

# 12-*O*-Tetradecanoylphorbol-13-acetate Induces Clonal Expansion of Potentially Malignant Keratinocytes in a Tissue Model of Early Neoplastic Progression<sup>1</sup>

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## ABSTRACT

Tumor promoters stimulate the selective expansion of initiated mouse keratinocytes in the two-stage model of skin carcinogenesis. However, it is not clear whether these promoters directly modulate the growth of initiated cells or rather permit clonal expansion of initiated cells by modifying the environment of adjacent normal cells. The goal of this study was to further understand the mechanism of action of tumor promotion during early neoplastic progression of human stratified epithelium. To accomplish this, we have established an organotypic culture model that mimics a preneoplastic tissue and contains mixtures of genetically marked ( $\beta$ -galactosidase), low-grade malignant keratinocytes (HaCaT-ras II-4) and normal human keratinocytes (NHKs) to monitor the fate and phenotype of these cells after treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In submerged culture, concentrations of 0.001–1  $\mu$ g/ml TPA were shown to limit the growth of NHKs yet had no effect on growth of II-4 cells. TPA (0.001  $\mu$ g/ml) was then added to organotypic cultures containing mixtures of NHK:II-4 cells at varying ratios to determine whether this agent could selectively stimulate clonal expansion of II-4 cells in a normal epidermal background. Immunofluorescence for  $\beta$ -galactosidase demonstrated that TPA caused a significant increase in the percentage of  $\beta$ -galactosidase-positive areas in 12:1 and 4:1 mixtures. This TPA-induced expansion of II-4 cells was associated with a marked decrease in proliferation of NHKs, suggesting that II-4 could selectively expand because of its growth advantage relative to NHKs. Clonal expansion of tumor cells was temporally linked to the decreased expression of filaggrin and keratin 1 expression in adjacent NHKs. These findings indicate that TPA may enable expansion of potentially malignant cells through the epigenetic modification of proliferation in NHKs and differentiation of NHK and II-4 cells.

## INTRODUCTION

The two-stage model of mouse skin carcinogenesis describes a two-step process of cell transformation requiring both initiation and promotion of murine keratinocytes (1, 2). Initiation is achieved by application of a single, subthreshold dose of a carcinogen causing an irreversible genetic change in the cellular genome, such as activation of the *ras* oncogene. Promoters are thought to induce tumor development in previously initiated cells by causing their selective expansion, but the mechanism of such clonal expansion remains to be determined (3). Moreover, it remains unclear whether tumor promotion would play a role in human skin carcinogenesis in light of the finding that TPA has not exhibited growth-stimulatory activity on human keratinocytes *in vitro* (4, 5).

TPA<sup>3</sup> is a phorbol ester tumor promoter that is thought to prefer-

entially stimulate proliferation of distinct subpopulations of mouse keratinocytes, leading to clonal expansion of initiated cells (3). Yuspa *et al.* (6) have theorized that a critical element in promotion requires the escape of initiated cells from the negative growth regulation imposed by normal cells adjacent to them. There are two mechanisms through which this may occur. It is possible that tumor promoters act directly on previously initiated cells by stimulating their growth (7, 8). Alternatively, the tumor promoter may act directly on the normal cell population, thereby altering their capacity to suppress the growth and expansion of the initiated cell population adjacent to them.

Factors controlling the selective expansion of potentially malignant human cells during early neoplastic progression in human epidermis have not been well established because it has not been possible to directly study clonal expansion in a stratified epithelium with neoplastic potential. In this study, we used a tissue model in which the early events in neoplastic progression can be monitored (9) by genetically marking malignantly transformed keratinocytes (10, 11) and mixing them with normal keratinocytes in organotypic culture to follow the behavior and fate of these cells. Using this tissue model of premalignancy, it has been determined previously that normal cell context and tissue architecture limits early neoplastic progression in stratified epithelium (9). It appears that for early neoplastic progression to occur, these low-grade malignant cells need first to overcome a restrictive microenvironment of normal cell context, which limits their growth and clonal expansion.

The goal of this study was to determine whether a tumor promoter such as TPA plays a permissive role in enabling cells with neoplastic potential to selectively expand in the context of normal keratinocytes. We examined how this tumor promoter enables such expansion by determining the phenotype of both normal and potentially malignant cells after TPA exposure. We determined that TPA may enable expansion of potentially malignant cells through the epigenetic modification of proliferation and differentiation of normal cells adjacent to them.

## MATERIALS AND METHODS

**Cell Culture.** NHKs were cultured from newborn foreskin by the method of Rheinwald and Green (12) in keratinocyte medium described by Wu *et al.* (13). Cultures were established through trypsinization of foreskin fragments and grown on irradiated 3T3 fibroblasts. 3T3 cells were maintained in DMEM containing 10% bovine calf serum. The keratinocyte cell line HaCaT-ras (clone II-4) was grown in DMEM containing 5% FCS. Organotypic cultures were prepared by adapting the protocol developed by Organogenesis, Inc. (Canton, MA; Ref. 14). Briefly, early passage human dermal fibroblasts were added to neutralized type I collagen (Organogenesis, Canton, MA) to a final concentration of  $2.5 \times 10^4$  cells/ml. Three ml of this mixture were added to each 35-mm polycarbonate insert of a six-well plate and incubated for 4 to 6 days, until the collagen matrix showed no further shrinkage. At this time, a total of  $5 \times 10^5$  keratinocytes, II-4-gal cells, or mixtures of these two cell types were plated on the contracted collagen gel. Cultures were maintained submerged in low calcium EGM (Organogenesis, Canton, MA) for 2 days, submerged for 2

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<sup>3</sup> The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NHK, normal human keratinocyte; EGM, epidermal growth medium; BrdUrd, bromodeoxyuridine;

X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; FIL, filaggrin; K1, keratin 1; LI, labeling index;  $\beta$ -gal,  $\beta$ -galactosidase.

