An Activating β1 Integrin Mutation Increases the Conversion of Benign to Malignant Skin Tumors

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Abstract

Identifying the physiologic relevance of cancer-associated genetic polymorphisms is a major challenge. Several changes in the coding sequence of β integrin subunits have now been described in human tumors. One of these, T188Iβ1, was identified as a heterozygous mutation in a poorly differentiated squamous cell carcinoma (SCC) and shown to activate extracellular matrix adhesion and inhibit keratinocyte differentiation *in vitro*. To study its contribution to tumor development, we overexpressed the mutant or wild-type (WT) human β1 subunit in the basal layer of mouse epidermis using the keratin 14 promoter. The transgenic integrins were expressed at the cell surface and were functional, with the T188Iβ1 subunit promoting cell spreading to a greater extent than WTβ1. Epidermal proliferation and differentiation were unaffected and no expansion of the stem cell compartment was detected. During chemical carcinogenesis, both transgenes increased papilloma formation, but only the T188Iβ1 transgene stimulated the conversion of papillomas to SCCs. Papillomas bearing the mutation showed increased Erk activity and reduced differentiation. SCCs expressing T188Iβ1 were less well-differentiated than those expressing WTβ1. These observations establish that the expression of a genetic variant in the I-like domain of β1 integrins does not affect normal epidermal homeostasis, but increases tumor susceptibility and influences tumor type. [Cancer Res 2009;69(4):1334–42]

Introduction

Integrin extracellular matrix receptors regulate many aspects of epidermal cell behavior (1). Within the basal layer of the human epidermis, stem cells express higher levels of integrins than cells that are committed to undergo terminal differentiation (2–4). Integrin signaling to Erk/mitogen-activated protein kinase (MAPK) plays a role in the maintenance of the stem cell compartment whereas signaling through Akt is involved in initiation of differentiation (5, 6). Studies with cultured human epidermal cells show that integrin ligation suppresses the onset of terminal differentiation, with ligated integrins sending a "do not differentiate" signal to the cells (7, 8). In mouse models, epidermal-specific deletion of specific integrins or modulation of integrin function lead to a range of phenotypes, from epidermal blistering to a failure to maintain the hair follicles (1, 9, 10).

Integrins are implicated in a variety of epidermal diseases (1, 11–16). Integrin expression or signaling is often altered in squamous cell carcinomas (SCC). In studies of chemically induced skin carcinogenesis, overexpression of integrins in the suprabasal layers alters susceptibility to tumor development (17–19).

We previously identified a heterozygous mutation in the β1 integrin subunit in cells from a poorly differentiated human SCC of the tongue (20). The mutation, T188I, lies in the specificity loop of the I-like domain (21, 22) and results in constitutive activation of ligand binding, thereby stimulating cell spreading at low extracellular matrix concentrations (20). Although expression of the mutation in cultured cells does not affect cell migration or invasion, it does lead to enhanced activation of Erk/MAPK signaling (20). In contrast to the wild-type β1 subunit, unligated T188Iβ1 does not trigger the initiation of terminal differentiation in human keratinocytes (20).

Sequencing of the β1 I–like domain in 124 human head and neck SCCs has revealed several additional single nucleotide changes, one of which results in a change in amino acid sequence (A239V; ref. 23). The same single nucleotide changes are present in normal tissue from patients, indicating germ line polymorphisms. In addition, sequencing by the Sanger Cancer Genome Project[4] has identified cancer-associated amino acid changes in the I-like domain of the β6 and β7 integrin subunits. We conclude that changes in the amino acid sequence of β integrin I–like domains are found at a low frequency in tumors and that they are probably polymorphisms rather than somatic mutations. In order to investigate the physiologic role of the T188I mutation, we expressed the human T188Iβ1 mutant or the wild-type human subunit in the epidermis of transgenic mice.

Materials and Methods

Generation of transgenic mice. Human wild-type and T188I mutant β1 integrin subunit cDNAs (24) were subcloned into the *Bam*HI restriction site of the keratin 14 (K14) promoter cassette kindly provided by E. Fuchs (Howard Hughes Medical Institute, Rockefeller University, New York, NY), and were injected into the male pronucleus of day 1–fertilized FVB/N mouse eggs. Potential founder lines were screened by PCR using one primer specific for β-globin (TACTCTGAGTCCAAACCGGGC) and one specific for the human integrin β1 subunit (CAATTTGGCCCTGCTTGTATACATTCTCCA). K14T188Iβ1 founder lines were 4826B, 4828B (low), and 4828B (high). K14WTβ1 founders were 4898A and 4837A. Founder lines 4828B (low) and 4837A had lower transgene copy numbers than the other lines.

