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Loss of expression of transforming growth factor β in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion

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ABSTRACT **Mouse skin carcinomas arise from a small subpopulation of benign papillomas with an increased risk of malignant conversion. These papillomas arise with limited stimulation by tumor promoters, appear rapidly, and do not regress, suggesting that they differ in growth properties from the majority of benign tumors. The transforming growth factor β (TGF-β) proteins are expressed in the epidermis and are growth inhibitors for mouse keratinocytes *in vitro*; altered TGF-β expression could influence the growth properties of high-risk papillomas. Normal epidermis, tumor promoter-treated epidermis, and skin papillomas at low risk for malignant conversion express TGF-β1 in the basal cell compartment and TGF-β2 in the suprabasal strata. In low-risk tumors, 90% of the proliferating cells are confined to the basal compartment. In contrast, the majority of high-risk papillomas are devoid of both TGF-β1 and TGF-β2 as soon as they arise; these tumors have up to 40% of the proliferating cells in the suprabasal layers. Squamous cell carcinomas are also devoid of TGF-β, suggesting that they arise from the TGF-β-deficient high-risk papillomas. In some high-risk papillomas, TGF-β1 loss can occur first and correlates with basal cell hyperproliferation, while TGF-β2 loss correlates with suprabasal hyperproliferation. Similarly, TGF-β1-null transgenic mice, which express wild-type levels of TGF-β2 in epidermis but no TGF-β1 in the basal layer, have a hyperproliferative basal cell layer without suprabasal proliferation. In tumors, loss of TGF-β is controlled at the posttranscriptional level and is associated with expression of keratin 13, a documented marker of malignant progression. These results show that TGF-β expression and function are compartmentalized in epidermis and epidermal tumors and that loss of TGF-β is an early, biologically relevant risk factor for malignant progression.**

In multistage chemical carcinogenesis of the mouse epidermis, malignant conversion of a benign papilloma to a squamous cell carcinoma is a rare event (1). While most papillomas and carcinomas generated by initiation with 7,12-dimethylbenz[*a*]anthracene (DMBA) have an A → T mutation at codon 61 of the Ha-*ras* allele (2, 3), there is evidence for subpopulations of papillomas with distinct risks for malignant conversion. Standard protocols utilizing DMBA initiation and continuous promotion with phorbol 12-myristate 13-acetate (PMA) produce a large number of papillomas, the majority of which arise after 10 weeks and have a malignant conversion frequency of 3–5% (4). Many of these tumors are promoter-dependent and regress rapidly when PMA treatment is terminated (5). In contrast, DMBA initiation followed by 5 weeks of PMA promotion, or continuous promotion with the weak promoter mezerein, yields fewer papillomas, which arise within 6–8 weeks, do not regress in the absence of promotion, and have a conversion frequency of 15–25% (4). This suggests that most carcinomas arise from a small population of papillomas that have relatively autonomous growth properties from the earliest stages.

Changes in growth factor expression could contribute to the altered growth properties of the high-risk papillomas. Transforming growth factor β1 (TGF-β1) and TGF-β2 are 25-kDa dimeric growth-regulatory proteins (6) that act as potent growth inhibitors for normal and initiated keratinocytes *in vitro* (7). TGF-β1 protein is increased suprabasally in the mouse epidermis after a single PMA treatment (8, 9) and in human epidermis after wounding (10). TGF-β2 is expressed by differentiating keratinocytes both *in vitro* (11) and *in vivo* (7). Dysplastic skin papillomas and carcinomas express high levels of TGF-β1 mRNA but do not express TGF-β1 protein (9), suggesting that significant alterations in TGF-β expression may occur in later stages of chemical carcinogenesis. To examine the potential role of TGF-β as a risk factor in tumor progression and malignant conversion, we have compared expression of TGF-β1 and TGF-β2 between low- and high-risk papillomas at early and late times after tumor appearance. Using 5-bromo-2′-deoxyuridine (BrdUrd) labeling, we have correlated changes in TGF-β expression with altered proliferation in low- and high-risk tumors and with the expression of known markers of malignant progression. To confirm a fundamental relationship between changes in TGF-β expression and epidermal cell proliferation, we have also examined cell proliferation in the epidermis of TGF-β1-null mice (12).

