

All-Trans Retinoic Acid Suppresses Stat3 Signaling during Skin Carcinogenesis

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Abstract

Squamous cell carcinoma (SCC) of the skin is the most clinically aggressive form of non-melanoma skin cancer. We have determined the effects of all-trans retinoic acid (ATRA), a naturally occurring chemopreventive retinoid, on signal transducer and activator of transcription 3 (Stat3) signaling during the development of skin SCC. Stat3 is a transcription factor that plays a critical role in cell proliferation and survival, and it is constitutively active in several malignant cell types. We have previously shown that Stat3 is required for the initiation, promotion, and progression of skin SCC. ATRA is a highly efficient suppressor of tumor formation in the two-stage mouse skin carcinogenesis model and we have shown that this effect correlates with the suppression of the B-Raf/Mek/Erk signaling pathway. In this study, we have determined the pattern of Stat3 phosphorylation throughout the course of the two-stage protocol, both in the presence and absence of ATRA. We have used both SENCAR mice and K5.Stat3C transgenic mice, which express the Stat3C protein, a constitutively active form of Stat3, in the skin. Using Western blotting and immunohistochemical staining with phosphospecific antibodies, we show that coadministration of ATRA suppressed the 12-O-tetradecanoylphorbol-13-acetate-induced phosphorylation of Stat3 in both models, but was only able to suppress tumor formation in the SENCAR mice. Surprisingly, ATRA actually enhanced tumor formation in 12-O-tetradecanoylphorbol-13-acetate-treated K5.Stat3C mice. We hypothesize that ATRA blocks tumor formation, at least in part, by targeting events upstream of Stat3, such as the B-Raf/Mek/Erk pathway, and that in the K5.Stat3C mice, in which Stat3 activity is constitutive, it cannot suppress tumor formation.

Nonmelanoma skin cancer is the most common cancer in the United States, with over a million new cases of the two most common forms, squamous cell carcinoma (SCC) and basal cell carcinoma, anticipated annually (1). The more clinically aggressive form is SCC (2), which has been increasing in incidence since the 1960s at annual rates from 4% to as much as 10% in recent years. Advanced disease-related and treatment-related morbidity

have a profound effect on the patients' quality of life. Unlike basal cell carcinoma, which bears a single genetic hallmark of disruption of the patched-sonic hedgehog signaling pathway, the genetic alterations leading to SCC seem more complex and varied, and are poorly understood (3). Better control of advanced skin SCC is clearly necessary, and will be greatly helped by improving our understanding of the molecular basis for skin carcinogenesis and of the action of chemopreventive drugs.

The two-stage mouse skin chemical carcinogenesis model is one of the best studied and most informative with regard to understanding molecular mechanisms and the evolution of cancer cells (4). It has proven to be ideal for the study of events leading to the transition from initiation, to promotion, and then progression to carcinoma. Molecular analysis of multistage human cancers such as prostate and colon cancer have shown a high level of genetic and biological similarity to mouse skin in the two-stage model. (5). The SENCAR (sensitive to carcinogen) mouse strain was developed for this assay due to its high susceptibility to chemical-induced tumor formation, relative to most other strains of mice tested (4). Skin tumors can be readily induced by the sequential application of a sub-threshold dose of carcinogen such as 7,12-dimethylbenz(*a*)anthracene (DMBA), referred to as the initiation stage, followed by repetitive treatment with a noncarcinogenic tumor promoter such as 12-O-tetradecanoylphorbol-13-acetate (TPA), referred to as the promotion stage.

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Retinoids comprise a class of chemical compounds that includes active metabolites of vitamin A (retinol), as well as a diverse array of synthetic derivatives. Retinoids modulate several cellular processes, including proliferation, differentiation, homeostasis, malignant transformation, and apoptosis (6). Retinoids also act pharmacologically to restore the regulation of differentiation and growth in certain premalignant and malignant cells *in vitro* and *in vivo*. Consequently, retinoids are being studied extensively for their potential as therapeutic and chemopreventive agents for a variety of cancers including skin SCC (7). It is now well established that retinoids exert their effects primarily through nuclear retinoid receptor proteins. Retinoid receptors comprise two families of ligand-dependent, DNA binding, transcriptional transactivators, retinoic acid receptors and retinoid X receptors, both members of the steroid hormone receptor superfamily (6, 8). All-trans retinoic acid (ATRA) has long been known as one of the most effective suppressors of tumor formation in the two-stage skin carcinogenesis model (9, 10). Recent studies using mice genetically engineered for keratinocyte-specific knockout of multiple nuclear receptors, indicates that it is the retinoid X receptor- α isoform, when heterodimerized with another nuclear receptor family member, peroxisome proliferator-activated receptor- γ , that mediates tumor suppression in the two-stage model (11).

Signal transducers and activators of transcription (Stat) proteins, a family of latent cytoplasmic transcription factors, are expressed in many cell types and, in response to a wide variety of extracellular signals, regulate the transcription of a broad spectrum of genes that are critically involved in normal physiologic processes including cytokine signaling (12), cell proliferation and development (13), and abnormal tumorigenesis (14–18). Among the seven known members of mammalian Stat family, Stat3 has been the most strongly implicated in tumorigenesis (14–18). Elevated levels of Stat3 activity have been observed in a number of human cancers and cancer cell lines (15). Stat3 is constitutively activated in head and neck SCC (19), breast cancer cell lines (20, 21), ovarian cancer cell lines (19), and lung cancer cell lines (22). In particular, Stat3 plays a critical role in the development of skin cancer (23). In the two-stage model, in collaboration with the DiGiovanni laboratory, we show that Stat3 is constitutively activated in TPA-treated skin and tumors (24), and in studies using temporally controlled skin-specific knockout of Stat3, that activated Stat3 is indispensable for both the initiation and the promotion stages of epithelial carcinogenesis (refs. 25, 26; see also refs. 27, 28 for reviews). The critical role of Stat3 in skin tumor development was further supported by data obtained from the K5-Stat3C transgenic mouse model in which we have expressed the Stat3C protein in skin under the control of the cytokeratin-5 promoter (29). Stat3C is a constitutively active mutant of Stat3 that dimerizes through the formation of covalent disulfide linkages between cysteines instead of phosphotyrosines (14). These mice have a skin phenotype closely resembling psoriasis in humans and, when subjected to the two-stage protocol, rapidly developed carcinomas, bypassing the papilloma stage that is normally observed in this model (29, 30).