Experimental procedures on mice. Experiments were subject to Cancer Research UK ethical review and were performed under the terms of a U.K. Government Home Office license. Bromodeoxyuridine (BrdUrd) injections were performed as previously described (25).

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[4] http://www.sanger.ac.uk/genetics/CGP/Studies/studies.shtml

Chemical carcinogenesis experiments were performed on 7-week-old female K14WTβ1 (line 4898A) and K14T188Iβ1 (4828B, high) mice, and nontransgenic littermates (25 animals/group), essentially as previously described (17–19). Mice received one topical application of 100 nmol of (25 μg) 7,12-dimethylbenz(*a*)anthracene (DMBA; Sigma-Aldrich) in 200 μL of acetone followed by twice weekly applications of 6 nmol of (3.7 μg) 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich) in 200 μL acetone. As controls, transgenic and nontransgenic littermates (5–10 animals/group) were subjected to the same protocol but substituting DMBA or TPA with acetone. Papillomas and SCC were recorded once a week for up to 54 weeks after the start of promotion. Tumor sections were graded as described previously (17).

Tissue processing. For sections, tissue was either fixed in 10% neutral-buffered formalin and embedded in paraffin or frozen in liquid nitrogen–cooled isopentane and then embedded in OCT embedding matrix (Raymond A Lamb, UK). Epidermal whole mounts of tail skin were prepared as described previously (25), with minor modifications.

Antibodies. The following monoclonal antibodies were used: AIIB2, TS2/16, DH12, and P5D2 (anti-human β1 integrin subunit; refs. 26, 27); LHK15 (anti-keratin 15; ref. 25), anti-BrdUrd (clone 3D4; BD Pharmingen), MB1.2 (anti-mouse β1 integrins; ref. 25), and GoH3 (anti-integrin α6 chain; BD Pharmingen). Rabbit antibodies were anti-phosphorylated p44/42 MAPK (Thr^{202}/Tyr^{204}) and anti-p44/42 MAPK (Cell Signaling Technology, Inc.), anti-mouse K14 (Covance Research Products, Inc.), anti-laminin (Sigma-Aldrich), and anti-Ki67 (Neomarkers). AlexaFluor 488– or 594–conjugated secondary antibodies were obtained from Invitrogen, Corp. Donkey anti-rabbit biotin antibody was from The Jackson Laboratory.

Immunohistochemistry and *in situ* hybridization. Ki67, laminin, and phosphorylated Erk were detected with the Bond Intense R Detection kit (Leica Microsystems) in formalin-fixed, paraffin-embedded sections following antigen retrieval. Sections were photographed and analyzed using the Ariol SL-50 system (Applied Imaging, Corp.).

For immunofluorescence staining, frozen sections were fixed in 4% PFA/PBS for 10 min; paraffin sections were dewaxed and rehydrated. Sections were permeabilized in 0.3% Triton X-100 for 5 min and blocked with 2% bovine serum albumin (BSA), 0.02% fish skin gelatin, and 10% FCS for 2 h in PBS at room temperature. Sections were incubated in primary antibody overnight at 4°C, washed in PBS and incubated with secondary antibody for 45 min at room temperature. Epidermal whole mounts were labeled as described previously (25). *In situ* hybridization was performed as previously described (26).

Keratinocyte culture. Keratinocytes were isolated from 7- to 8-week-old mouse dorsal skin (28) and cultured on confluent J2-3T3 feeders in type I collagen-coated flasks in calcium-free FAD medium (three parts DMEM medium and one part F12 medium supplemented with 1.8×10^{-4} mol/L adenine) supplemented with 10% FCS, hydrocortisone, insulin, cholera toxin, and epidermal growth factor. Spontaneously immortalized lines arose after approximately 10 passages.