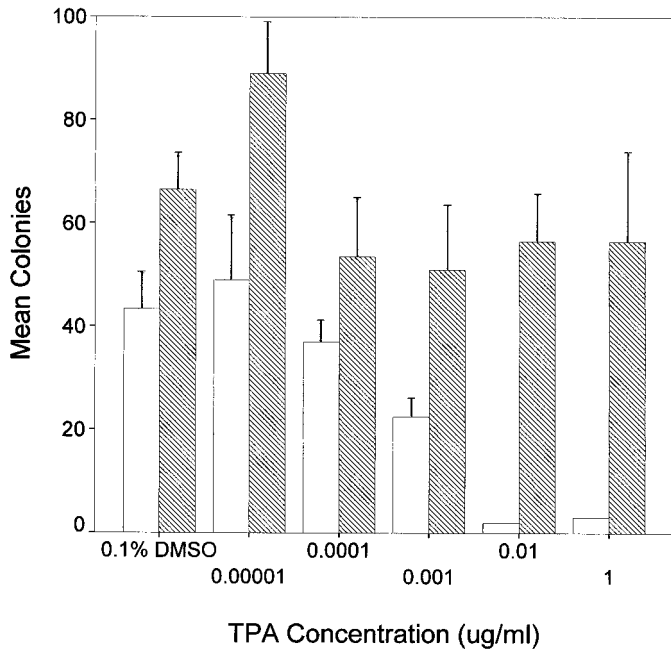


Fig. 1. The effect of TPA on the clonal growth of normal and II-4 human keratinocytes. One hundred NHKs (□) or II-4 keratinocytes (▨) were plated per 60-mm dish. After 1 day, cultures were fed with medium containing different concentrations of TPA prepared in 0.1% DMSO or with normal medium with 0.1% DMSO. NHK cultures were fixed and stained with rhodamine on day 15, whereas II-4 cultures were stained with X-gal. Colonies of five or more cells were counted. Results represent the mean of two determinations; bars, SD. The effect of TPA was found to be significant for NHK (ANOVA,  $F = 15.785$ ,  $P < 0.05$ ) and nonsignificant for II-4 keratinocytes (ANOVA,  $F = 1.287$ ,  $P > 0.05$ ).

days in normal calcium EGM, and raised to the air-liquid interface by feeding from below with normal calcium EGM for 3 days. Cultures were treated with TPA when raised to an air-liquid interface and were exposed for 8, 36, or 72 h. For proliferation assays, BrdUrd (Sigma Chemical Co., St. Louis, MO) was added to organotypic cultures at a final concentration of 10  $\mu$ M and incubated for 8 h before harvesting.

**Retroviral Vectors and Transduction of Keratinocytes.** The MFG-gal vector is a Moloney murine leukemia virus-based vector that contains the gene for bacterial  $\beta$ -gal (15). Transduction of II-4 cells with this vector was performed as described previously (16). Briefly, II-4 cells were transduced 24 h after plating  $1 \times 10^6$  cells in a 10-cm dish using fresh, filtered (0.45 unit; Gelman, Ann Arbor, MI) supernatant from confluent amphotropic producer cells making the MFG-gal vector. Transduced II-4 keratinocytes were passaged at clonal density, and clones were screened for persistence of transgene expression through three passages. Only clones maintaining transgene expression in 100% of cells were expanded and used for organotypic culture and grafting experiments. No deleterious effect on cell growth or phenotype were seen after transduction. Producer lines were maintained in 10% bovine calf serum.

**Clonal Growth of NHK and II-4 Keratinocytes in Response to TPA.** Human epidermal keratinocytes and II-4 cells were plated at clonal densities by plating 100 cells in p60 dishes. At 24 h or 7 days after plating, various concentrations of TPA (0.00001–1  $\mu$ g/ml) were dissolved in 0.1% DMSO and media containing 0.1% DMSO and were added to cultures. After 2 weeks, dishes were stained with X-gal (Sigma Chemical Co., St. Louis, MO) to detect the genetically marked II-4 colonies and rhodamine (Sigma Chemical Co.) to detect NHK. NHK colonies were easily distinguished by their pink color, which differed from the II-4, which appeared purple because of the combination of the pink (rhodamine) and blue (X-gal) stains seen only in these colonies. Colony size and number were determined for each cell type by counting colonies and measuring colony area.

**Immunofluorescence.** Specimens were frozen in embedding media (Triangle Biomedical, Durham, NC) in liquid nitrogen vapors after being placed in 2 M sucrose for 2 h at 4°C. Tissues were serial sectioned at 6  $\mu$ m and mounted onto gelatin-chrome alum-coated slides (Sigma). Tissue sections were washed

with PBS and blocked with 10 mg/ml goat IgG, 0.05% goat serum, and 0.2% BSA, v/v in PBS without fixation. Sections were incubated with rabbit polyclonal antiserum to bacterial  $\beta$ -gal (Cortex Pharmaceuticals, San Leandro, CA), which was detected with FITC-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Double-stain immunofluorescence was performed by double staining using either a monoclonal antibody to BrdUrd (Boehringer Mannheim, Indianapolis, IN) or to human FIL (Biosearch Technologies, San Rafael, CA) or to K1 (Biosearch Technologies), which were detected with Texas Red-conjugated horse anti-mouse IgG (Vector Laboratories). Slides were coverslipped with Vectashield containing 1  $\mu$ g/ml 4',6-diamidino-2-phenylindole (Vector Laboratories). Fluorescence was visualized using a Nikon OptiPhot microscope, and double exposure photomicroscopy was performed using FITC and Texas Red channels. For routine light microscopy, tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, and 4- $\mu$ m sections were stained with H&E. Analysis of immunostained slides was done using UTHSCA ImageTool (Windows, version 1.27), and the mean percentage of  $\beta$ -gal-positive area for serial sections of 12:1, 4:1, and 1:1 mixtures was calculated for both TPA-treated and non-TPA-treated cultures. Proliferation was measured as the percentage of BrdUrd-positive basal and suprabasal BrdUrd-positive cells, because II-4 cells demonstrated proliferation throughout the tissue. Five serial sections from random areas in the specimen were stained.

**RESULTS**

**TPA Causes Differential Growth Suppression of NHK and II-4 Cells in Submerged Cultures.** Keratinocytes were plated at clonal density and grown for 2 weeks at various TPA concentrations or in control media containing 0.1% DMSO to determine whether growth of NHK and II-4 cells was altered. At low TPA concentrations ( $10^{-4}$  and  $10^{-5}$   $\mu$ g/ml), no decrease in NHK colony number was seen when compared with controls (Fig. 1). At TPA concentrations  $\geq 0.001$   $\mu$ g/ml, a statistically significant decrease in NHK colony number was observed (Fig. 1). TPA had similar effects on established keratinocyte