In the present study, we have for the first time determined the effect of ATRA treatment on Stat3 activity in the two-stage model and tested its ability to block TPA-induced tumor formation in the K5.Stat3C mice. We found that whereas ATRA could block Stat3 phosphorylation in both SENCAR and K5.

Stat3C mice, it could not suppress tumor formation in the K5.Stat3C mice.

Materials and Methods

Two-stage mouse skin chemical carcinogenesis protocol

Young female outbred SENCAR mice were obtained from the National Cancer Institute (Frederick, MD) and were housed in a temperature-controlled and humidity-controlled Association for Assessment and Accreditation of Laboratory Animal Care International facility with a 12 h light/dark cycle. K5.Stat3C mice were generated and characterized previously (29). All procedures were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee in accordance with NIH guidelines. Mice were maintained on AIN-76A diet (Dyets), and allowed access to food and water *ad libitum*. Mice were treated as follows: for SENCAR mice, groups of five mice per treatment were shaved on the dorsal side and 2 d later initiated with 2.56 μ g (10 nmol/L) of DMBA in 200 μ L of high-performance liquid chromatography-grade acetone, followed by twice weekly applications of 1 μ g (1.62 nmol/L) of TPA in 200 μ L of acetone, or TPA plus 5 μ g of ATRA (16.67 nmol/L) in 200 μ L of acetone, ATRA alone, or acetone solvent only for 30 wk. The K5.Stat3C mice were treated the same as the SENCAR mice except that initiation was with 6.4 μ g of DMBA (25 nmol/L) and promotion was with 2.1 μ g of TPA (3.4 nmol/L) per application, as previously described (31). Tumor incidence (number of mice bearing tumors/total number of mice in treatment group) and the tumor multiplicity (average number of tumors per mouse) were recorded weekly until the 30-wk time point. ATRA, DMBA, and TPA were obtained from Sigma Chemical, Co.

Immunohistochemical examination of mouse skin

Dorsal skin from mice in each of the treatment groups was isolated 6 h after the last TPA treatment and fixed in formalin and embedded in paraffin prior to sectioning. Paraffin sections of 4 μ m were deparaffinized thrice in xylene for 7 min at room temperature, and rehydrated by stepwise washes in a decreasing ethanol/H₂O ratio (100% to 50%, followed by soaking in water). Sections were cut and stained with H&E. For immunohistochemical staining, sections were incubated in Superblock (Pierce Biotechnology, Inc.) blocking reagent for 1 h at room temperature to block nonspecific antigen sites. After washing thrice in PBS, slides were incubated overnight at 4°C with antibodies against Tyr⁷⁰⁵ and Ser⁷²⁷ phosphorylated forms of Stat3 (pStat3-Tyr⁷⁰⁵ and pStat3-Ser⁷²⁷), total Stat3 (Stat3-T), cyclin D1, proliferating cell nuclear antigen (PCNA), survivin, phosphorylated B-Raf (pB-Raf-Ser⁴⁴⁵), phosphorylated Mek1/2 (pMek1/2-Ser^{217/221}), and phosphorylated Erk1/2 (pErk1/2-Thr²⁰²/Tyr²⁰⁴; Cell Signaling Technologies, Inc.). Slides were photographed on a Nikon TE300 fluorescence microscope under oil immersion at 600 \times magnification with a CCD camera (Roper Scientific). Images were processed with IPLabs v3.55 software (Scanalytics, Inc.).

Western blotting

Mouse epidermis was scraped from isolated dorsal skin 6 h after the last TPA treatment and homogenized on ice with a Polytron for three 10-s bursts in radioimmunoprecipitation assay lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate] supplemented with 40 μ L of Complete protease inhibitor cocktail (Roche) according to manufacturer-provided instructions. Extracted protein was quantified using the Bio-Rad Protein Assay kit. Proteins were separated by SDS acrylamide gel electrophoresis (12%) and transferred to nitrocellulose membranes (Schleicher&Schuell). Blots were blocked with 5% bovine serum albumin for 1 h at room temperature, followed by incubation overnight

with the same antibodies as above against phosphorylated forms of Stat3 (Tyr⁷⁰⁵ and Ser⁷²⁷), total Stat3, cyclin D1, PCNA, phosphorylated Erk1/2 (Thr²⁰²/Tyr²⁰⁴), as well as mouse β -actin (all from Cell Signaling Technology, except for β -actin which is from Santa Cruz Biotechnology). Blots were washed with TBS/0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, followed by three additional washes with TBS/0.1% Tween 20. Chemiluminescence detection was done according to the instructions of the manufacturer (Pierce), followed by exposure to X-ray film.

Surgical specimens

Surgical specimens of aggressive skin SCC came from the Department of Otolaryngology, Head and Neck Surgery at Louisiana State University Health Sciences Center-Shreveport. Seven specimens from seven patients were selected to include tumor and adjacent nonmalignant tissue. The specimens were processed by routine fixation in 10% buffered formalin and embedded in paraffin. All specimens were cut to 5 μ mol/L sections and mounted on lysine-coated slides.