MATERIALS AND METHODS

Chemical Induction of Tumors. Tumors were induced in outbred female SENCAR or CD-1 mice as described (4). Shaved dorsal skin was treated with DMBA (20 μg) in acetone (200 μl). Low-risk papillomas were generated by continuous promotion with PMA (2 μg), given once per week until sacrifice. High-risk papillomas were treated with PMA (2 μg) weekly for 5 weeks or treated continuously with mezerein (4 μg) twice weekly until sacrifice. Tumors from continuous promotion protocols were isolated 48 hr after the last promoter treatment. For each promotion protocol, all visible papillomas were isolated from 5–20 animals at a specific time point and frozen together in optimal-cutting-temperature (OCT) compound (Miles) with adjacent normal

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Abbreviations: BrdUrd, 5-bromo-2′-deoxyuridine; DMBA, 7,12-dimethylbenz[*a*]anthracene; PMA, phorbol 12-myristate 13-acetate; TGF-β, transforming growth factor β.

skin. Carcinomas were isolated from animals after >30 weeks of promotion from these and similar tumor-induction experiments.

TGF-β Immunodetection. Immunodetection of TGF-β on 5-μm frozen sections was done without hyaluronidase treatment, by using Vectastain Elite (Vector Laboratories) and Histomark Orange metal-enhanced diaminobenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) stains. Sections were counterstained with Mayer's hematoxylin. TGF-β1 antibodies were anti-CC(1-30-1), which is directed against a peptide in the mature TGF-β1 and detects predominantly the extracellular molecule (13), and anti-pre(266-278-1), which recognizes a peptide in the precursor domain (14). Staining occurred with anti-CC(1-30-1) in tumors when frozen sections were pretreated briefly in Bouin's fixative. TGF-β2 antibodies were anti-LC(50-75-2) (15) and anti-352-376 (Santa Cruz Biotech), both directed against peptides in the mature TGF-β2 molecule, and anti-pre(197-212-2), directed against a peptide in the TGF-β2 precursor domain. Similar staining was seen with antibodies to both the precursor and the mature TGF-β2 molecule. All TGF-β antibodies were used at 2 μg/ml. Immunohistochemical analysis of low- and high-risk groups was done simultaneously with identical development times and repeated at least three times. Negative staining was judged against sections incubated with corresponding blocking peptide and normal skin controls. Results were confirmed by indirect immunofluorescence. All photomicrographs were taken with a Zeiss Axiophot microscope.

BrdUrd Labeling and Detection. One hour prior to sacrifice mice were injected with BrdUrd (0.2 mg/g) in saline (0.9% NaCl, 300 μl). BrdUrd incorporation was localized on serial frozen sections of papillomas previously characterized for TGF-β expression by using a monoclonal anti-BrdUrd antibody (Becton Dickinson) and horseradish peroxidase-labeled second antibody (16). The labeling index was calculated from enlargements of low-power photomicrographs as the number of BrdUrd-positive cells in either the basal or suprabasal compartments per total basal nuclei. Regions containing 100–300 total basal cells were used for each count.

Keratin Immunodetection. Keratin expression was localized on serial frozen sections by using monospecific rabbit antibodies to keratins 1, 10, and 13 (17).

***In Situ* Hybridization.** Frozen sections (5 μm) of week 22 papillomas were prepared for hybridization, prehybridized, and hybridized *in situ* (18) to ^{35}S-labeled TGF-β2 antisense and sense RNA probes (2×10^7 cpm/ml) generated from a fragment of the mouse TGF-β2 coding sequence subcloned in pBluescript KS (Stratagene) (19). Sections were hybridized for 4 hr at 52°C in hybridization solution as described (18). The sections were rinsed and treated with RNases A and T1 for 30 min at 37°C, subjected to a final wash in 30 mM NaCl/3 mM sodium citrate, pH 7/50% (vol/vol) formamide at 60°C for 15 min, and processed for autoradiography with Kodak NBT-2 emulsion. Slides were exposed 12 days and examined under dark-field conditions with a Zeiss Axiophot microscope.