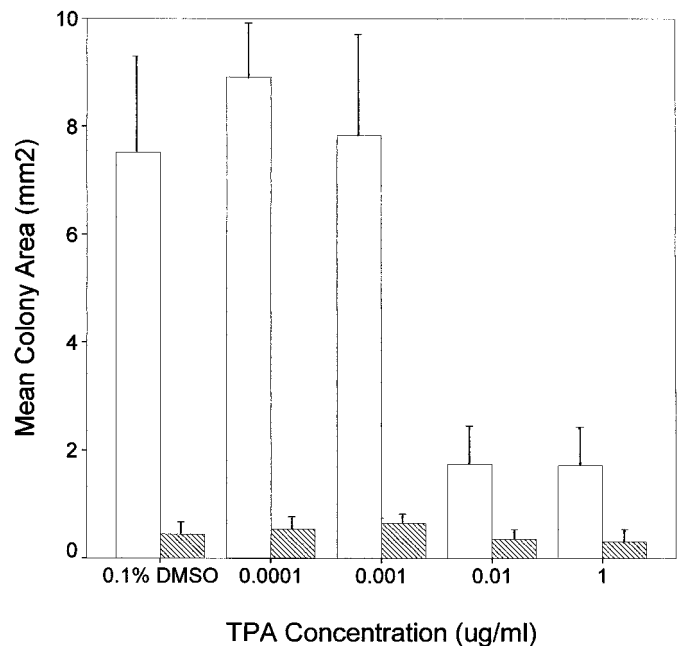
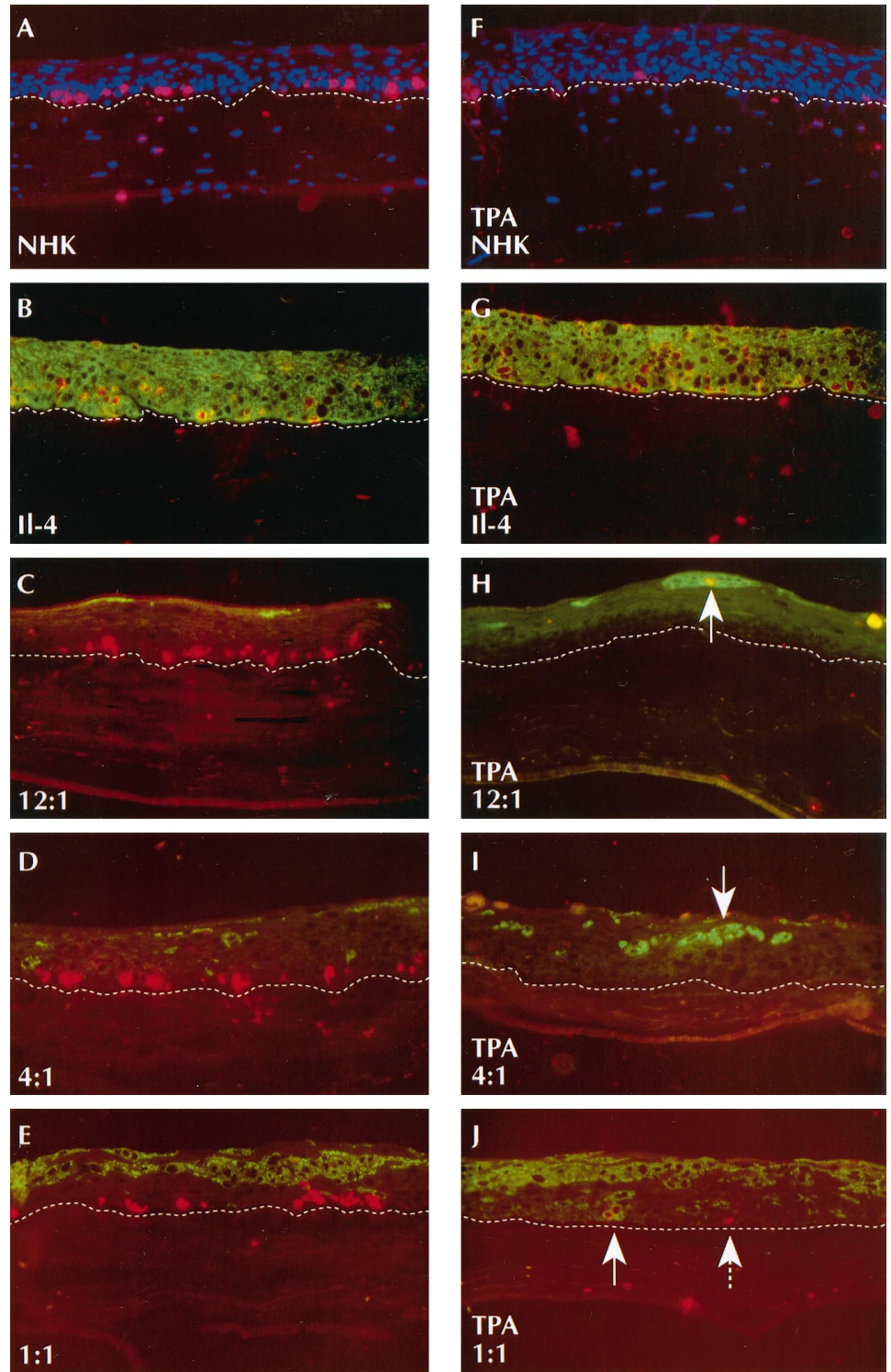


Fig. 2. The effect of TPA on the size of normal and II-4 human keratinocytes. One hundred NHKs (□) or II-4 keratinocytes (▨) were plated per 60-mm dish. After 1 day, cultures were fed with medium containing different concentrations of TPA prepared in 0.1% DMSO or with normal medium with 0.1% DMSO. NHK cultures were fixed and stained with rhodamine on day 15, whereas II-4 cultures were stained with X-gal. Colonies of five or more cells were counted; bars, SD. The effect of TPA was found to be significant for NHK (ANOVA,  $F = 8.925$ ,  $P < 0.05$ ) and nonsignificant for II-4 keratinocytes (ANOVA,  $F = 2.839$ ,  $P > 0.05$ ).