Results

ATRA suppresses Stat3 phosphorylation in mouse skin during the two-stage carcinogenesis protocol

We have previously shown that ATRA suppressed TPA-induced hyperproliferation in the skin of SENCAR mice at an early time point (3 weeks) in the two-stage protocol (32). In the present study, paraffin sections of mouse skin isolated 6 hours after a single application, or 6 hours after the final twice-weekly application at 30 weeks of TPA, ATRA, or their combination, were probed with an antibody to phos-

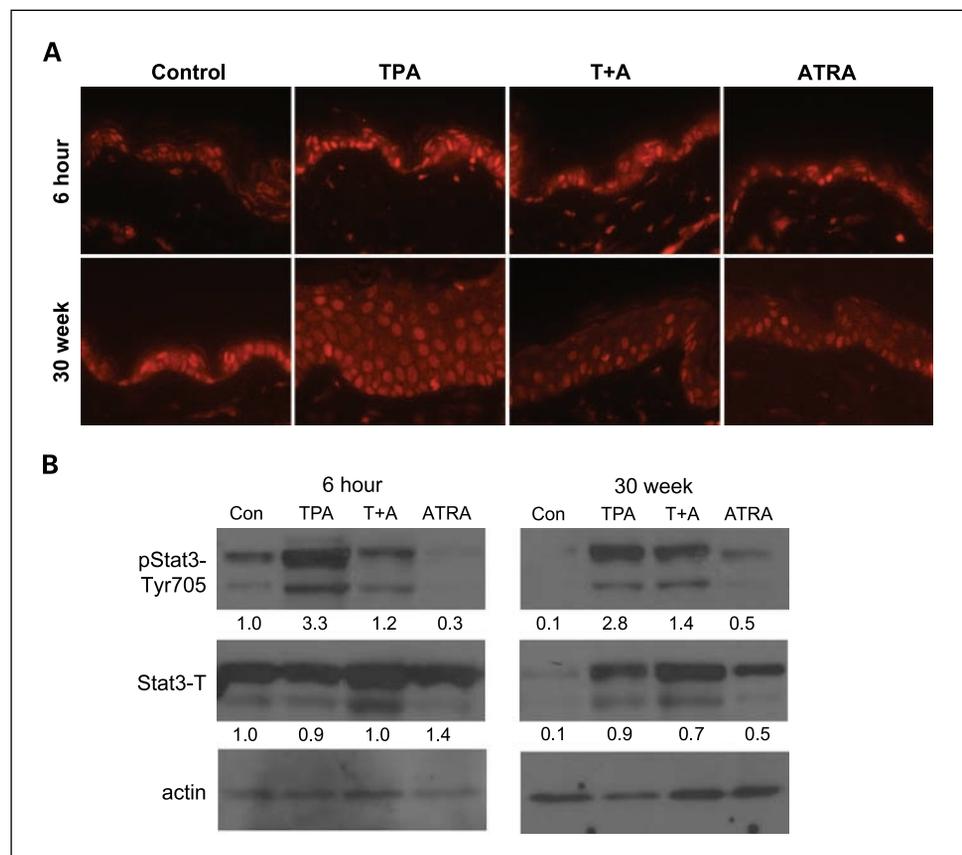
photyrosine 705 of Stat3 (pStat3-Tyr⁷⁰⁵; Fig. 1A). ATRA coadministration with TPA suppressed TPA-induced Tyr⁷⁰⁵ phosphorylation of Stat3 at both time points. This effect was confirmed by Western blotting of scraped epidermal cell protein lysates from pooled mouse skin ($n = 5$) at the same time points (Fig. 1B). At 30 weeks, the suppression was less pronounced than at 6 hours (Fig. 1B, numbers below panels indicate relative expression, normalized to β -actin). Interestingly, the total amount of Stat3 protein, as detected by an antibody to total Stat3 (Stat3-T), was reduced in the skin of control mice at 30 weeks compared with the 6-hour controls (Fig. 1B, Stat3-T).

There was also an incomplete suppression of TPA-induced hyperproliferation by 30 weeks of treatment (Fig. 1A, *bottom*, compare TPA with T+A). None of the ATRA control mice developed tumors, but these mice did have a low level of hyperproliferation (Fig. 1A, 30 week ATRA panel).

Constitutive activation of Stat3 in skin of K5.Stat3C mice overcomes ATRA suppression of tumor induction by TPA

Recently, the DiGiovanni laboratory, in collaboration with our laboratory, has generated transgenic mice expressing the constitutively active Stat3C protein under the control of the cytokeratin-5 promoter (29). K5.Stat3C mice express high levels of Stat3C in the skin and develop skin lesions closely resembling psoriasis. When subjected to the two-stage protocol, these mice develop carcinomas, which seem to bypass the papilloma stage that is normally observed in this model

Fig. 1. A, immunohistochemical staining of SENCAR mouse skin from the two-stage skin carcinogenesis model. Paraffin sections of SENCAR mouse skin treated with acetone (*Control*), TPA, TPA +ATRA (*T+A*), and ATRA alone were probed with a Stat3 phosphorylated Tyr⁷⁰⁵ antibody (pStat3-Tyr⁷⁰⁵) followed by an Alexa546-labeled secondary antibody. Magnification, $\times 600$. B, Western blot analysis of SENCAR mouse skin from the two-stage skin carcinogenesis model. Epidermal protein lysates of scraped SENCAR mouse skin treated with acetone (*Con*), TPA, TPA+ATRA (*T+A*), and ATRA alone were run on a 10% SDS-polyacrylamide gel and probed with pStat3-Tyr⁷⁰⁵, Stat3-T, and β -actin antibodies. Numbers (*bottom*) indicate scanned density values divided by the β -actin band intensity for each sample, and then normalized to the 6-h control band intensity, which was assigned a relative value of 1. Pooled epidermal lysates were run ($n = 5$), such that the intensities represent an average for five mice.



(29, 30). We have decided to test the tumor-suppressive effect of ATRA for the two-stage protocol in the K5.Stat3C mice. As observed previously (30), there were abundant carcinomas in the TPA-treated mice. Somewhat unexpectedly, we observed that ATRA treatment did not suppress tumor formation, but rather correlated with increased tumor incidence. We also observed other lesions resembling papillomas as well as bumps with a wart-like appearance (Supplementary Fig. S1A). The average incidence of all lesions for TPA-treated and TPA+ATRA-treated K5.Stat3C mice was 5.2 and 11.4, respectively, after 25 weeks (Supplementary Fig. S1B). We also note that the K5.Stat3C mice had a thickened psoriatic area of skin starting at the base of the tail and extending anteriorly as far as halfway along the length of the back (Supplementary Fig. S2A, *top*). The occurrence of all types of lesions was similar in both the psoriatic and nonpsoriatic fields. In order to determine whether ATRA altered the phenotype of the tumors, all lesions from TPA-treated and TPA+ATRA-treated mice were harvested 6 hours after the last TPA treatment and were subjected to histopathologic analysis. Lesions were ranked as either having a preinvasive epithelial proliferative phenotype or as invasive SCC. There were 66% (55 out of 83 lesions scored) and 71% (43 out of 60 lesions scored) invasive SCCs among the TPA-treated and TPA+ATRA-treated lesions, respectively, indicating that ATRA did not markedly alter tumor phenotype. Interestingly, two out of five K5.Stat3C mice treated with DMBA followed by ATRA treatment alone had multiple lesions (9 and 15, for an average of 4.8 tumors per mouse in a group of five mice) that included SCCs. This indicates that ATRA could act as a tumor promoter in the context of high levels of Stat3C expression.