PCR Analysis of TGF-β1-Null Mice. Targeted deletion of TGF-β1 was done with replacement vectors as described (12). Genotypes were determined from PCR analysis of tail DNA. Wild-type alleles were identified with a primer pair from the first exon and intron of the mouse TGF-β1 gene (5′-CGTGCGCCTGTCGCTTTCTG-3′ and 5′-GCGGACTACTATGCTAAAG-3′). Null alleles were identified with a primer from exon 1 of the TGF-β1 locus (5′-AGGGAGCTGGTGAAACGGAA-3′) and from the phosphoglycerate kinase 1 promoter of the neomycin-resistance cassette (5′-TCCATCTGCACGAGACTAGT-3′). The PCR mixture contained 3 mM Mg^{2+}, and the annealing temperature was 60°C.

RESULTS

Compartmentalized Expression of TGF-β in Normal Epidermis and Low-Risk Papillomas. TGF-β1 and TGF-β2 were localized on frozen sections with specific anti-peptide polyclonal antibodies directed against either the mature or the precursor region of the TGF-β molecule. TGF-β1 was present exclusively in the basal layer of normal mouse epidermis, whereas TGF-β2 was exclusive to the suprabasal differentiating layers (Fig. 1 *a* and *b*). A similar pattern was found in epidermis chronically treated with PMA (Fig. 1 *d* and *e*). Benign papillomas with a low risk for malignant conversion (4) were generated with DMBA initiation and continuous PMA promotion. This protocol generates a few papillomas between weeks 6 and 8 which are the high-risk type and a large number of papillomas between weeks 9 and 11 which are of the low-risk type. At 11 weeks from the onset of promotion, all papillomas were isolated from several animals; 90% of these tumors appeared between weeks 9 and 11. Eleven of 13 (85%) of these predominantly low-risk tumors had compartmentalized TGF-β expression identical to that found in normal epidermis (Fig. 1 and Table 1), although the level of TGF-β1 staining in the basal layer of the papillomas was reduced. Similarly, in a second experiment in which sections were analyzed after 22 weeks of PMA promotion, the majority of this benign tumor group remained positive for compartmentalized expression of both TGF-β isoforms (Table 1). However, in tumors from long-term continuous PMA promotion (>30 weeks), many tumors had either focal loss of TGF-β1 only or focal loss of both isoforms.

Loss of TGF-β Expression in High-Risk Papillomas and Squamous Cell Carcinomas. Benign papillomas with a high risk for malignant conversion were generated by DMBA initiation and either limited promotion with PMA (5 weeks) or promotion with the weak promoter mezerein (4). Tumors were isolated 8 weeks after the start of promotion—i.e., 1–2 weeks after first detection and when only 1–2 mm in diameter. At this time point there was 0.2–0.5 papilloma per mouse. Of these early-appearing, high-risk papillomas, 14/23 (60%) were devoid of immunodetectable TGF-β1 or TGF-β2 (Fig. 1 and Table 1), while 3 of 23 expressed only TGF-β2 and 5 of 23 had a low-risk TGF-β phenotype (Table 1). The difference between low- and high-risk groups was maintained at 22 weeks of promotion with mezerein (Table 1), with 60% of the high-risk tumors devoid of either TGF-β isoform. Both TGF-β-positive and TGF-β-negative papillomas were present in tumor populations withdrawn from promotion at week 11 and examined at weeks 22 and 33 (data not shown), indicating independence of both phenotypes from acute effects of promotion. Significantly, of 22 squamous cell carcinomas analyzed, 20 also had the TGF-β-deficient phenotype, suggesting that most carcinomas are derived from the TGF-β-deficient high-risk papillomas. *In situ* hybridization analysis showed that papillomas deficient in TGF-β2 protein still expressed TGF-β2 mRNA (Fig. 2). Similar results were obtained for TGF-β1 in dysplastic papillomas and carcinomas (9), suggesting that a posttranscriptional block of expression is responsible for loss of both isoforms in high-risk papillomas and carcinomas.