Fig. 3. TPA induces clonal expansion and proliferation of II-4 cells in 12:1 and 4:1 mixtures, which is associated with the decreased proliferation of NHKs. Double immunofluorescence stain demonstrating superimposed fluorescent signals for  $\beta$ -gal (FITC channel, green) and BrdUrd (Texas red channel, red). *A*, NHK cultures grown without TPA, demonstrating BrdUrd-positive nuclei limited to the basal layer and no  $\beta$ -gal expression. All nuclei counterstained with 4',6-diamidino-2-phenylindole, blue channel. *B*, pure II-4 cell cultures grown without TPA, demonstrating that all II-4 cells express  $\beta$ -gal and the presence of BrdUrd-positive nuclei in basal and suprabasal layers. *C*, mixture of NHK:II-4 cells (12:1), demonstrating individual  $\beta$ -gal cells in a suprabasal position and BrdUrd-positive nuclei limited to the normal basal keratinocytes. Individual  $\beta$ -gal cells lack colocalization of  $\beta$ -gal and BrdUrd, demonstrating withdrawal of II-4 cells from the cell cycle in the absence of TPA. *D*, mixture of NHK:II-4 cells (4:1), demonstrating individual  $\beta$ -gal cells in a suprabasal position and BrdUrd-positive nuclei limited to the normal basal keratinocytes. *E*, mixture of NHK:II-4 (1:1), showing large  $\beta$ -gal-positive II-4 cell clusters and BrdUrd-positive nuclei limited to the normal basal keratinocytes. *F*, NHK cultures grown with 0.001  $\mu$ g/ml TPA, demonstrating decreased numbers of BrdUrd-positive nuclei limited to the basal layer and no  $\beta$ -gal expression. *G*, pure II-4 cell cultures grown with 0.001  $\mu$ g/ml TPA, demonstrating continued proliferation as evidenced by presence of BrdUrd-positive nuclei in all strata. *H*, mixture of NHK:II-4 cells grown with 0.001  $\mu$ g/ml TPA, demonstrating increased size of  $\beta$ -gal-positive II-4 cells. Note positive cell cluster, which is also BrdUrd-positive (arrow), as seen by the yellow nucleus and green cytoplasm (arrow) in the center of an expanding cluster. *I*, mixture of NHK:II-4 (4:1) grown with 0.001  $\mu$ g/ml TPA, demonstrating increased numbers of  $\beta$ -gal-positive II-4 cells (arrow). *J*, mixture of normal keratinocytes:II-4 (1:1), showing large clusters of  $\beta$ -gal cells, an occasional BrdUrd-positive NHK nucleus (dotted arrow), and a BrdUrd-positive II-4 cell nucleus (solid arrow). White dotted line, the dermal-epithelial interface.



colonies that had grown for 1 week (data not shown). No decrease or increase in colony number was demonstrated for the II-4 cells at any TPA concentration. This suggested that at TPA concentrations of 0.001  $\mu$ g/ml or greater, the growth of II-4 cells and NHKs was differentially affected. A similar effect was observed when the mean colony size for both NHK and II-4 cells was determined at various TPA concentrations. Fig. 2 shows a decrease in NHK colony size with increasing concentrations of TPA, whereas there was no such diminution in size of II-4 colonies at any TPA concentration. It was clear that the growth potential of NHKs was suppressed above a critical

TPA concentration (0.001  $\mu$ g/ml), whereas that of II-4 cells was unaffected.

**TPA Suppresses Proliferation of NHK and Enables Clonal Expansion of II-4 Cells in Organotypic Cultures.** Because a differential growth response between NHK and II-4 cells was seen in submerged culture at elevated TPA concentrations, it was studied whether similar TPA doses could induce clonal expansion of II-4 in organotypic cultures containing mixtures of these cell types. When 12:1, 4:1, and 1:1 cell mixtures (NHK:II-4) were exposed to 0.001  $\mu$ g/ml TPA for 3 days, distinct, enlarged  $\beta$ -gal-positive clusters were

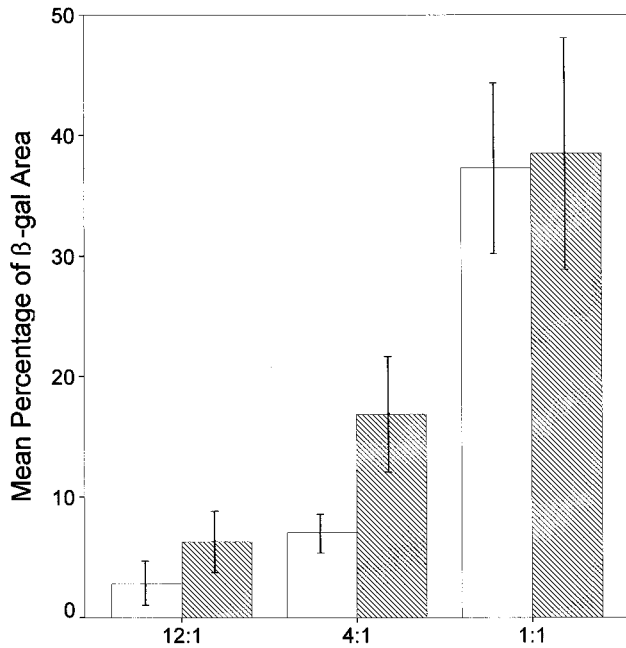


Fig. 4. The percentage of  $\beta$ -gal-positive area in mixtures of normal and II-4 keratinocytes with and without exposure to TPA. Mixtures (12:1, 4:1, and 1:1) of NHK:II-4 were grown in organotypic culture for 4 days and cultured in the presence of 0.001  $\mu$ g/ml TPA (▨) or 0.1% DMSO (□) when raised to the air-liquid interface. Cultures were grown for 3 additional days and stained for  $\beta$ -gal expression. The total mean  $\beta$ -gal-positive area as detected by immunofluorescence was determined using the UTHSCA Image Tool. Bars, SD.

visualized in both 12:1 (Fig. 3H) and 4:1 (Fig. 3I) mixtures when compared with control cultures not treated with TPA (Fig. 3, C and D). This demonstrated that II-4 cells underwent expansion because of TPA treatment. Upon quantification of a  $\beta$ -gal-positive area, a 2-fold increase was observed in 12:1 and 4:1 mixtures in the presence of TPA compared with control cultures (Fig. 4). On the other hand, 1:1 mixtures did not show an increase in the  $\beta$ -gal-positive area (Fig. 3, E and J, and Fig. 4), demonstrating that no additional expansion was induced in these mixtures by the addition of TPA.

To study the association of such expansion with proliferation, organotypic cultures were double stained by immunofluorescence for BrdUrd and  $\beta$ -gal. Colocalization of  $\beta$ -gal and BrdUrd in II-4 cells in TPA-treated 12:1 mixtures (Fig. 3H, arrow) suggested that the TPA-associated increase in clonal expansion of II-4 was accompanied by their proliferation. No such BrdUrd-positive nuclei were seen in  $\beta$ -gal-positive cells in non-TPA-treated 12:1 or 4:1 cultures (Fig. 3, C and D). In contrast, proliferation of II-4 cells in pure cultures was not significantly altered when the percentage of total BrdUrd-positive nuclei was determined with and without TPA (Fig. 3, B and G, and Fig. 5). In contrast, a significant decrease in the LI of NHKs was seen in TPA-treated organotypic cultures of pure NHKs (Fig. 3, A and F, and Fig. 5). Lis of NHK in non-TPA-treated 12:1 and 4:1 mixtures varied from 25 to 40%, whereas Lis of TPA-treated cultures ranged from 8 to 10% (Fig. 5). All mixtures (1:1, 4:1, and 12:1) showed a statistically significant decrease in BrdUrd-positive basal NHKs when compared with the DMSO control (Fig. 5).