The growth rates of immortalized lines were compared by plating 5×10^4 cells per well in six-well plates in complete keratinocyte serum-free medium (Life Technologies) without feeders. Wells were precoated with 10 μg/mL of human plasma fibronectin. Triplicate wells were harvested for each time point. Cell number was determined using the CellTiter 96 AQueous One Solution cell proliferation assay kit from Promega, measuring absorbance at 490 nm.

To determine colony-forming efficiency, 400 or 2,000 primary keratinocytes were plated per well in six-well type I collagen-coated plates (BD Pharmingen). After 14 days, cultures were fixed and stained with 1% rhodamine B and 1% Nile blue (Acros Organic; ref. 2). Colony-forming efficiency was defined as the percentage of plated cells that formed a colony of three or more cells.

Flow cytometry. Single cell suspensions were incubated for 20 min on ice with anti–β1 integrin antibodies diluted in prechilled PBS. After washing in chilled PBS, cells were incubated with the appropriate secondary antibodies as before. Flow cytometric analysis was performed using the FACSCalibur (BD FACSCalibur System, BD Biosystems) and FlowJo software (Tree Star, Inc), excluding dead, 7AAD-positive cells, and differentiated cells with high forward and side scatter.

Cell adhesion assays. Microtiter plates (96-well) were coated with 50 μL of human plasma fibronectin (Chemicon) or human placenta laminin (Sigma-Aldrich) overnight at 4°C and blocked with 1% heat-denatured BSA in PBS. 2×10^4 cells were added per well and incubated at 37°C for 30 min in serum-free medium containing 0.5% BSA. After washing, cells were fixed with 4% PFA in PBS and stained with Diff-Quik (International Reagents, Japan). Spread cells (defined as cells in which the long axis was more than twice the diameter of the nucleus) were counted in three independent fields per well. In some experiments, cells were incubated with 10 μg/mL of P5D2 antibody for 20 min at room temperature prior to plating.

To visualize filamentous actin, cells were fixed with 4% PFA in PBS for 10 min, permeabilized with 0.1% Triton X-100/PBS for 5 min and stained with phalloidin-conjugated Alexa-555 (Invitrogen, Corp.).

Integrin turnover time. Adherent subconfluent keratinocytes were surface-labeled with 1 mg/mL of EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in PBS (pH 8.0) for 15 min at room temperature. Cells were washed twice with serum-free, calcium-free FAD medium and incubated in complete medium at 37°C for 0, 4, 8, or 20 h. Cells were harvested with trypsin/EDTA, incubated with P5D2 antibody for 30 min on ice and washed twice with ice-cold PBS. Pellets containing 4×10^6 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mmol/L Tris-HCl (pH 8.0), and 150 mmol/L NaCl] with proteinase inhibitors (Roche). Cell lysates were clarified by centrifugation, then 20 μL of a 50% slurry of UltraLink Immobilized Protein G Plus Gel (Pierce) was added and incubated for 1 h at 4°C with gentle agitation. Beads and immune complexes were washed thrice with 0.5% Nonidet P-40, 0.6 mol/L of NaCl, 50 mmol/L of Tris-HCl (pH 8.3), resuspended in SDS-PAGE sample buffer without added reducing agent, boiled for 3 min, and resolved on 4% to 12% gradient polyacrylamide gels.

Following SDS-PAGE, separated proteins were transferred to polyvinylidene difluoride membranes, blocked with 5% skimmed milk in TBS for 30 min and incubated with extrAvidin-peroxidase (Sigma-Aldrich) in RIPA buffer for 1 h at room temperature. After washing, extrAvidin-peroxidase was visualized with enhanced chemiluminescence reagents (Amersham).

Erk signaling. Cells were starved overnight in serum-free, calcium-free FAD, and were plated (3.5×10^5 cells) in a 60 mm dish coated with 10 μg/mL of fibronectin in the same medium supplemented with 0.5% BSA. Protein lysates were prepared in RIPA buffer containing protease and phosphatase inhibitors. Lysates were examined by Western blotting with anti Erk antibodies, essentially as described above, except that nitrocellulose membrane was used and the blocking buffer consisted of 2.5% skimmed milk powder and 0.05% Tween 20.