We examined keratin expression in the high-risk papillomas to relate loss of TGF-β expression to other biochemical changes relevant for tumor progression. Keratins 1 and 10, expressed in the differentiating layers of normal epidermis, are lost during tumor progression and commonly replaced by keratin 13 (16, 17). Nearly all of the early TGF-β-positive papillomas expressed keratins 1 and 10 but not keratin 13, whereas 10 of 14 of the 8-week TGF-β-deficient papillomas expressed keratin 13 and had reduced or absent keratins 1 and 10 (Fig. 1). Thus, loss of TGF-β is associated with early

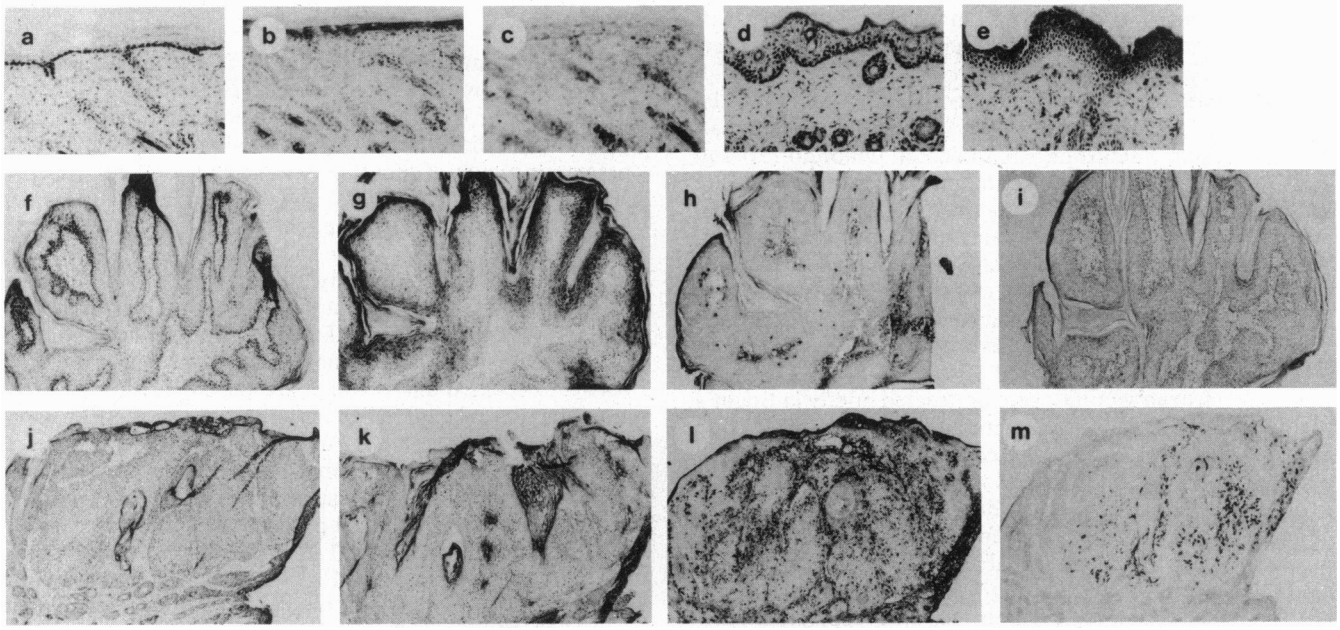

FIG. 1. Immunohistochemical analysis of TGF-β expression, BrdUrd incorporation, and keratin expression in normal skin and papillomas. (*a–c*) Normal epidermis. (*d* and *e*) Normal epidermis promoted 8 weeks with PMA (2 μg/200 μl of acetone once per week). (*f–i*) Serial sections of a low-risk papilloma initiated with 20 μg of DMBA and isolated after promotion for 11 weeks with PMA (2 μg/200 μl of acetone once per week). Tumors were isolated 48 hr after the last PMA treatment. (*j–m*) Serial sections of a high-risk papilloma initiated with 20 μg of DMBA and promoted 8 weeks with mezerein (4 μg/200 μl of acetone twice per week). Tumors were isolated 48 hr after the last mezerein treatment. *a*, *d*, *f*, and *j* show staining with TGF-β1 antibody anti-pre(266-278-1). *c* shows normal skin incubated with anti-pre(266-278-1) and blocking peptide 266-278-1. Blocking was also observed with incubation of the TGF-β2 antibodies with the respective peptide antigens, and for both TGF-β1 and TGF-β2 on the tumor sections (data not shown). *b*, *e*, *g*, and *k* show staining with the TGF-β2 antibody anti-pre(197-212-2); *h* and *l*, staining with anti-BrdUrd; and *i* and *m*, staining with anti-keratin 13 antibody. Similar staining patterns were observed with antibodies to both the precursor and mature forms of TGF-β1 and TGF-β2. (*a–e*, ×160; *g–j*, ×80; *h–n*, ×40.)

expression of biochemical markers strongly associated with malignant progression.