We also note that the K5.Stat3C mice were generated on an FVB background. The wild-type littermates in this two-stage experiment did not develop tumors in the 25-week time frame (with one exception) using these DMBA and TPA concentrations (data not shown).

ATRA suppresses Stat3 signaling in K5.Stat3C mice

We next determined whether ATRA could suppress TPA-induced Tyr⁷⁰⁵ phosphorylation of Stat3 in K5.Stat3C mouse tumors and skin, as was observed above in SENCAR mice. Somewhat unexpectedly, ATRA suppressed TPA-induced Tyr⁷⁰⁵ phosphorylation in papillomas and carcinomas from K5.Stat3C mice (Fig. 2A). This suppression was also observed in wild-type skin and K5.Stat3C normal-appearing and psoriatic skin after 30 weeks of treatment (Supplementary Fig. S2A, compare TPA with TPA+ATRA). It has been shown that phosphorylation of Stat3 at Ser⁷²⁷ could also positively modulate Stat3 activity (33–35). We therefore tested the effect of TPA and TPA+ATRA treatments on Ser⁷²⁷ phosphorylation using an antibody to phosphorylated Ser⁷²⁷ of Stat3 (pStat3-Ser⁷²⁷). Stat3 Ser⁷²⁷ phosphorylation was also suppressed by ATRA in K5.Stat3C tumors and in wild-type and K5.Stat3C skin (Fig. 2B; Supplementary Fig. S2B). We note that the basal levels of Tyr⁷⁰⁵ and Ser⁷²⁷ phosphorylation in untreated K5.Stat3C mouse skin were also higher than in wild-type mice, suggesting that expression of Stat3C could have an effect on Stat3 phosphorylation at these sites (Supplementary Fig. S2A and B, control). Interestingly, expression of total Stat3 (Stat3-T) was high in the papillomas and carcinomas of K5.Stat3C mice, and this was suppressed by ATRA (Fig. 2C). This suppression

of total Stat3 expression by ATRA was also observed in the psoriatic skin of K5.Stat3C mice (Supplementary Fig. S2C, compare columns labeled “normal app” and “psoriatic”).

In order to further verify the effects of TPA and ATRA on Stat3 activity, we determined the expression of cyclin D1 and survivin, two known transcriptional targets of Stat3 that regulate proliferation and cell survival, respectively (36, 37). Cotreatment with ATRA and TPA resulted in lower expression levels of both of these proteins compared with TPA treatment alone in both tumors and skin, and a similar result was also observed for another proliferation marker, PCNA (Fig. 2D-F; Supplementary Fig. S3A-C).

Western blotting of scraped epidermal cell protein lysates from wild-type and K5.Stat3C mice, using the same antibodies as for immunohistochemistry, confirmed the relative levels of expression of pStat3-Tyr⁷⁰⁵, pStat3-Ser⁷²⁷, Stat3-T, cyclin D1, and PCNA for TPA-treated and TPA+ATRA-treated skin (Fig. 3).

Stat3 signaling and Raf/Mek/Erk pathway interaction

We have previously shown that the B-Raf/Mek/Erk signaling pathway is activated in SENCAR mouse skin in response to TPA treatment, and that this activation is suppressed by coadministration of ATRA (32, 38). It has also been previously shown that Erk1/2 can directly phosphorylate Stat3 at Ser⁷²⁷ (39), raising the possibility that at least part of the effect of ATRA on Stat3 activity could be mediated through its suppression of the B-Raf/Mek/Erk signaling pathway. In order to assess the activity of this pathway in the K5.Stat3C mice and their nontransgenic littermates, we stained paraffin sections of control, TPA-treated, and TPA+ATRA-treated skin similar to Supplementary Fig. S2, with antibodies to phosphorylated forms of B-Raf, Mek1/2, and Erk1/2. In agreement with our previous findings in SENCAR mice, TPA induced the phosphorylation of B-Raf, Mek1/2, and Erk1/2 in both wild-type and K5.Stat3C skin and this effect was blocked by coadministration of ATRA (Fig. 4A, B and C, TPA and TPA+ATRA). The suppression of phosphorylated Erk1/2 by ATRA was confirmed by Western blotting of scraped epidermal cell protein lysates, using the same pERK1/2-Thr²⁰²/Tyr²⁰⁴ antibody (Fig. 3). We note that levels of pB-Raf-Ser⁴⁴⁵ and pErk1/2 staining were also higher in control psoriatic skin of K5.Stat3C mice than in the control normal-appearing skin. This could be related to the higher proliferation index in the psoriatic skin. The relationship between B-Raf/Mek/Erk signaling and the psoriatic phenotype will require further study.

pStat3-Tyr⁷⁰⁵ can be detected in the cell membrane and nucleus in human skin SCC and adjacent hyperplastic skin

In order to determine whether our observations of Stat3 expression in mouse skin have relevance to human skin SCC, we probed serial paraffin sections of human skin SCC tumor samples ($n = 7$), which include nonmalignant margins, with the same pStat3-Tyr⁷⁰⁵ antibody used above. We observed a range of staining intensities and patterns, which included prominent nuclear staining, as expected, as well as membrane staining (Fig. 5, *bottom*). Interestingly, intense nuclear staining in basal and suprabasal cells was often observed in the nonmalignant skin margins, indicating high Stat3 activity there (Fig. 5, *bottom right*). The membrane staining was not observed in all samples. These findings are consistent with an earlier study from

