

several phorbol ester analogues. Therefore, the protein level and tyrosine phosphorylation status of Stat3 was examined by Western blot analysis using epidermal lysates from mice after treatment with TPA, PDD, and 4- $\alpha$ -PDD. As shown in Fig. 2B, there was no significant alteration in the protein level of Stat3 associated with any of the phorbol ester treatments. However, the level of Stat3 tyrosine phosphorylation followed the tumor-promoting activities of TPA and the two structural analogues, with PDD being less potent than TPA and 4- $\alpha$ -PDD being inactive (16). Quantitation of Stat3 tyrosine phosphorylation relative to protein level is presented as numeric values below each lane in the Western blot shown in Fig. 2B.

**EGFR-Mediated Activation of Stat3 during Skin Tumor Promotion.** It has been proposed that Stat3 activation during human cancer development is caused by aberrant growth factor signaling (10). Our laboratory has previously shown activation of the EGFR (18, 20) concomitant with an elevated expression of its ligands (18, 19, 21) in mouse epidermis treated with TPA. In addition, these changes are constitutive in skin papillomas generated by the two-stage DMBA-TPA regimen (20, 21). Therefore, we examined whether the EGFR pathway is involved in the activation of Stat3 during tumor promotion and multistage skin carcinogenesis. Initially, the Stat3 status in adult primary keratinocytes in response to exogenous EGF was examined. Five min after EGF stimulation, there was a marked increase of tyrosine phosphorylated Stat3 detected in whole cell lysates (Fig. 3A). In addition, tyrosine phosphorylated Stat3 began to appear in the nuclear fraction (see again Fig. 3A). By 15 min after EGF stimulation, both total Stat3 protein as well as tyrosine phosphorylated Stat3 were markedly elevated in the nuclear fraction. The nuclear translocation of Stat3 after EGF stimulation of primary keratinocytes was confirmed by immunofluorescence staining. As shown in Fig. 3B, Stat3 was mostly localized in the cytoplasm of untreated keratinocytes, with a basal level of Stat3 in the nucleus (Fig. 3B, ii). However, as early as 5 min after EGF stimulation of mouse keratinocytes, the majority of Stat3 was localized in the nucleus (Fig. 3B, iii). The localization of Stat3 in the nucleus decreased 15 min after EGF stimulation, and Stat3 began to reappear in the cytoplasm (Fig. 3B, iv). Keratinocytes were stained with FITC-conjugated secondary antibody only as a control for nonspecific staining (Fig. 3B, i).

To further examine the role of the EGFR signaling pathway in regulating Stat3 activity in mouse epidermis *in vivo*, we determined the status of Stat3 in the epidermis of transgenic mice overexpressing TGF- $\alpha$  under the control of the human keratin 14 promoter (K14.TGF $\alpha$  mice) (29). Epidermal lysates were isolated from transgenic mice and age-matched littermates, and the status of Stat3 was analyzed by Western blot analysis. As shown in Fig. 3C, Stat3 was constitutively activated in the epidermis of K14.TGF- $\alpha$  mice as assessed by elevated tyrosine phosphorylation.

The results shown in Fig. 3 suggested that EGFR activation in mouse keratinocytes leads to activation of Stat3 both *in vitro* and *in vivo*. To explore the relationship between EGFR activation and Stat3 during mouse skin tumor promotion, SENCAR mice were treated topically with the EGFR kinase specific inhibitor AG1478 (34) in the presence and absence of TPA. As shown in Fig. 4A, AG1478 significantly inhibited the TPA-induced tyrosine phosphorylation of Stat3 (~30% inhibition as determined by densitometry). As a second approach to establish a link between EGFR activation and Stat3 activation during tumor promotion *in vivo*, we used transgenic mice expressing a dominant negative form of EGFR in the basal compartment of the epidermis, where EGFR autophosphorylation is completely abrogated (35). After treatment of these mice with TPA, the level of Stat3 tyrosine phosphorylation was reduced (~37%) compared with nontransgenic mice (Fig. 4B). Previous studies showed that the EGFR can directly interact with and phosphorylate Stat3 *in vitro* (36).