Progressive Hyperproliferation Associated with Loss of Compartmentalized TGF-β Expression in Tumors. To determine the relationship between TGF-β expression and cell proliferation, mice were injected with BrdUrd, and serial sections were analyzed for TGF-β expression and BrdUrd incorporation by immunoperoxidase staining. This analysis and double indirect immunofluorescence assay of TGF-β and BrdUrd (data not shown) revealed a striking inverse correlation between TGF-β1 expression and proliferation in the basal layer and between TGF-β2 expression and suprabasal proliferation. Normal epidermis, with the highest expression of both isoforms, had a BrdUrd labeling index of <5%; proliferation was exclusively in the basal layer (20). In 11-week low-risk TGF-β-positive papillomas, the labeling index increased 3- to 4-fold, but only 11% of the proliferating cells were suprabasal (Fig. 3). In high-risk papillomas where TGF-β1 was reduced or absent but TGF-β2 was highly expressed, the labeling index was further increased, but only 17% of the proliferating cells were in the suprabasal layer (Fig. 3). However, in high-risk papillomas where both isoforms were absent, the total labeling index increased to 66%, and 40% of the proliferating cells were in the suprabasal compartment. This represents a 25-fold increase in suprabasal proliferation compared with TGF-β-positive papillomas (Fig. 3). Thus the expansion of the proliferative compartment, a hallmark of tumor progression, occurs early in high-risk papillomas and is directly related to altered TGF-β2 synthesis.

Table 1. Difference in TGF-β expression between high- and low-risk papillomas

Promotion protocol	Weeks after initiation	No. of papillomas		
		$\beta1^-\beta2^-$	$\beta1^-\beta2^+$	$\beta1^+\beta2^+$
High risk	8	14	3	5
	22	6	2	2
Low risk	11	2	0	11
	22	2	3	8

The difference in the number of TGF-β-deficient papillomas between the week 8 high-risk papillomas and the week 11 low-risk papillomas is significant ($P < 0.02$, Fisher–Irwin exact test). High-risk papillomas were generated by DMBA initiation and promotion either once weekly with PMA for 5 weeks or with continuous twice weekly treatments with mezerein, as described in *Materials and Methods*. Low-risk papillomas were generated by DMBA initiation and continuous weekly promotion with PMA. All tumors from groups receiving continuous promotion were isolated 48 hr after the last treatment. Since sustained PMA treatment promotes both papilloma subclasses, the continuous PMA promotion group contains ≈20% of high-risk papillomas. Papillomas were classified as 0–25%, 50%, and 75–100% of tissue section positive for each isoform. Papillomas with 0–25% positive staining for both isoforms were considered $TGF\text{-}\beta1^-\beta2^-$. The majority of high-risk papillomas had no detectable positive staining. Papillomas that did not stain for TGF-β1 but were >50% positive for TGF-β2 were considered $TGF\text{-}\beta1^-\beta2^+$. Papillomas with >50% positive staining for both isoforms were considered $TGF\text{-}\beta1^+\beta2^+$. The majority of low-risk papillomas were 75–100% positive for each isoform.

Epidermal Hyperproliferation in TGF-β1-Null Mice. To support a causal relationship between TGF-β1 expression and epidermal cell proliferation, we determined the labeling index of the epidermis of 5-week-old transgenic mice homozygous for a targeted disruption of the TGF-β1 locus (12). These animals have wild-type expression of TGF-β2 (data not shown). The labeling index of the TGF-β1-null epidermis was 3- to 5-fold higher than that of wild-type littermates (Table 2), without evidence of suprabasal proliferation or hyperplasia. Although the basal and suprabasal layers in normal adult epidermis are in close proximity, basal cells may not be able to activate or respond to TGF-β2. Since keratins