Results

Wild-type and mutant human β1 integrin subunits are expressed at similar levels in transgenic epidermis. We used the K14 promoter to target transgene expression to the known locations of epidermal stem cells (29). We initially compared three transgenic lines expressing the T188I mutant β1 subunit (T188Iβ1) and two expressing the human wild-type subunit (WTβ1). Mice from all founder lines were viable and fertile and none had any gross phenotypic abnormalities. We selected one founder line expressing each transgene for further analysis.

Transgene expression was confirmed by radioactive *in situ* hybridization using a probe specific for the human β1 integrin. No signal was detected in sections of nontransgenic mice (Fig. 1*A*). In mice expressing either the wild-type human β1 subunit (K14WTβ1) or the mutant subunit (K14T188Iβ1), the transgene was detected in the basal layer of the interfollicular epidermis, the sebaceous gland and hair follicle outer root sheath (Fig. 1*A*). Using antibodies specific for the human (Fig. 1*B*) or mouse β1 integrins

(Fig. 1*C*), we confirmed that the transgene-encoded integrins were coexpressed with the endogenous β1 integrins. However, in the K14WTβ1 epidermis, staining for endogenous β1 integrin was reduced (Fig. 1*C*).

Cell surface β1 integrin levels were examined in primary keratinocyte cultures or immortalized keratinocyte lines. Cells were labeled with TS2/16 or DH12, two monoclonal antibodies to the human β1 integrin, or MB1.2, specific for mouse β1 integrins (Fig. 1*D*). Flow cytometry established that cells from K14WTβ1 and K14T188Iβ1 transgenic mice expressed similar levels of human β1 integrins, with the caveat that the T188I mutation might affect the epitopes detected by the antibodies (Fig. 1*D*). In cells expressing either transgene, the level of surface mouse β1 integrins was decreased relative to transgene-negative control cells, the effect being more marked in cells expressing the wild-type transgene (Fig. 1*C* and *D*).

Down-regulation of the endogenous mouse β1 integrin is expected because surface levels of the β1 subunit are dependent on the availability of endogenous α subunit partners (30). However, the fact that mouse β1 was not completely lost from the cell surface indicates that the T188Iβ1 mutant integrin was coexpressed on the cell surface with wild-type (mouse) β1 (20).

To compare the turnover time of the transgene-encoded human β1 integrins (31), adherent keratinocytes were surface-labeled with biotin, chased for different periods of time, and immunoprecipitated with P5D2 (Fig. 2*A*). Consistent with the flow cytometry data, the levels of expression of WTβ1 and T188Iβ1 were similar. Two bands were detected by immunoprecipitation, corresponding to the mature β1 subunit and its α partners. Between 0 and 8 hours, the levels of the wild-type and mutant human β1 subunits were unchanged. By 20 hours, surface levels of both mutant and wild-type subunits had decreased to a similar extent. P5D2 displayed weak cross-reactivity with the endogenous mouse β1 integrin, and immunoprecipitation of surface-labeled transgene-negative keratinocytes revealed that the kinetics of loss from the cell surface were similar to those of the transgene-encoded integrins (Fig. 2*A*).

The T188Iβ1 mutation promotes Erk/MAPK signaling, cell spreading, and actin cytoskeletal assembly, but not proliferation. To examine Erk/MAPK activation in transgenic keratinocytes, starved cells were plated on fibronectin for up to 150 minutes (Fig. 2*B*). The kinetics of Erk/MAPK activation were similar in nontransgenic and K14WTβ1 transgenic cells. However, in K14T188Iβ1 transgenic cells, Erk/MAPK activation was greater at all time points examined (20). The ratio of phosphorylated Erk to total Erk band intensity at 40 minutes was 0.34 for nontransgenic cells, 0.27 for K14WTβ1 cells, and 1.63 for K14T188Iβ1 cells. In spite of the increased Erk activation, the T188I mutation did not increase the growth rate of immortalized keratinocytes on fibronectin-coated dishes under feeder-free, low calcium culture conditions (Fig. 2*C*).