Recently, specific tyrosine residues in the cytoplasmic terminus of the EGFR have been identified that are essential for Stat3 activation (37). Using immunoprecipitation analyses, we further examined the relationship between EGFR activation and Stat3 activation during tumor promotion and multistage carcinogenesis. These experiments were designed to provide evidence for a direct interaction between the EGFR and Stat3. As shown in Fig. 4C, immunoprecipitation of the EGFR in protein lysates from TPA-treated epidermis and from papillomas followed by Western blotting with antibody against Stat3 showed the presence of elevated levels of Stat3 protein. In addition, there was also an increased level of tyrosine phosphorylated Stat3 associated with the EGFR in protein lysates from TPA-treated mouse epidermis and in skin papillomas, compared with protein lysates from control mice (see again Fig. 4C).

**Stat3 Deficiency Leads to a Significant Reduction in TPA-Induced Epidermal Hyperproliferation.** To additionally elucidate the functional role of Stat3 during mouse skin tumor promotion, we used tissue-specific Stat3-deficient mice (30). As shown in Fig. 5A, the Stat3-deficient mice used in the current experiments had a significantly reduced level of epidermal Stat3. After topical treatment with TPA, there was a significant reduction in the epidermal proliferative response of Stat3-deficient mice compared with control littermates as seen by the reduced number of 5-bromo-2'-deoxyuridine-positive basal cells (Fig. 5, C and B, respectively). As shown in Fig. 5D, the epidermal labeling index was reduced 64% in Stat3-deficient mice as compared with control littermates after TPA treatment.

## DISCUSSION

In the current study, we have examined the possible role of STATs and in particular Stat3 in skin tumor promotion and multistage skin carcinogenesis. Our initial experiments characterizing the early stage of tumor promotion showed that several STATs (1, 3, and 5) were activated in mouse epidermis after treatment with the phorbol ester, TPA. Stat3 was also activated after treatment with diverse classes of tumor promoters, and in addition, Stat3 activity was shown to correlate with the tumor-promoting activity of several phorbol esters. In skin papillomas, representing the late stage or end point of tumor promotion, Stat1, Stat3, and Stat5 were found to be constitutively activated in these premalignant lesions. Additional data showed that Stat3 was constitutively activated in malignant SCCs. Previous studies have shown that the EGFR is activated (20) and the levels of its ligands (both mRNA and protein; Refs. 18, 19, 21) are elevated in tumor promoter-treated mouse epidermis and in skin papillomas. Through a series of experiments whereby EGFR function was modulated, we demonstrated that Stat3 activity was regulated, at least in part, by EGFR signaling during skin tumor promotion. Furthermore, in a mouse model where Stat3 is deficient in keratinocytes, we showed that Stat3 deficiency led to significant reduction in the epidermal proliferative response of these mice to TPA treatment. The current results demonstrate for the first time that the Stat1, Stat3, and Stat5 are activated during the tumor promotion stage of mouse skin carcinogenesis. In addition, the data suggest an important role for Stat3 in both the production and maintenance of epidermal hyperproliferation during this step of multistage skin carcinogenesis. The constitutive activation of STATs in papillomas and SCCs suggest a role for these transcription factors in the autonomous growth and progression of epithelial tumors in this model system. Finally, the results suggest that the activation of Stat3 during tumor promotion may be attributed, at least in part, to activation of the EGFR signaling pathway.

In the current study, the generality of Stat3 activation during tumor promotion was investigated by examining whether this phenomenon occurred after treatment with diverse skin tumor promoters. Notably,