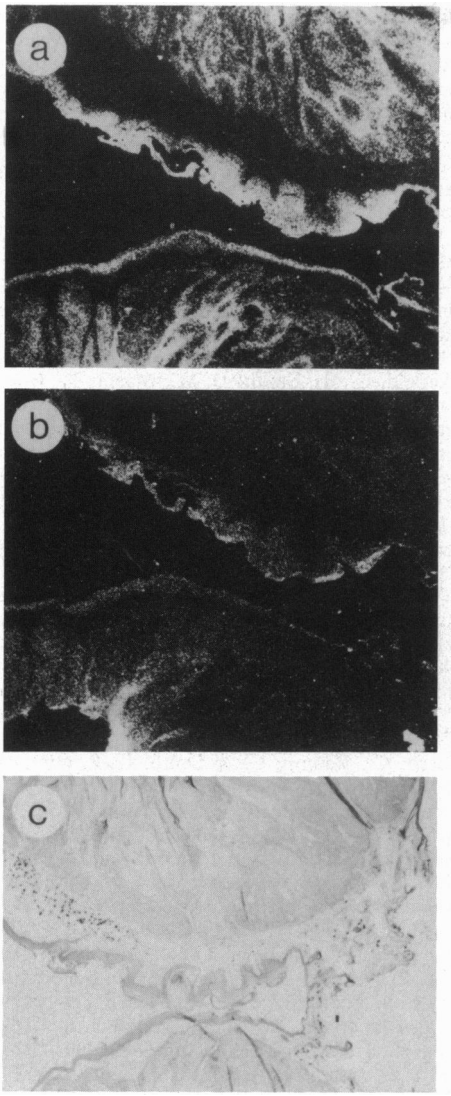

FIG. 2. TGF-β2 mRNA expression in papilloma devoid of TGF-β2 peptide. (*a*) *In situ* hybridization of TGF-β2 antisense RNA probe to TGF-β2-deficient papilloma isolated after 22 weeks of mezerein promotion. Similar hybridization is seen for the TGF-β2-deficient papilloma tissue and overlying TGF-β2-positive normal epidermis. (*b*) Section adjacent to *a*, hybridized with TGF-β2 sense RNA probe. (*c*) Section adjacent to *a*, showing immunolocalization of TGF-β2. Immunodetection of TGF-β2 was performed as described in *Materials and Methods*. (×50.)

1 and 10 were expressed normally in the mutant epidermis, and keratin 13 was not expressed (data not shown), altered expression of these markers in tumors was not directly caused by loss of TGF-β1.

DISCUSSION

These data show that TGF-β expression and response are compartmentalized, that each isoform may have distinct functions *in vivo*, and that the loss of TGF-β in epidermis is causally associated with hyperproliferation and tightly linked to a high risk for malignant conversion. Loss of responsiveness to TGF-β (21, 22) may also be important during carcinogenesis and may be represented by the few TGF-β-positive carcinomas detected in this study. Loss of TGF-β is global in early high-risk papillomas, suggesting that the phenotype commences with initiation and may be the result of multiple mutations, variable expression of oncogenes, or the developmental phenotype of the target cell. However, the

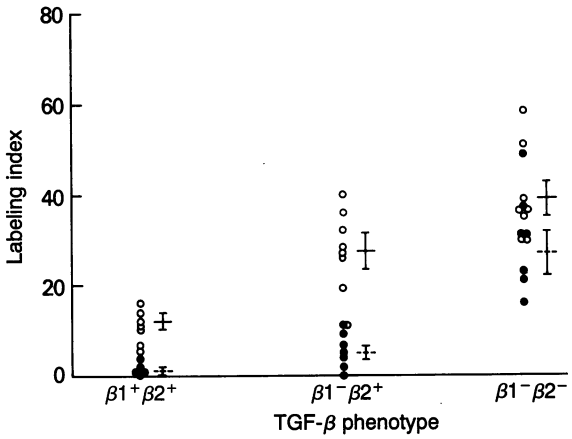

FIG. 3. Relationship of BrdUrd labeling index to TGF-β expression in basal and suprabasal compartments of early high- and low-risk papillomas. Open circles, basal-layer labeling index of individual tumors; filled circles, suprabasal-layer labeling index of individual tumors. Solid and broken horizontal lines represent the mean basal and suprabasal labeling indices, respectively. Error bars show SEM. The mean labeling indices and SEMs for the $\beta1^+\beta2^+$ group were 10.8 ± 1.36 (basal), and 1.3 ± 0.5 (suprabasal); for the $\beta1^-\beta2^+$ group, 27.3 ± 4.3 (basal) and 5.5 ± 1.2 (suprabasal); and for the $\beta1^-\beta2^-$ group, 39.8 ± 3.8 (basal) and 26.7 ± 4.9 (suprabasal).

focal loss of TGF-β in most papillomas subject to long-term promotion supports the concept of similar pathways of progression in all tumors (23), but at vastly different rates.