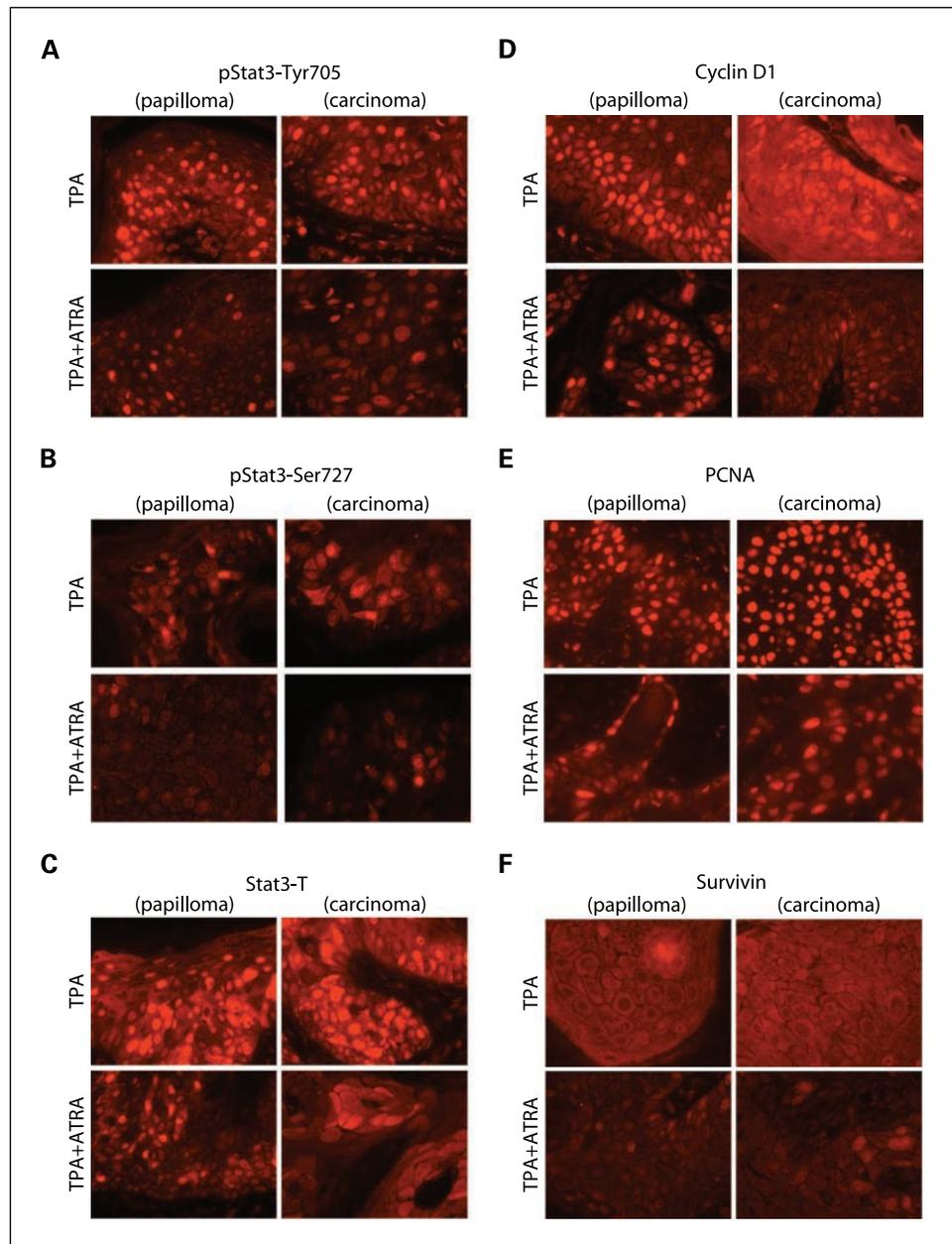


Fig. 2. Immunohistochemical staining of K5.Stat3C-derived skin tumors. Serial sections of papillomas (*left*) and carcinomas (*right*) isolated from K5.Stat3C skin treated with TPA or TPA+ATRA, were probed with antibodies to pStat3-Tyr⁷⁰⁵ (A), pStat3-Ser⁷²⁷ (B), Stat3-T (C), cyclin D1 (D), PCNA (E), and survivin (F) as in Fig. 1. Magnification, $\times 600$.

our laboratory in which we observed strong staining for total Stat3 α and Stat3 β in skin and skin SCCs (40).

Discussion

ATRA and other retinoids have been studied in the clinic as therapeutic and chemopreventive agents for a variety of cancers, including skin cancer (7, 41–43). In spite of the tremendous promise for retinoids in therapy and prevention of cancer, clinical results have often been disappointing due to hypervitaminosis A-related side effects, leading to the discontinuation of treatment. Our group has attempted to understand the chemopreventive mechanism of ATRA in order to more efficiently use retinoids in the clinic, either alone or in combination with other drugs.

We have previously used the mouse skin two-stage chemical carcinogenesis protocol to test the ability of ATRA, along with

other clinically important retinoids, to suppress skin tumor formation (44). In the two-stage protocol, SENCAR mice developed benign papillomas beginning at ~ 9 weeks of TPA treatment, which convert to malignant SCCs at a very low frequency after 30 weeks of TPA treatment. This process is completely blocked by topical administration of ATRA (10). We have also shown that ATRA suppression of TPA-induced hyperproliferation is lost after the first few weeks of treatment, even though tumor suppression is maintained (32). In the present study, we extend this observation to show that ATRA suppression of pStat3-Tyr⁷⁰⁵ is also greatly reduced at the 30-week time point (Fig. 1B), indicating a positive correlation between pStat3-Tyr⁷⁰⁵ levels and hyperproliferation. Thus, ATRA can still suppress tumor formation in hyperproliferative skin in which pStat3-Tyr⁷⁰⁵ levels are high, suggesting that other ATRA effects in addition to Stat3 inhibition are important for its tumor-suppressive activity (see Fig. 6 for proposed model).