**Temporal Sequence of TPA-induced Changes in Organotypic Culture.** Because the increased size of  $\beta$ -gal-positive, II-4 clusters and decreased NHK proliferation was seen after 3 days of continuous TPA exposure, it was important to determine the temporal sequence leading to these changes. To do so, NHK, II-4, and mixtures of these cells were studied by light microscopy and by double immunofluorescence staining for  $\beta$ -gal and BrdUrd at 8, 36, and 72 h after

addition of 0.001  $\mu$ g/ml TPA. The presence of a stratified epithelium at 8 and 72 h for both NHKs (Fig. 6, a and e) and II-4 (Fig. 6, b and f) in the presence of TPA showed that this agent was not toxic to either cell type in organotypic cultures. Pure NHK cultures were partly stratified at the 8-h time point and showed complete morphological differentiation at the 72-h time point without TPA (Fig. 6, a and e). In contrast, NHK cultures treated with TPA were thinner and showed less morphological differentiation (Fig. 6, a and e). TPA-treated 12:1 mixtures showed similar morphological changes (Fig. 6, c and g), whereas TPA-treated 4:1 mixtures were of similar thickness as cultures not treated with TPA (Fig. 6, d and h). In addition, these 4:1 cultures demonstrated clusters of morphologically distinct cells (Fig. 6h, arrows). On the other hand, TPA did not induce significant changes in tissue architecture of II-4 cells at any time points when compared with non-TPA-treated controls (Fig. 6, b and f).

BrdUrd labeling of organotypic cultures demonstrated a 60–80% decrease in NHK proliferation in 12:1 and 4:1 mixtures and pure NHK cultures treated with TPA. This growth suppression was evident as early as 8 h and remained at similar levels at 36 and 72 h (Fig. 7). In contrast, significant expansion of  $\beta$ -gal-positive cells was seen only at the 36-h time point in 12:1 mixtures and at the 72-h time point in 4:1 mixtures (Fig. 8). By 72 h, 12:1 mixtures treated with TPA demonstrated a similar percentage of  $\beta$ -gal area as 4:1 mixtures not treated with TPA, because of the clonal expansion of II-4 cells in the TPA-treated cultures. These findings demonstrated a temporal delay in expansion of II-4 cells relative to the suppression of NHK growth and suggested that expansion of II-4 cells may occur in response to changes in NHK proliferation. Because proliferation of II-4 cells was not significantly altered at any time point by TPA (Figs. 5 and 7), it appeared that II-4 cell expansion occurred as a result of the growth advantage of these cells in relation to the suppressed NHK cells rather than being caused by the direct stimulation of II-4 growth.

**TPA-induced Suppression of Differentiation of NHK in Cell Mixtures Is Temporally Linked to Clonal Expansion.** Because NHK proliferation was dramatically reduced after exposure to TPA for 8, 36, and 72 h, cultures were double-stained for  $\beta$ -gal and either K1 or FIL to determine whether concomitant changes in keratinocyte

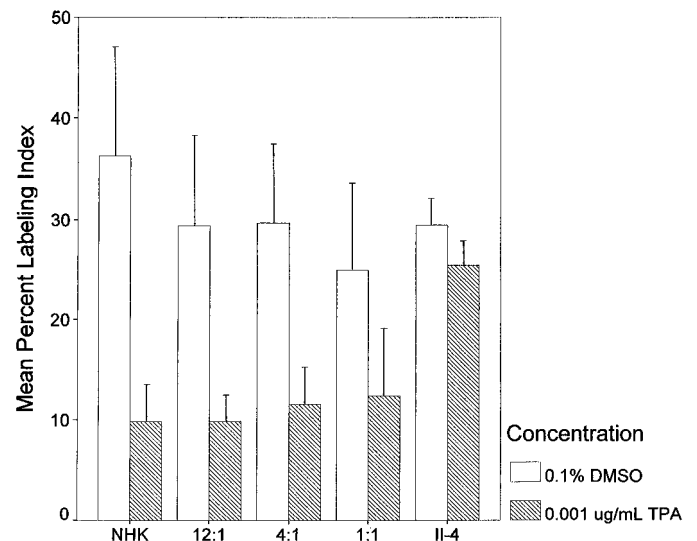


Fig. 5. Proliferation indices in pure and mixed cultures of normal (NHK) and II-4 keratinocytes with (▨) and without (□) TPA. Cells were plated with 10  $\mu$ M BrdUrd for 6 h, and sections were stained by immunofluorescence using an anti- $\beta$ -gal antibody as described in “Materials and Methods.” Proliferation of NHKs was measured as the percentage of BrdUrd-positive basal NHK nuclei (LI), and II-4 cell proliferation was calculated by counting basal and suprabasal BrdUrd-positive cells, because II-4 cells demonstrated proliferation throughout the tissue. Bars, SD.