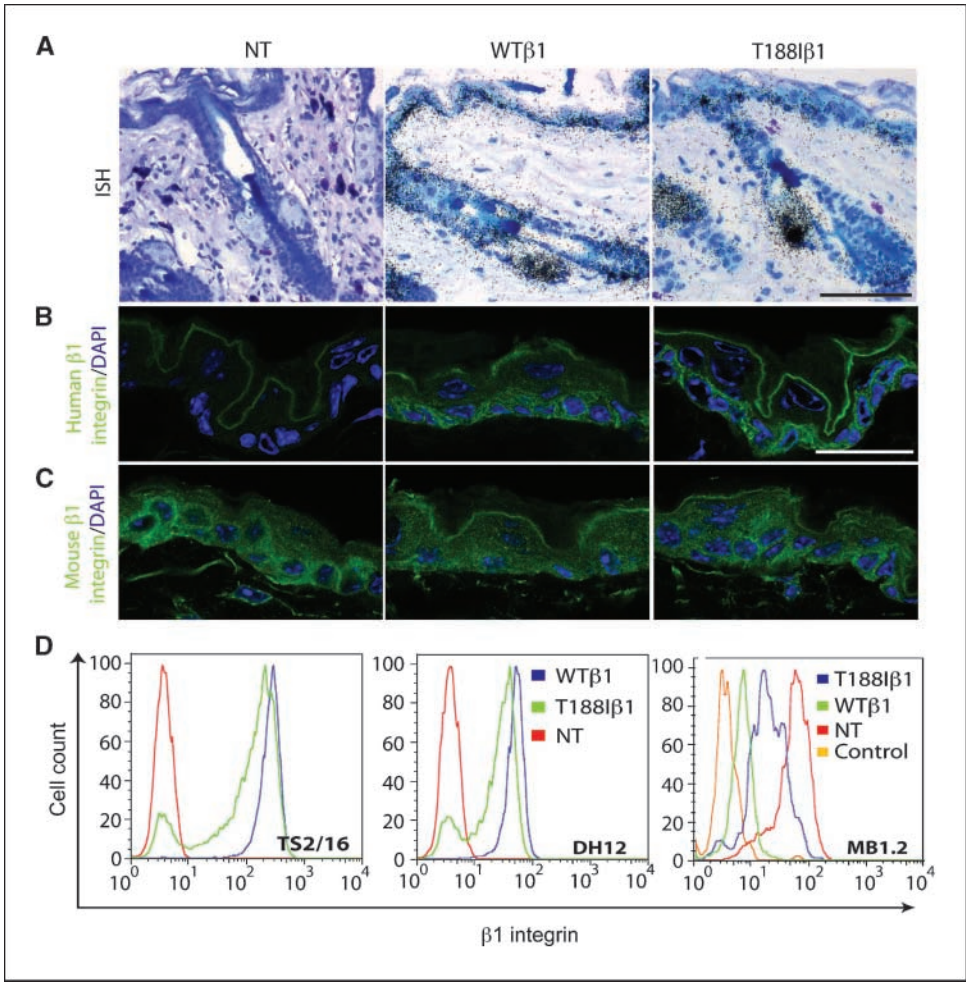

Figure 1. Transgene expression in adult dorsal epidermis and cultured keratinocytes. *A, in situ* hybridization (*ISH*) with probe specific for human β1 integrin subunit. *B* and *C,* immunofluorescence staining for human (*green*; *B*) and mouse (*green*; *C*) β1 integrins with 4′,6-diamidino-2-phenylindole (*DAPI*) nuclear counterstain (*blue*). *Bars,* 60 μm (*A*), 25 μm (*B* and *C*). *D,* flow cytometry of viable basal keratinocytes labeled with the antibodies shown. *Yellow line*, NT cells labelled with secondary antibody only. *NT,* nontransgenic; *WTβ1,* K14WTβ1 transgenic; *T188Iβ1,* K14T188Iβ1 transgenic.