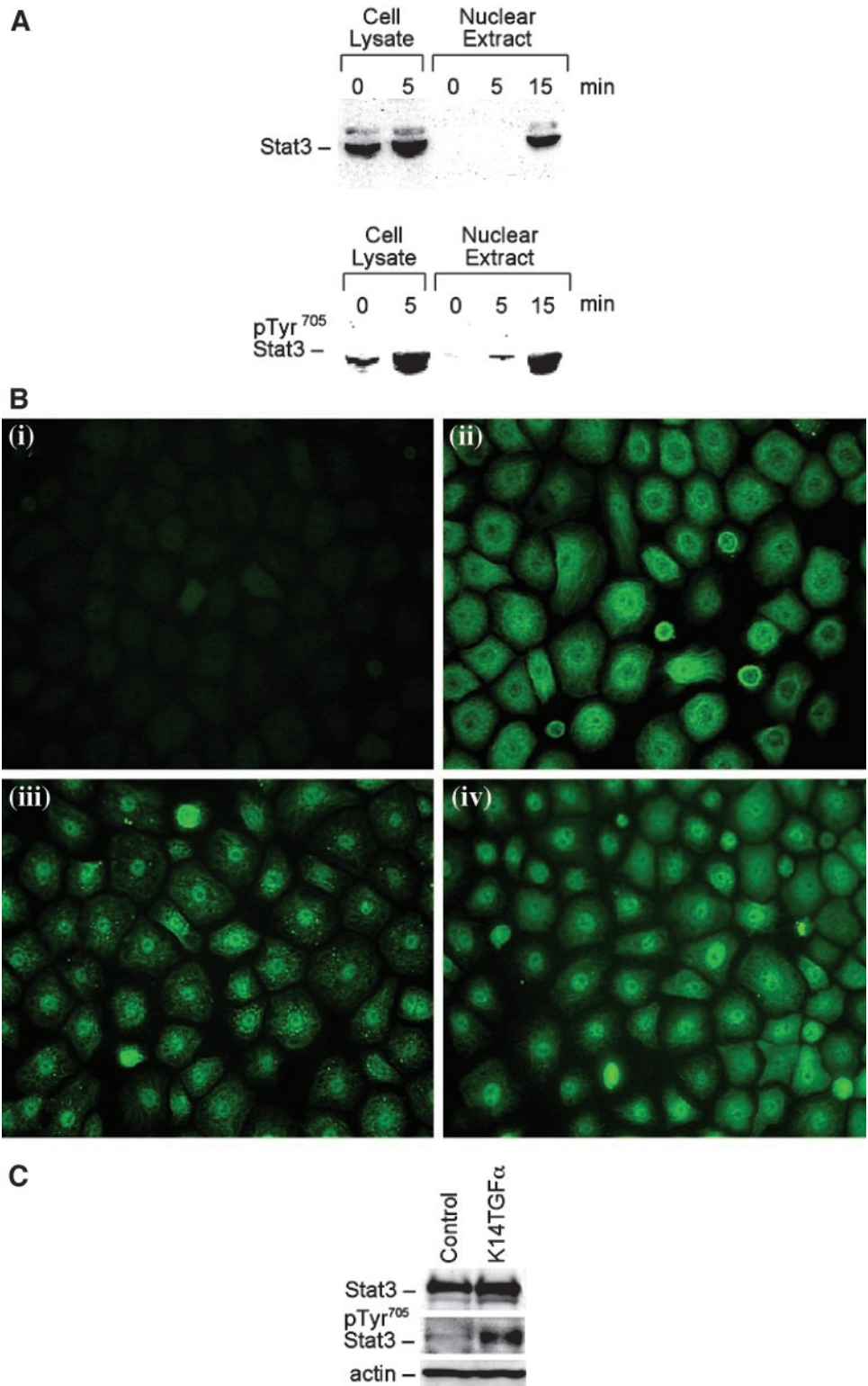


Fig. 3. Epidermal growth factor receptor (EGFR) signaling and Stat3 activation *in vitro* and *in vivo*. Primary cultures of mouse keratinocytes were serum starved, stimulated with EGF (100 ng/ml), and collected at various time points as indicated. *A*, whole cell lysates and nuclear extracts were collected at the times indicated and subjected to Western blot analyses with anti-Stat3 and an anti-pTyr<sup>705</sup> Stat3 antibody; *B*, keratinocytes were treated with EGF and stained with anti-Stat3 antibody followed by a FITC-conjugated secondary antibody. *i*, control, secondary antibody only; *ii*, no treatment; *iii*, treatment with EGF for 5 min; and *iv*, treatment with EGF for 15 min. *C*, epidermal lysates were collected from K14.TGF $\alpha$  transgenic mice and subjected to Western blot analysis with either anti-Stat3 or anti-pTyr<sup>705</sup> Stat3 antibody.