The inverse relationship of TGF-β expression to labeling index in papillomas and TGF-β1-null mice and the low level of suprabasal proliferation in TGF-β1-deficient papillomas suggest that the action of each isoform is compartmentalized along with expression and that distant paracrine influences are marginal. TGF-β1 is likely to be a negative regulator of proliferation in the basal layer, while TGF-β2 may act to inhibit DNA synthesis or induce apoptosis (24, 25) in the suprabasal terminally differentiating compartment. Since the TGF-β1-null epidermis has a TGF-β phenotype similar to that found in some papillomas, factors in addition to hyperproliferation, such as altered differentiation, must be responsible for the hyperplasia found in papillomas (26).

The uncoupling of isoform loss in certain papillomas suggests that loss of TGF-β1 may be the initial alteration, and

Table 2. Hyperproliferation of TGF-β1-null epidermis

TGF-β1 genotype	BrdUrd labeling		
	Litter 1	Litter 2	Litter 3
−/−	11.2 ± 2.1	10.1 ± 1.2	13.8 ± 4.0
−/+	8.2 ± 2.6	2.4 ± 1.4	4.1 ± 2.1
		1.7 ± 0.4	
+/+	3.7 ± 1.2	—	5.3 ± 1.5

The difference in labeling index between TGF-β1-null and wild-type epidermis was significant ($P < 0.016$, litter 1; $P < 0.004$, litter 3; Wilcoxon rank sum test). Mice were injected intraperitoneally with BrdUrd (0.2 mg/g of body weight) 1 hr prior to sacrifice. BrdUrd staining was done as described in *Materials and Methods*. Labeling index was calculated as the number of BrdUrd-positive cells per total basal nuclei, determined from double fluorescence with Hoechst dye 33258. At least five counts of 100–200 total nuclei from two sections were made for each epidermis. Each number represents a single animal. At time of sacrifice, mice in litters 1 and 2 were 5 weeks old, and mice in litter 3 were 3 weeks old. No evidence of inflammation in the epidermis was seen in hematoxylin/eosin-stained tissue sections. The increased labeling index in the heterozygote animal of litter 1 is paralleled by a decrease in TGF-β1 fluorescence intensity relative to wild type. Epidermis of heterozygotes in litters 2 and 3 had TGF-β1 immunofluorescence staining similar to that of wild-type epidermis.

that the posttranscriptional block of expression is not necessarily coordinate. Posttranscriptional regulation of TGF-β expression has been observed in several cell culture systems (7, 27) and has been reported for TGF-β1 in dysplastic papillomas and carcinomas (9). While both low- and high-risk papillomas initiated with DMBA have identical *ras* mutations (28), high-level expression of the v-Ha-*ras* oncogene in cultured keratinocytes is sufficient to cause a posttranscriptional block of TGF-β2 expression (7). Thus differences in expression levels of the mutant Ha-*ras* allele between low- and high-risk papillomas could contribute to this phenotype.

These data support the concept that TGF-β is an antipromoter in this tissue (7, 9). Reduced expression of both TGF-β isoforms in papillomas compared to normal skin or chronically promoter-treated skin indicates that papillomatous outgrowth requires an altered balance between positive and negative growth signals (7, 29). The extent of expression of TGF-β in expanding initiated clones could explain the growth properties and promotion requirements of different papilloma populations.

Previous studies (30–33), and the benign histology of high-risk papillomas, indicate that multiple genetic changes in papillomas are required for malignant conversion. Documented effects of the TGF-β family on metalloproteinase expression (6) and suppression of tumorigenesis (21, 34) suggest that absence of TGF-β could contribute directly to the malignant phenotype. Additionally, aneuploidy and specific oncogenic changes due to chromosome instability are characteristic of progression in epidermal carcinogenesis (23, 31, 35). Although specific mutations may be required to create an unstable genome, it is possible that the increased cell proliferation in TGF-β-deficient papillomas enhances the probability for generation of aneuploid clones, producing some with changes relevant for malignancy. The enhanced sensitivity of high-risk papillomas to malignant conversion by additional carcinogen treatment (28) supports this hypothesis.

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