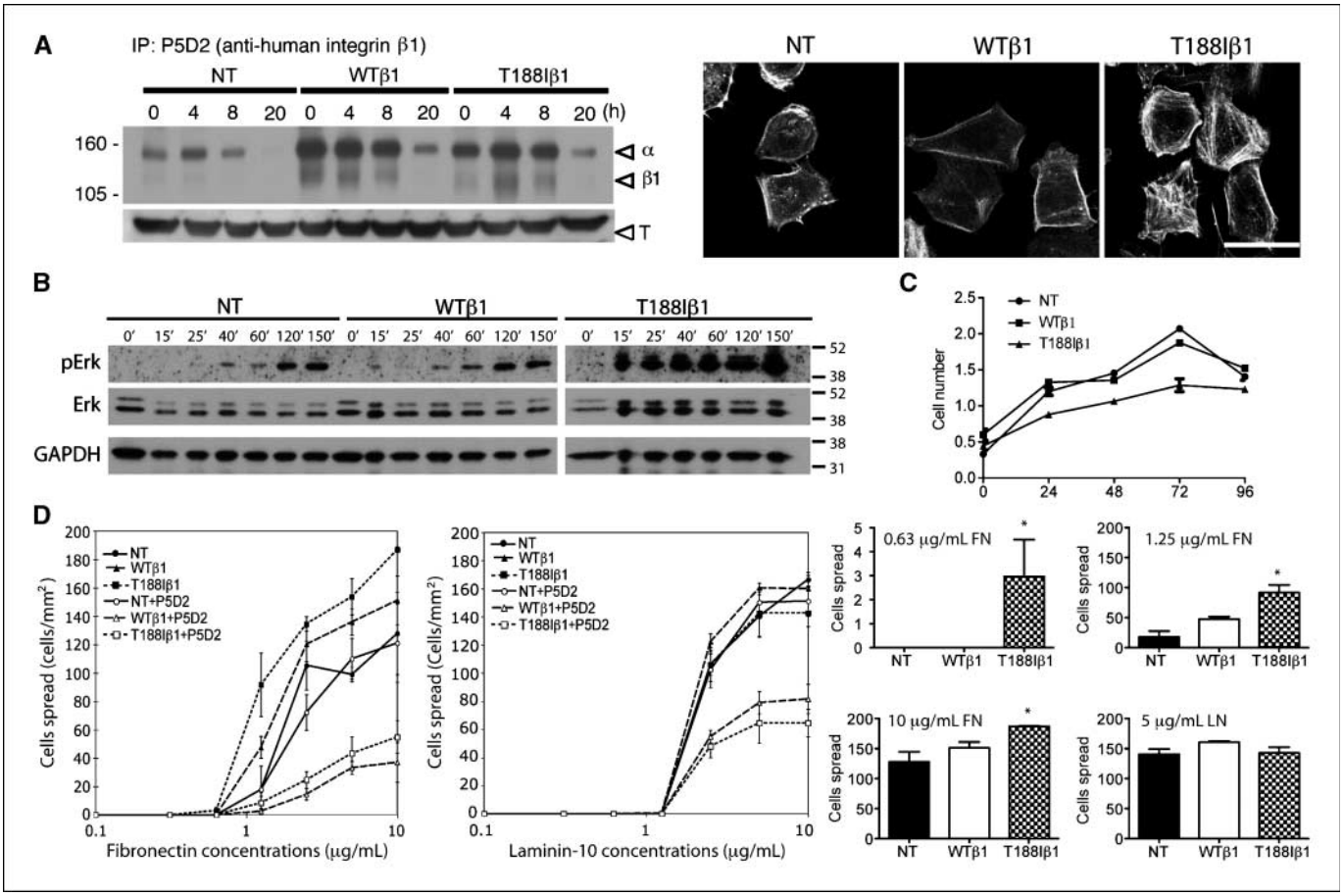

Figure 2. Effects of transgenic integrins on keratinocyte signaling, adhesion, and proliferation. *A, left,* immunoprecipitation of surface-biotinylated β1 integrins in cells harvested immediately after labeling (0 h) or after the chase periods shown. *Arrows,* α and β1 integrin subunits. *Bottom,* β-tubulin immunoblot of total cell lysates (*T*). *Right,* Cells plated for 30 min on 20 μg/mL of fibronectin stained with phalloidin-conjugated Alexa-555. *Bar,* 50 μm. *B,* Western blot of keratinocytes seeded on 10 μg/mL of fibronectin for the number of minutes indicated. Time zero, single cell suspension prior to plating. Blots were probed with the antibodies indicated. *A* and *B,* molecular weights (kDa) are indicated. *C,* proliferation of keratinocytes on fibronectin-coated dishes. *Y*-axis, absorbance at 490 nm. *Points,* mean; *bars,* SE. *D,* immortalized keratinocytes were plated for 30 min on the concentrations of fibronectin or laminin-10 shown. Statistical significance of number of spread cells was evaluated (*histograms*). *, $P < 0.05$ (unpaired Student's *t* test). *NT,* nontransgenic; *WTβ1,* K14WTβ1 transgenic; *T188Iβ1,* K14T188Iβ1 transgenic.