Stat3 was activated in mouse epidermis by different classes of skin tumor promoters, including TPA, okadaic acid, and chrysarobin. TPA is the most commonly used phorbol ester tumor promoter that is known to directly activate protein kinase C as one of its primary mechanisms (38). Okadaic acid is an inhibitor of protein phosphatases 1 and 2A (33). Chrysarobin appears to work through a mechanism involving autooxidation and the generation of free radicals (28). Thus, okadaic acid and chrysarobin represent nonphorbol ester tumor pro-

motors whose initial mechanism of action appears to be different from that of the phorbol esters. Regardless, there are pathways of convergence whereby most if not all tumor promoters produce similar effects. Up-regulation of EGFR ligands and subsequent activation of this cell surface receptor tyrosine kinase appears to be one common pathway among diverse types of promoting stimuli (20, 21). Our finding that Stat3 is activated by all these tumor promoting agents suggests that the Stat3 signaling pathway may be a common and

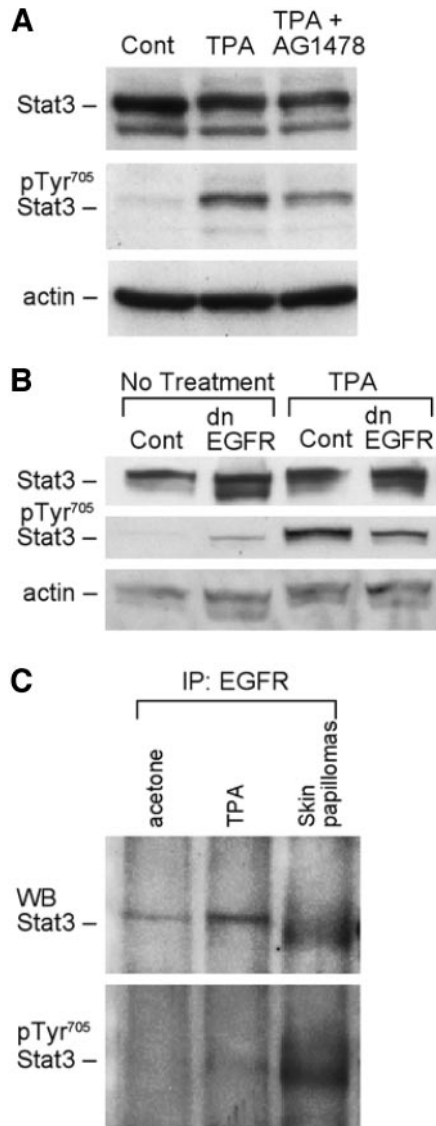


Fig. 4. Evidence for epidermal growth factor receptor (EGFR) signaling in Stat3 activation during mouse skin tumor promotion. **A**, groups of at least three SENCAR mice were treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the presence and absence of the EGFR kinase-specific inhibitor AG1478. Epidermal lysates were collected 3 h after TPA treatment and subjected to Western blot analysis using Stat3 antibodies as shown. **B**, groups of at least three mice each [nontransgenic littermates or dominant negative EGFR (dnEGFR)] were treated with TPA, and epidermal lysates were collected 3 h after TPA treatment. Western blot analysis was performed using Stat3 antibodies as shown. **C**, epidermal lysates from SENCAR mice treated with TPA and lysates from papillomas were immunoprecipitated with an anti-EGFR antibody and separated by gel electrophoresis under reducing conditions. After transfer to membranes, blots were probed with an anti-Stat3 antibody.

critical event for mediating some aspect of the tumor-promoting effects of diverse chemicals. In addition, these findings also led us to further examine the role of the EGFR in Stat3 activation during skin tumor promotion.