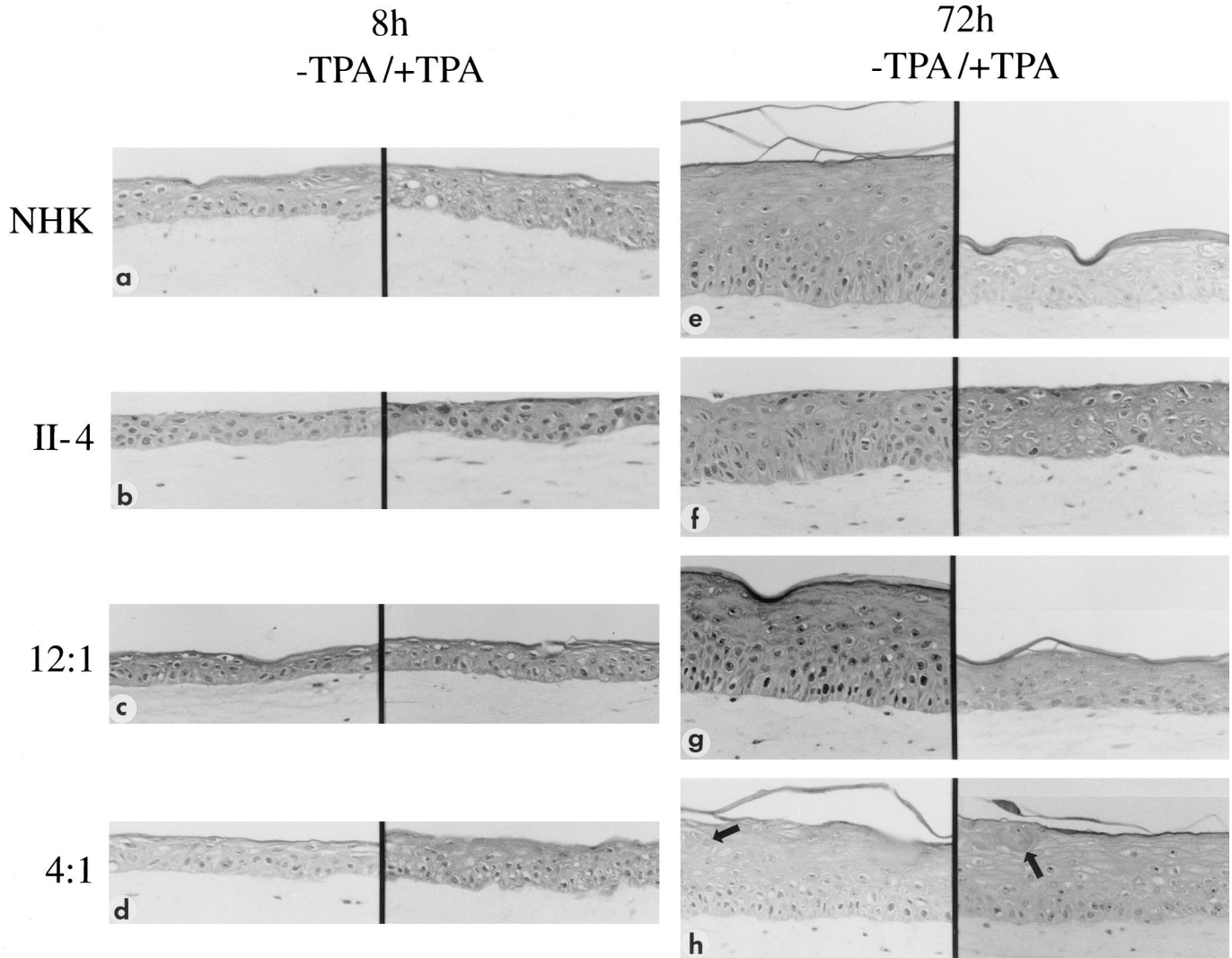


Fig. 6. The effects of TPA on the morphology of normal keratinocyte, II-4, 12:1 and 4:1 mixed cultures grown in the presence of TPA for either 8 or 72 h in organotypic culture. A comparison of organotypic cultures grown with and without TPA at 8 h (a–d) and at 72 h (e–h) demonstrates a decreased thickness and absence of *Stratum granulosum* for NHK and 12:1 mixtures when cultures are exposed to TPA for 72 h (e and g). TPA induced only mild alterations in pure II-4 and 4:1 mixtures. Clusters of II-4 cells demonstrating an altered morphology are seen in 4:1 mixtures (h). Pure cultures of NHK show complete morphological differentiation at 72 h (e), whereas II-4 cultures are dysplastic and show no morphological differentiation (f). TPA has induced little change in epithelial morphology or thickness at the 8-h time point (a–d).

differentiation could be detected. After 72 h of TPA treatment, K1 expression in NHK of TPA-treated mixtures was completely suppressed (Fig. 9b), whereas non-TPA-treated mixtures demonstrated expression of K1 in nearly all suprabasal NHK cells (Fig. 9a). In addition, control cultures of pure II-4 cells did not coexpress K1 and  $\beta$ -gal either with or without TPA (Fig. 9, c and d). In contrast, isolated II-4 cells with a flattened morphology coexpressed K1 and  $\beta$ -gal in 4:1 mixed cultures not treated with TPA (Fig. 9e) but not in II-4 cells in such mixtures treated with TPA (Fig. 9f). It was concluded that only II-4 cells surrounded by differentiated cells had the capacity to undergo terminal differentiation. By limiting the differentiation of NHK in 4:1 mixtures, TPA may lead to II-4 cell clonal expansion by preventing II-4 from undergoing terminal differentiation.

A similar pattern of altered differentiation was seen for FIL after 72 h of TPA exposure. FIL was expressed in the four to five uppermost cell layers of the epithelium in non-TPA-treated, pure NHK cultures (Fig. 9g), whereas only a punctate pattern of expression in the most superficial strata was visible when pure NHK were treated with TPA (Fig. 9h). Similarly, TPA treated 4:1 mixtures demonstrated sparse, patchy FIL staining which was confined to the uppermost layer

of the NHK in this epithelium (Fig. 9i, dotted arrow). This reduction in FIL may be associated with the fewer strata seen in TPA-treated cultures (Fig. 6). Importantly, there was no colocalization of FIL and  $\beta$ -gal in II-4 cells in these mixtures, suggesting that these cells did not undergo differentiation as observed in the non-TPA-treated cultures (Fig. 9k). A similar pattern of suppression of K1 and FIL was seen at 36 h after exposure to TPA (data not shown). Because it was determined that II-4 expansion began at 36 h, it became apparent that the TPA-induced suppression of NHK differentiation was temporally linked to the expansion of II-4 cells and that both of these events were preceded by the differential suppression of NHK proliferation.

## DISCUSSION

The two-stage model of mouse skin carcinogenesis involves two well-characterized early events. The first event generates a small number of keratinocytes harboring a genetic lesion (initiation), and the second leads to clonal expansion of these initiated cells (promotion; Refs. 1 and 17). Initiated cells are preferentially stimulated to grow in the presence of a promoter, but the mechanism of such clonal expansion during early

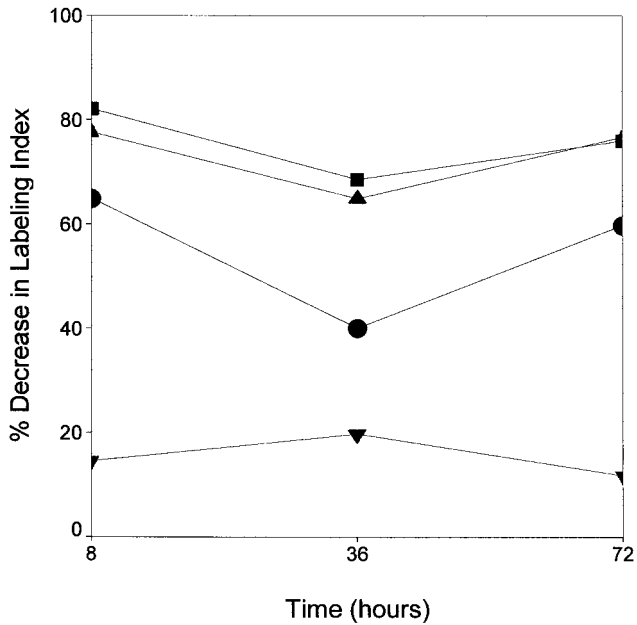


Fig. 7. Decrease in LI of NHKs after exposure to TPA for 8, 36, and 72 h. The percentage decrease in LI before and after addition of TPA was calculated as (% decrease = LI of NHK - LI of NHK with TPA). For example, a significant decrease in proliferation (60–80%) was seen for NHKs in the 12:1 (▲), 4:1 (●), and pure (■) NHK cultures when treated with TPA. II-4 (▼) cells showed only an 18–20% decrease in LI when TPA was added (not significant at  $P < 0.5\%$ ).