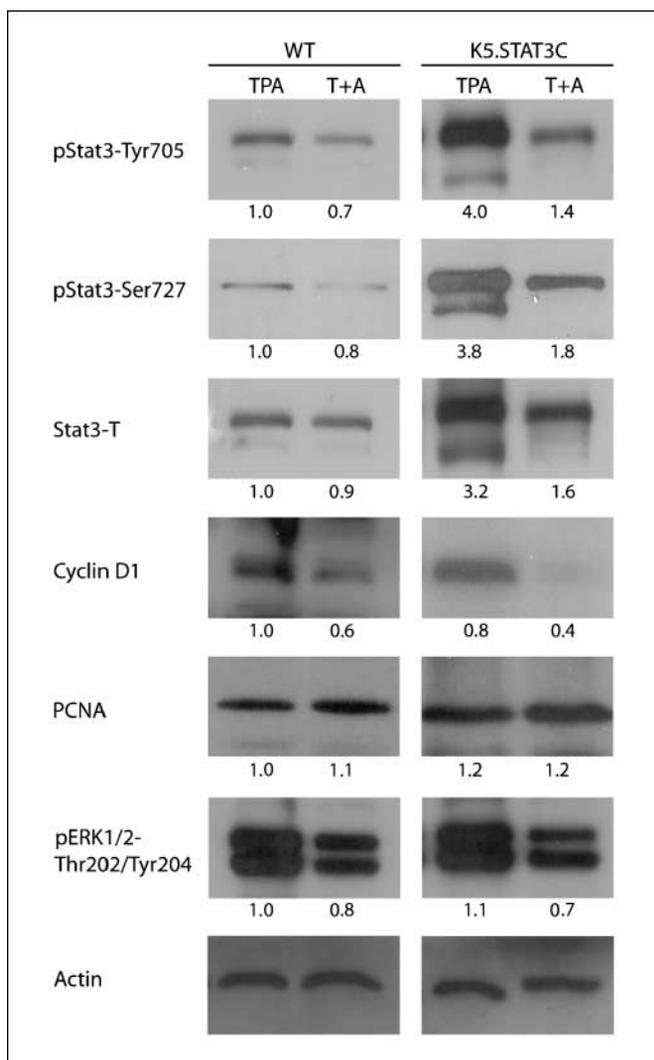


Fig. 3. Western blot analysis of TPA-treated and T+A-treated wild-type and K5.Stat3C mouse skin. Epidermal protein lysates from scraped wild-type and K5.Stat3C mouse skin were subjected to SDS-PAGE and Western blotting as in Fig. 1A. Blots were probed with the same pStat3-Tyr⁷⁰⁵, pStat3-Ser⁷²⁷, Stat3-T, cyclin D1, PCNA, and pERK-202/204 antibodies that were used for immunohistochemistry. Numbers (*bottom*) indicate scanned density values divided by the β -actin band intensity for each sample, and then normalized to the wild-type TPA band intensity, which was assigned a relative value of 1. Representative of at least three different sets of epidermal lysate samples.

Unlike SENCAR mice, the K5.Stat3C mice subjected to the two-stage protocol begin to develop invasive SCCs in as early as 4 weeks of TPA treatment, with lesions bypassing the papilloma stage (30). We hypothesized that if ATRA intervenes in steps downstream of Stat3 activity in blocking the carcinogenic process, it would suppress SCC formation in the K5.Stat3C mice subjected to the two-stage protocol. However, if ATRA instead acts on targets upstream of Stat3 in the process, it should not be able to block SCC formation in this model. Our finding that ATRA does not block TPA-induced tumor formation in the K5.Stat3C mice (Supplementary Fig. S1) indicates that the latter case is the most likely. A strong candidate for an upstream activator of Stat3 is the B-Raf/Mek/Erk pathway. We have previously shown that this pathway is activated in SENCAR mouse skin in the two-stage model, and that this activation is sup-

pressed by ATRA, and in the present study, we observed a similar result for the K5.Stat3C mice (Figs. 3 and 4). Interestingly, Erk1/2 kinase, the distal kinase in the pathway, has been shown to phosphorylate Stat3 at Ser⁷²⁷ (39). This has led to an extension of our original hypothesis whereby we suggest that ATRA exerts its tumor-suppressive effect, at least in part, through repression of B-Raf/Mek/Erk signaling, resulting in subsequent reduction of Stat3 activity. This repression is likely to involve receptor tyrosine kinases such as the epidermal growth factor receptor, that can activate both the B-Raf/Mek/Erk pathway and can directly activate Stat3 (Fig. 6). In fact, ATRA has been shown to suppress epidermal growth factor receptor expression in human head and neck SCC cells and cultured human trophoblast cells (45, 46), and retinoid suppression of epidermal growth factor receptor-associated cell proliferation in HPV-immortalized ectocervical epithelial cells was shown to correlate with suppression of Erk1/2 activity (47).

A model in which the B-Raf/Mek/Erk pathway is one of the upstream activators of Stat3 is further supported by the following: (*a*) although there is some increased phosphorylated B-Raf and phosphorylated Erk1/2 in the psoriatic skin of K5.Stat3C mice compared with wild-type mice, we do not observe strong constitutive activation of the B-Raf/Mek/Erk pathway elsewhere in the skin of these mice, in which Stat3 is constitutively active (Fig. 4B and C); and (*b*) ATRA blocks B-Raf/Mek/Erk phosphorylation effectively in K5.Stat3C mice without suppressing tumor formation. This suggests that activities downstream of the B-Raf/Mek/Erk pathway, such as Stat3 activity, are sufficient to promote tumor development (Fig. 6). Current experiments are aimed at exploring the relationship between epidermal growth factor receptor, B-Raf/Mek/Erk signaling, and Stat3 signaling.

The suppression of TPA-induced phosphorylation of Tyr⁷⁰⁵ and Ser⁷²⁷ by ATRA in K5.Stat3C mice is unexpected because this would be predicted to correlate with a reduction in tumor size or number, and/or to increased latency to tumor formation, as was observed for SENCAR mice. Instead, there was a greater number of tumors in the TPA-treated K5.Stat3C mice that were coadministered ATRA. This and the finding that two out of five K5.Stat3C mice that received DMBA followed by ATRA treatment alone developed tumors, indicates a possible tumor-promoting activity of ATRA in the context of constitutively active Stat3 (Supplementary Fig. S1B). We note that in the K5.Stat3C wild-type littermates and SENCAR mice, in which only the native Stat3 exists, prolonged topical treatment of DMBA-initiated skin with ATRA does not produce tumors (32, 35). Although the preponderance of data indicates that ATRA suppresses tumor promotion, it has long been observed that topical application of ATRA to the skin stimulates basal keratinocyte proliferation, resulting in epidermal hyperplasia (45). Thus, the pro-proliferative action of ATRA, in the presence of the abnormal, constitutively active Stat3C variant, seems to contribute to tumor formation. This has the potential clinical implication that ATRA may be contraindicated for the treatment of skin tumors, and possibly other tumors, that have high Stat3 activity.