As noted in the "Introduction," it has been proposed that aberrant growth factor signaling may contribute to the activation of Stat3 seen in human tumors (10). In SCCs of the head and neck, where over ~80% of tumors show an activation of the EGFR pathway, targeted inhibition of EGFR signaling in SCCs of the head and neck can inhibit Stat3 activation as well as suppress cancer cell growth (14). In addition, in samples from breast cancer patients, there is a strong correlation between EGFR expression and nuclear Stat3 expression (15). Our *in vitro* data in primary cultures of keratinocytes stimulated

by EGF and *in vivo* data in mouse epidermis with forced expression of TGF- $\alpha$  clearly indicate that activation of EGFR in keratinocytes can lead to rapid Stat3 activation. Furthermore, we found enhanced Stat3 association with the EGFR in lysates from both TPA-treated epidermis and in skin papillomas. Finally, abrogation of EGFR function by various strategies led to partial inhibition of TPA-induced Stat3 activation. Taken together, these data suggest that the EGFR plays a role in Stat3 activation during mouse skin tumor promotion. Evidence in the literature suggests that other nonreceptor kinases such as Janus-activated kinase and c-Src may play a role in activating Stat3 in other model systems (5). It is possible that other kinases such as c-Src, which we previously found to be activated by TPA (29), may play a role in activating Stat3 during mouse skin tumor promotion. Additional work is necessary to determine the extent to which these

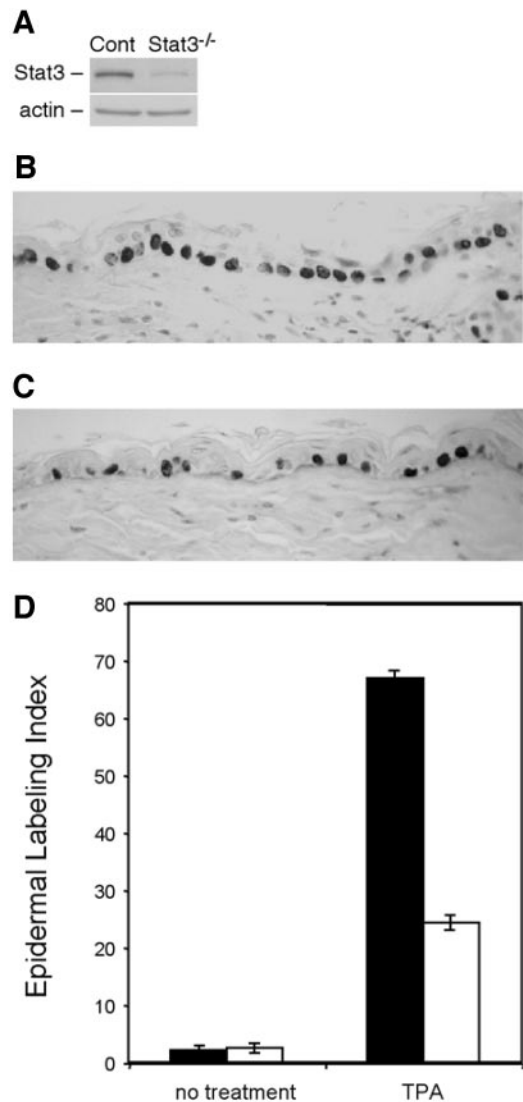


Fig. 5. Stat3 deficiency in mouse epidermis leads to a significant hyperreduction in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal hyperproliferation. Stat3-deficient mice and control littermates were treated with a single dose of TPA. Mice were injected with 5-bromo-2'-deoxyuridine (BrdUrd) 30 min before sacrifice, and mice were sacrificed 17 h after treatment (peak of epidermal cell proliferation). **A**, Western blot analysis of total Stat3 protein level in epidermal lysates from Stat3-deficient and control mice. **B**, BrdUrd stained skin section from control littermates. **C**, BrdUrd stained skin section from Stat3-deficient mice. **D**, percentage of BrdUrd-positive cells [labeling index (LI)] in epidermis of Stat3-deficient mice compared with control littermates. LI was determined as described previously (51). (■) represents control littermates, and (□) represents Stat3-deficient mice after TPA treatment was found to be statistically significant ( $P < 0.01$ ) by the Student *t* test.

and possibly other kinases contribute to Stat3 activation during carcinogenesis in this model of epithelial cancer.