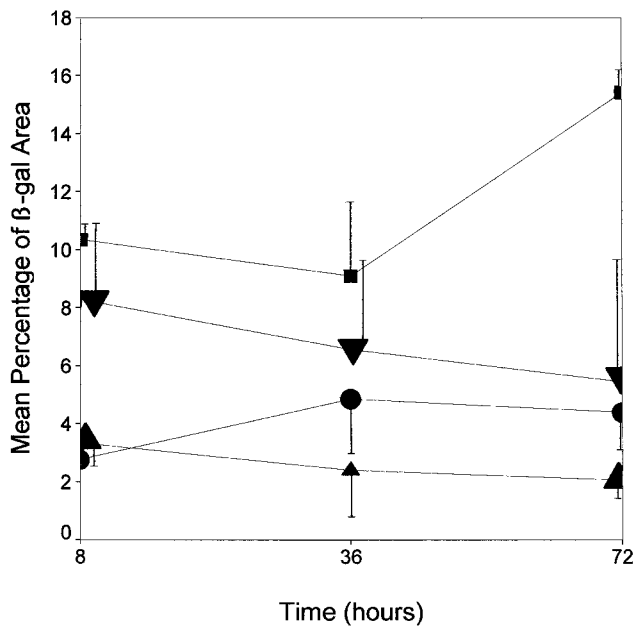


Fig. 8. Expansion of  $\beta$ -gal-positive cells in mixed organotypic cultures at 8, 36, and 72 h after TPA exposure. Mixtures (12:1 and 4:1) of NHK:II-4 were grown in organotypic culture for 4 days and then cultured with (●, 12:1 TPA; ■, 4:1 TPA) and without (▲, 12:1 Norm; ▼, 4:1 Norm) 0.001  $\mu$ g/ml TPA for 8, 36, or 72 h at the air-liquid interface. Cultures were stained for  $\beta$ -gal expression, and the total mean  $\beta$ -gal-positive area, as detected by immunofluorescence, was determined using the UTHSCSA Image Tool. The effect of TPA was found to be significant for II-4 expansion in both 12:1 and 4:1 cultures. For 12:1 (ANOVA,  $F = 13.317$ ,  $P < 0.05$ ) and for 4:1 (ANOVA,  $F = 20.539$ ,  $P < 0.05$ ).

neoplastic progression remains unclear. It is not known whether promoters directly stimulate the growth of initiated cells or rather permit clonal expansion of initiated cells by modifying normal cells adjacent to them. Furthermore, the relevance of the mouse skin model for human skin carcinogenesis is unclear, as are the tumor-promoting effects of TPA on human keratinocytes. The goal of this study was to further understand the

mechanism of action of tumor promoters during early neoplastic progression of human stratified epithelium. For this purpose, we have developed an *in vitro* skin model that simulates early neoplastic progression in stratified squamous epithelium.