It is also likely that sufficient levels of active Stat3 remain in the TPA+ATRA-treated skin to mediate the tumorigenic effects of TPA. Immunohistochemical staining indicates higher levels of Tyr⁷⁰⁵ and Ser⁷²⁷ phosphorylation in the TPA+ATRA-treated K5.Stat3C skin than in untreated controls (Supplementary Fig. S2A and B, compare TPA+ATRA with Controls). We also

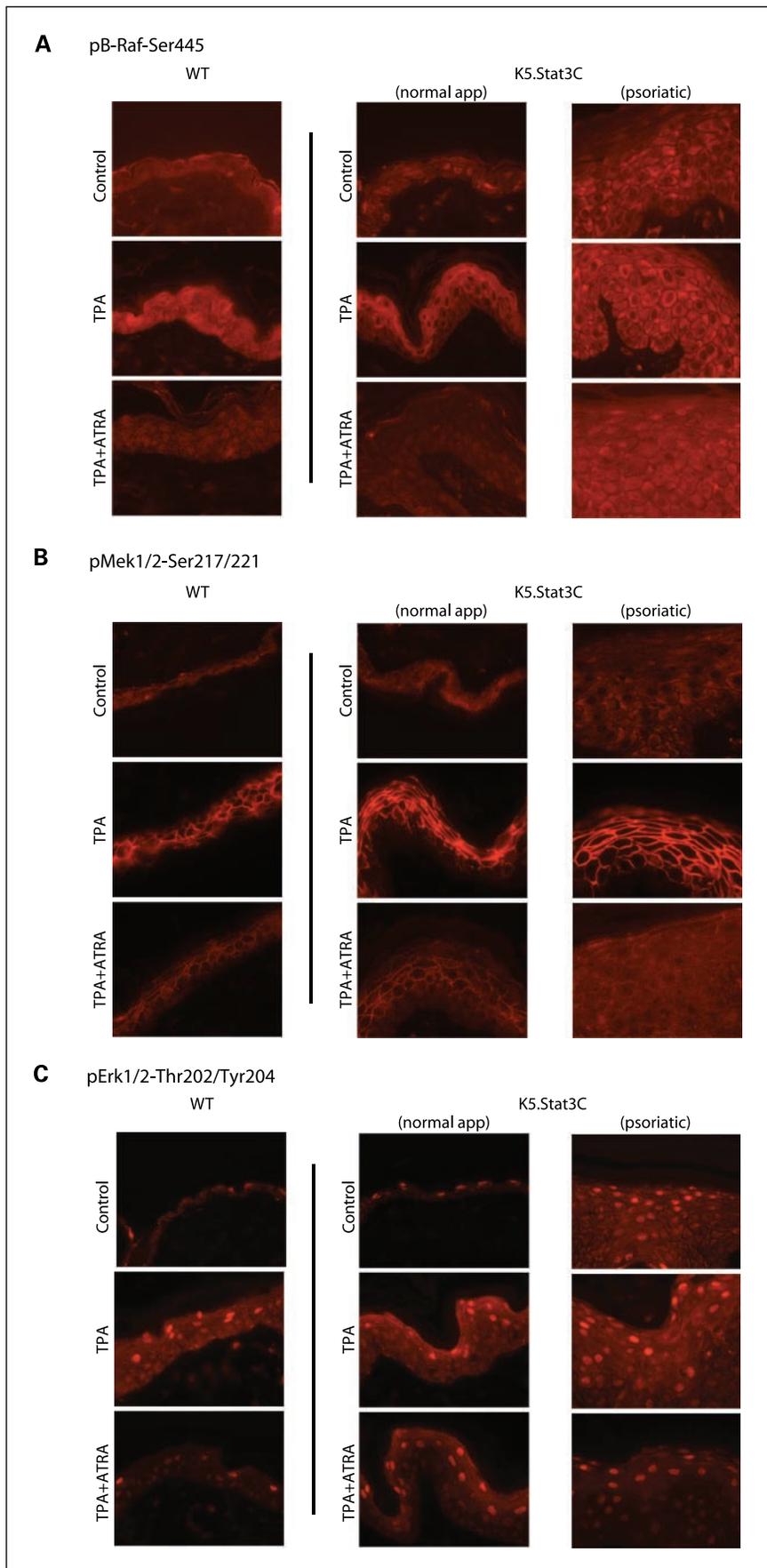


Fig. 4. Immunohistochemical staining of K5.Stat3C transgenic and wild-type mouse skin treated with acetone (*Control*), TPA, or TPA+ATRA. Immunohistochemistry was done on serial sections of the same tissue as in Fig. 2. Slides were probed with antibodies to pB-Raf-Ser⁴⁴⁵ (A), pMek1/2-Ser^{217/221} (B), and pErk1/2-Thr²⁰²/Tyr²⁰⁴ (C). Magnification, $\times 600$.