Tumor promoter-induced keratinocyte proliferation is a major mechanism contributing to the promotion of initiated cells into pre-malignant lesions (16, 17). The results presented in Fig. 5, using mice deficient in Stat3 in the epidermis clearly demonstrate a role for Stat3 in regulating TPA-induced epidermal hyperproliferation. Stat3 has been implicated in modulating several critical transcriptional targets that are involved in the regulation of cell cycle progression, including c-Myc and cyclin D1 (39). In skin, three separate groups have reported the generation of transgenic mice that overexpress c-Myc in the basal layer of epidermis and all exhibit a hyperproliferative phenotype (40–42). In addition, transgenic mice overexpressing cyclin D1 in epidermis in either sensitive (43) or resistant (44) background strain also showed an enhanced epidermal proliferation. These observations suggest that Stat3 may regulate TPA-induced epidermal hyperproliferation through its ability to regulate critical molecules governing cell cycle progression. Sano *et al.* (30) previously reported that Stat3 deficient keratinocytes possessed a defect in growth factor-induced migration while retaining normal [<sup>3</sup>H]thymidine incorporation in response to EGF in culture. However, Dlugosz *et al.* (45) previously showed relatively low growth stimulation in growth factor treated primary keratinocyte cultures. Therefore, growth factor treatment of cultured keratinocytes may not adequately reveal defects seen in mouse epidermis *in vivo*. On the other hand, Hauser *et al.* (46) reported that Stat3 activity correlated with p27 levels when keratinocytes were detached from adhesion by suspension. In addition, Stat3 activity was reduced in cultures of p27-deficient keratinocytes and in MK cells. The authors concluded that Stat3 activation was associated with keratinocyte differentiation. Clearly, additional studies will be necessary to fully understand the function of Stat3 in regulating keratinocyte proliferation and/or differentiation *in vivo*. Nevertheless, our current data provide evidence that tumor promoter-induced epidermal hyperproliferation *in vivo* involves Stat3 activation, at least in part, through activation of the EGFR.

In the early stages of skin tumor promotion, continuous TPA treatment is required to maintain papilloma growth. However, in SENCAR mice, by ~10 weeks of TPA promotion, the majority of papillomas that form have the ability to grow autonomously (*i.e.*, independent of tumor promoter treatments; Ref. 47). This ability to grow autonomously is likely caused by the acquisition of additional genetic changes that lead to alterations in the level or activity of growth regulatory molecules. We have shown that the mRNA, and protein levels of EGFR ligands (*e.g.*, TGF- $\alpha$ , amphiregulin) are elevated in papillomas (20, 21), suggesting a role for constitutive EGFR signaling in the autonomous growth of these tumors. Our finding that Stat3 is constitutively activated in both skin papillomas and SCCs suggests that Stat3 and its target genes may be critical for maintaining the autonomous growth of skin tumors that develop in this model system. Constitutive activation of Stat3 in SCCs may suggest an additional role for this transcription factor in tumor progression. Stat3 regulates genes that could contribute to tumor progression, including Bcl-xL (48) and vascular endothelial growth factor (49, 50). Additional work will be necessary to explore these and other aspects of Stat3 action during the later stages of skin carcinogenesis.

In conclusion, our current data demonstrate for the first time that Stat3 activation occurs during the tumor promotion stage of skin carcinogenesis and that Stat3 signaling is important for tumor promoter-induced epidermal hyperproliferation. Furthermore, constitutive activation of Stat3 may be necessary for the maintenance of autonomous growth in skin tumors (both papillomas and SCCs) and possibly play a role in the progression of papillomas to SCCs in this model of epithelial carcinogenesis. These data suggest that Stat3 and

its regulated genes may be excellent targets not only for the treatment of cancer but also for cancer prevention.

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