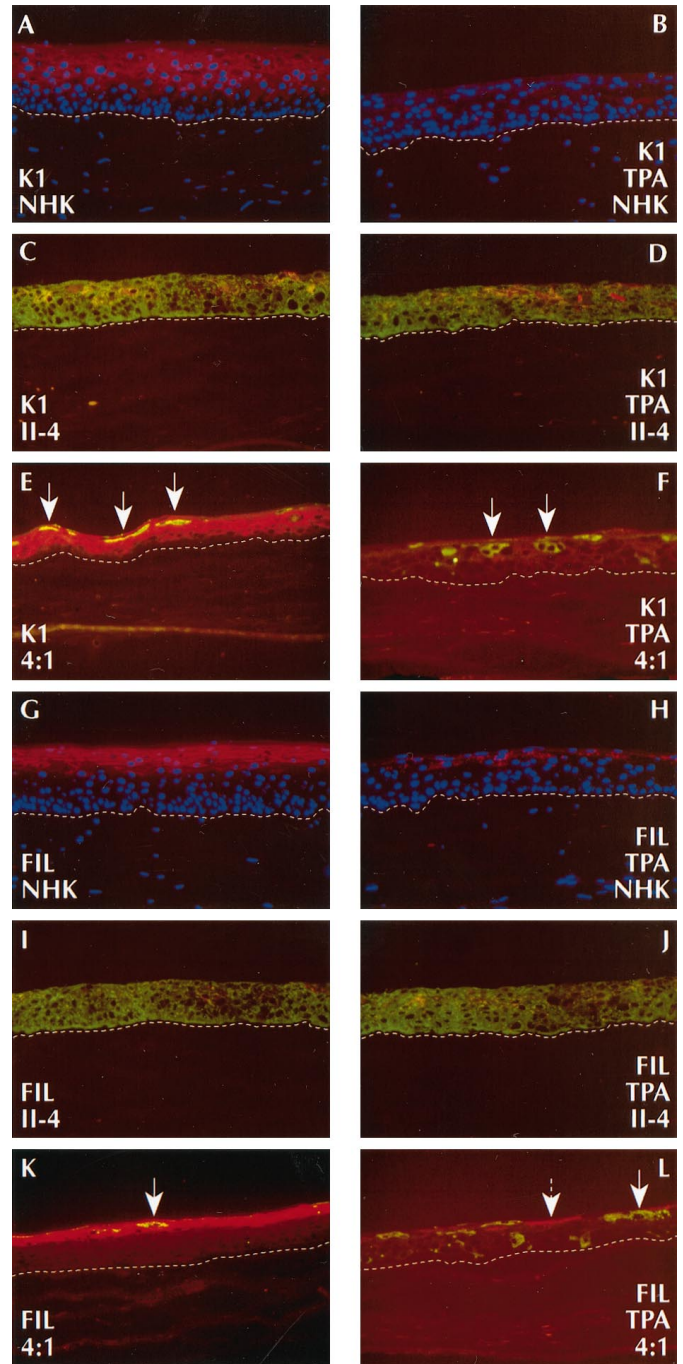


Fig. 9. Double immunofluorescence stain of organotypic cultures for  $\beta$ -gal and either K1 or FIL after TPA exposure. Superimposed immunofluorescence signals for  $\beta$ -gal (FITC channel, green) and K1 or FIL (Texas red channel, red). Normal keratinocytes were stained for K1, with (B) and without (A) TPA, and demonstrated the complete loss of K1 expression in the presence of TPA. II-4 cells stained for K1, with (D) and without (C) TPA showed no difference in expression. Mixtures (4:1; NHK:RII) treated with TPA demonstrated the complete loss of K1 expression (F) and the expansion of II-4 cells (F, arrows), whereas 4:1 mixtures not treated with TPA coexpressed K1 and  $\beta$ -gal in II-4 cells (E, yellow cells, arrows) and K1 in keratinocytes adjacent to them. Normal keratinocytes stained for expression of FIL after culture with (H) and without (G) TPA showed a decrease in FIL in the presence of TPA. II-4 cells stained for FIL, with (J) and without (I) TPA, showed a similar staining pattern. Mixtures (4:1; NHK:RII) treated with TPA demonstrated patchy FIL expression in the upper strata (L, broken arrow) and the expansion of II-4 cells (L, solid arrow), whereas 4:1 mixtures not treated with TPA demonstrated coexpression of FIL and  $\beta$ -gal in II-4 cells (K, arrow).

Previous studies have shown that TPA had such a differential effect on the growth of normal *versus* transformed mouse keratinocytes (7) and stimulated growth of some subpopulations of mouse keratinocytes while inducing differentiation of others (18). In addition, cultured human keratinocytes have been shown to undergo growth arrest in response to TPA (4, 5, 19). This differential sensitivity to TPA-induced growth suppression suggested that TPA may promote the clonal growth of potentially malignant cells by inducing their selective expansion at the expense of growth-inhibited normal keratinocytes. Here we studied such effects in an organotypic tissue model that more closely simulated the *in vivo* environment, where the role of normal cell context and normal tissue architecture could be analyzed.

We have found previously that interactions with NHKs suppress the neoplastic behavior of potentially invasive keratinocyte clones *in vitro* and *in vivo* (9). Here we show that the skin tumor promoter TPA modulates these cell interaction-mediated growth constraints of normal keratinocytes on tumorigenic cells. However, this effect was clearly dependent on the ratio of normal keratinocytes to tumor cells. We found a significant increase in the size of the II-4 clones in 12:1 and 4:1 mixtures when treated with 0.001  $\mu\text{g/ml}$  TPA, but TPA did not significantly increase the size of the II-4 clones in 1:1 mixtures. It is possible that II-4 cells continued to expand because intercellular contact with adjacent II-4 cells in clusters of sufficiently large size. It has been shown that contact between adjacent tumor cells results in suppression of a death signal (20), whereas loss of contact between cancerous cells induces their apoptosis and elimination (21). Regardless of mechanism, the results reported here clearly demonstrate that large clusters of II-4 cells, such as in 1:1 mixtures, are not susceptible to the induction of further clonal expansion by TPA.

The induction of II-4 expansion by TPA in 12:1 and 4:1 mixtures may be explained by the differential regulation of proliferation of NHK and II-4 cells in these cultures. TPA did not increase the size or the number of II-4 colonies in submerged culture and did not alter proliferation of II-4 cells in organotypic cultures. On the other hand, TPA limited the growth of NHKs in submerged culture and decreased proliferation by 2–4-fold in organotypic cultures. This suggested a direct suppression of normal keratinocyte growth by TPA and may explain the selective expansion of II-4 cells in the 12:1 and 4:1 mixtures because these cells, although not stimulated with TPA, continued to grow without hindrance. Induction of II-4 clonal expansion was therefore relative to the decreased proliferation induced by TPA on the surrounding NHKs.

The temporal sequence of inhibition of NHK proliferation and clonal expansion of II-4 cells, however, did not coincide. The marked decrease in NHK proliferation was evident after 8 h of TPA exposure, and proliferation remained at these levels after 36 and 72 h of TPA exposure as well. However, the significant increase in clonal expansion in 12:1 and 4:1 mixed cultures was seen only after a TPA exposure of 72 h. If TPA had directly mediated the stimulation of II-4 clones, expansion would likely have been seen shortly after TPA exposure. The fact that the immediate suppression of NHK proliferation did not occur simultaneously with the increase in II-4 cell expansion suggests that the TPA-induced expansion of II-4 cells might be dependent on additional changes induced in the NHK microenvironment rather than being caused by the direct stimulation of the II-4 cells by TPA.

We hypothesized that an alteration in NHK differentiation might be linked to expansion of II-4 cells. In fact, expression of K1 and FIL were dramatically altered in NHKs by TPA in organotypic mixtures. The decrease in expression of FIL in the superficial layers of the organotypic cultures may be associated with the decreased NHK proliferation seen in these cultures. Alternatively, the altered pattern of FIL expression seen in NHKs adjacent to expanding II-4 cells may

be associated with the fewer strata seen in TPA-treated cultures. Regardless of the reason for the lack of expression of these markers of differentiation, it appears that clonal expansion of II-4 cells is associated with the absence of K1 and FIL in NHKs adjacent to II-4 cells. Because this change was temporally coincident with expansion of II-4 cells, TPA-induced inhibition of the differentiated NHK phenotype may relieve the suppression of transformed cell growth. It has been shown previously that differentiated normal cells can suppress the growth of cells with malignant potential (22–24). In addition, normal tissue architecture and differentiation was found previously to limit II-4 cell expansion in our model of early neoplastic progression (9). Moreover, we have shown that inhibition of tumor cell growth is coincident with and possibly caused by induction of differentiation in these cells. Thus, inhibition of keratinocyte differentiation may reduce their growth-inhibitory capacity on tumor cells. It has been shown previously that TPA can overcome growth suppression induced by normal mouse fibroblasts in submerged culture. In this study, Dotto *et al.* (25) showed that *ras*-transformed mouse fibroblasts were suppressed by their normal neighbors in submerged culture but were stimulated to overcome this suppression by treatment with TPA. This may be due to the ability of tumor promoters to block intercellular communication, thereby resulting in escape of initiated cells from regulation by normal cells (26–28), because this promoter induces expansion of initiated clones to a critical size (23). Although the detailed mechanism of this intercellular signaling and growth regulation is not clear, our data clearly indicate that in addition to its regulation of proliferation, TPA is also effective in controlling NHK differentiation.

In summary, TPA may enable clonal expansion by altering the rate of growth and differentiation potential of normal cells, rather than by directly altering the phenotype of the potentially malignant cells. Expansion of II-4 cells by TPA is likely to be, therefore, secondary to epigenetic changes induced in normal cells in the microenvironment. By doing so, TPA may act to stimulate the early stages of neoplastic progression in human stratified epithelium by creating a microenvironment conducive for clonal expansion of previously suppressed, potentially malignant cells by permitting them to overcome the growth-suppressive effects of normal cell context.

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