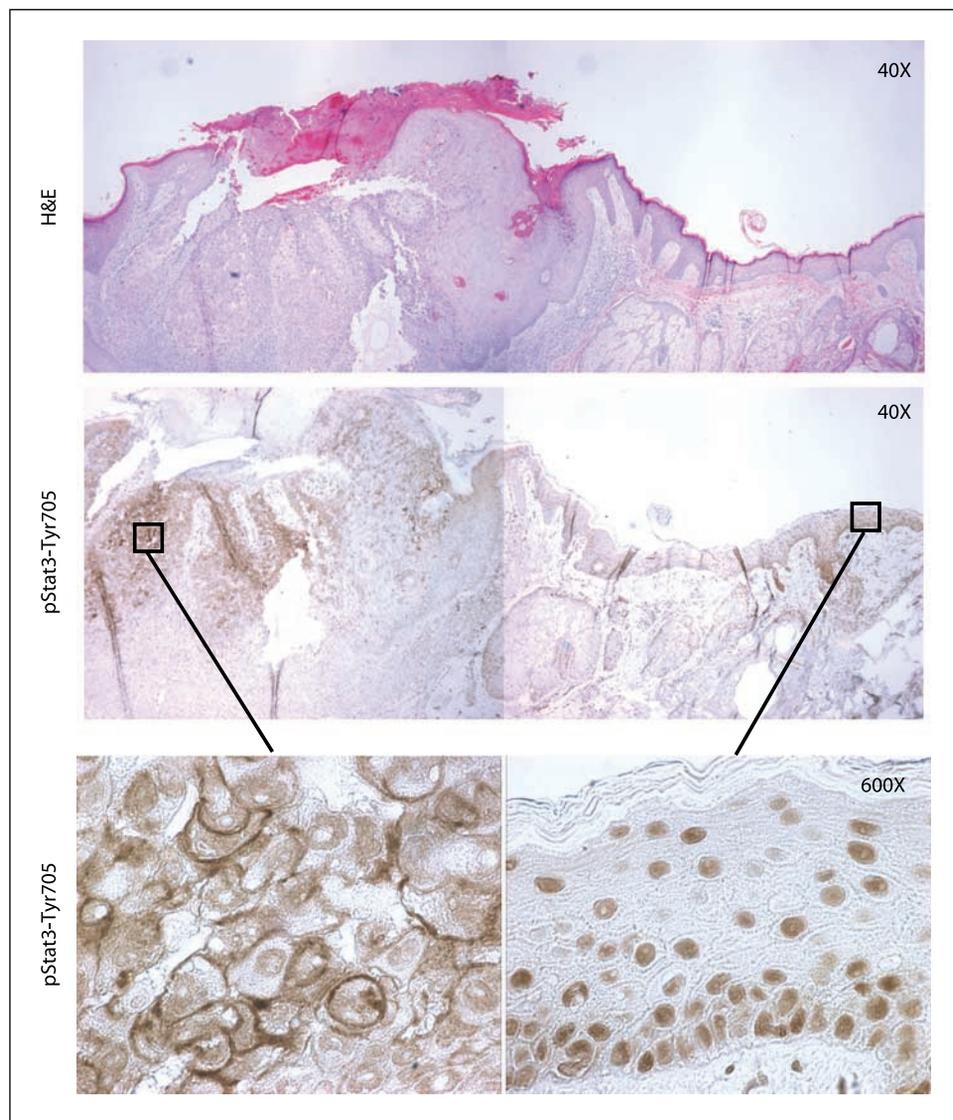


Fig. 5. pStat3-Tyr⁷⁰⁵ immunohistochemical staining of human skin SCC sections. Paraffin sections of human SCC with adjacent nonmalignant skin were either stained with H&E or probed with a phosphospecific pStat3-Tyr⁷⁰⁵, followed by a biotin-labeled secondary antibody. Magnification, $\times 40$ (top and middle); $\times 600$ (bottom).

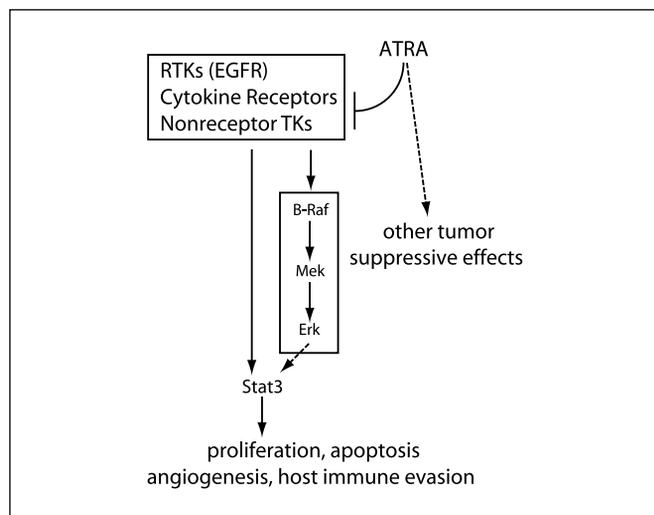


Fig. 6. Hypothesis for mechanism of ATRA suppression of Stat3 signaling. RTK, receptor tyrosine kinase; solid lines, established interactions; dotted lines, hypothetical ones.

observed higher levels of total Stat3 in TPA-treated K5.Stat3C skin, and in the psoriatic skin, even in the absence of TPA treatment (Supplementary Fig. S2C). Total Stat3 levels were reduced by ATRA treatment in the K5.Stat3C mice, indicating that some or all of the ATRA suppression of Tyr⁷⁰⁵ and Ser⁷²⁷ phosphorylation could be due to this reduction. Whether ATRA has a direct effect on Stat3 Tyr⁷⁰⁵ or Ser⁷²⁷ phosphorylation will require further study. To date, there is little direct evidence for an interaction between ATRA and Stat3 signaling pathways in cancer, except in acute promyelocytic leukemia, in which it has been shown that several oncogenic fusion proteins containing retinoic acid receptor- α could augment Stat3 transcriptional activity, and that treatment of acute promyelocytic leukemia cells with ATRA could block this augmentation (49). However, this block does not involve a suppression of Stat3 phosphorylation but rather is mediated by an inhibitory complex between the retinoic acid receptor- α fusion partner and Stat3.

We show, what is to our knowledge, the first example of the suppression of Stat3 activity, as measured by pStat3-Tyr⁷⁰⁵ levels, by ATRA *in vivo*, using the two-stage skin carcinogenesis model. Although this finding is striking, the suppression is not

sustained at the 30-week time point (Fig. 1), indicating that Stat3 inhibition alone is not sufficient for tumor suppression by ATRA. However, in the presence of a constitutively active form of Stat3, ATRA treatment proved insufficient to block TPA-induced tumorigenesis, indicating a dominant role for Stat3 in conferring the malignant phenotype. Continued study of the function of Stat3 during skin carcinogenesis, and the mechanism of action of ATRA and other retinoids in the suppression of skin carcinogenesis, is supported by these findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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