We next examined adhesion on fibronectin, which is mediated by α5β1, and laminin-10, which is mediated by α3β1 and α6β4 in combination (1). On fibronectin, transgene-positive keratinocytes adhered and spread to a greater extent than transgene-negative keratinocytes. The effect was greatest in cells expressing T188Iβ1 plated on low fibronectin concentrations (ref. 20; Fig. 2*D*). T188Iβ1 was also more effective than WTβ1 in promoting the polymerization of the actin cytoskeleton in cells plated on fibronectin, as evidenced by more intense labeling with phalloidin (Fig. 2*A*). Adhesion of transgenic keratinocytes was markedly reduced by incubation with the anti-human β1 antibody P5D2. In contrast, P5D2 had no effect on transgene-negative keratinocytes (Fig. 2*D*).

Keratinocyte adhesion and spreading on laminin-10 were similar in transgene-negative and transgene-positive keratinocytes, as predicted because of the role of α6β4 (ref. 1; Fig. 2*D*). Nevertheless, the transgene-encoded β1 integrins did contribute because adhesion of transgenic keratinocytes to laminin-10 could be partially inhibited by P5D2 (Fig. 2*D*).

The T188Iβ1 integrin mutation does not disturb epidermal organization or homeostasis. Comparison of the histology of adult back skin from transgenic mice and transgene-negative littermate controls did not reveal any differences in the interfollicular epidermis, hair follicles, or sebaceous glands, and there was no evidence of a dermal inflammatory infiltrate (Fig. 3*A*). Proliferation was evaluated by staining sections of adult back skin for Ki67 (data not shown) and by measuring BrdUrd incorporation into S phase cells in tail epidermal whole mounts (Fig. 3*B*). K14WTβ1 and K14T188Iβ1 transgenic mice exhibited a similar number of proliferative cells in the back and tail interfollicular epidermis and hair follicles to transgene-negative littermates.

Keratin 15 (K15) and DNA label retention are markers of bulge stem cells (25, 32). No differences in K15 expression were observed between transgenic and nontransgenic epidermis examined in whole mount preparations of tail (Fig. 3*C*). In addition, there were no differences in the number of label retaining cells, either within the hair follicles or interfollicular epidermis (Fig. 3*D*).

As a further stem cell assay, we determined the colony-forming efficiency of primary adult transgene-positive and transgene-negative keratinocytes in culture (Fig. 3*D*; ref. 33). Whereas cells from nontransgenic and K14T188Iβ1 mice gave rise to a similar percentage of colonies, keratinocytes from K14WTβ1 mice had a decreased ability to form colonies (Fig. 3*D*).

WTβ1 and T188Iβ1 transgene expression stimulates papilloma development. To determine whether the T188Iβ1 integrin mutation influenced skin carcinogenesis, transgenic mice and their respective transgene-negative littermates were subjected to two-stage carcinogenesis with DMBA (to induce Ha-Ras mutations) and TPA (to promote tumor formation; ref. 17; Fig. 4). Macroscopically, papillomas were identified as pedunculated or sessile mushroom-like lesions, whereas SCCs presented as "craters" with signs of dermal ingrowth.

Papillomas first emerged in all groups between 7 and 11 weeks after the start of TPA promotion, and the maximum number of papillomas was reached by 20 weeks (Fig. 4*A* and *B*; data not shown). No papillomas developed in mice treated with DMBA or TPA only (data not shown).

The proportion of mice that developed papillomas was not increased by expression of either transgene (29 of 50 for T188Iβ1 versus 32 of 50 for nontransgenic mice and 46 of 50 for wild-type β1 versus 50 of 50 for nontransgenic mice). However, both K14WTβ1 and K14T188Iβ1 transgenic mice developed significantly more papillomas than nontransgenic mice (Student's t test, $P < 0.0001$; Fig. 4*A* and *B*).

The T188Iβ1 integrin mutation stimulates malignant conversion. In K14WTβ1 mice and nontransgenic littermates, SCC first emerged between 16 and 20 weeks, reaching a plateau by

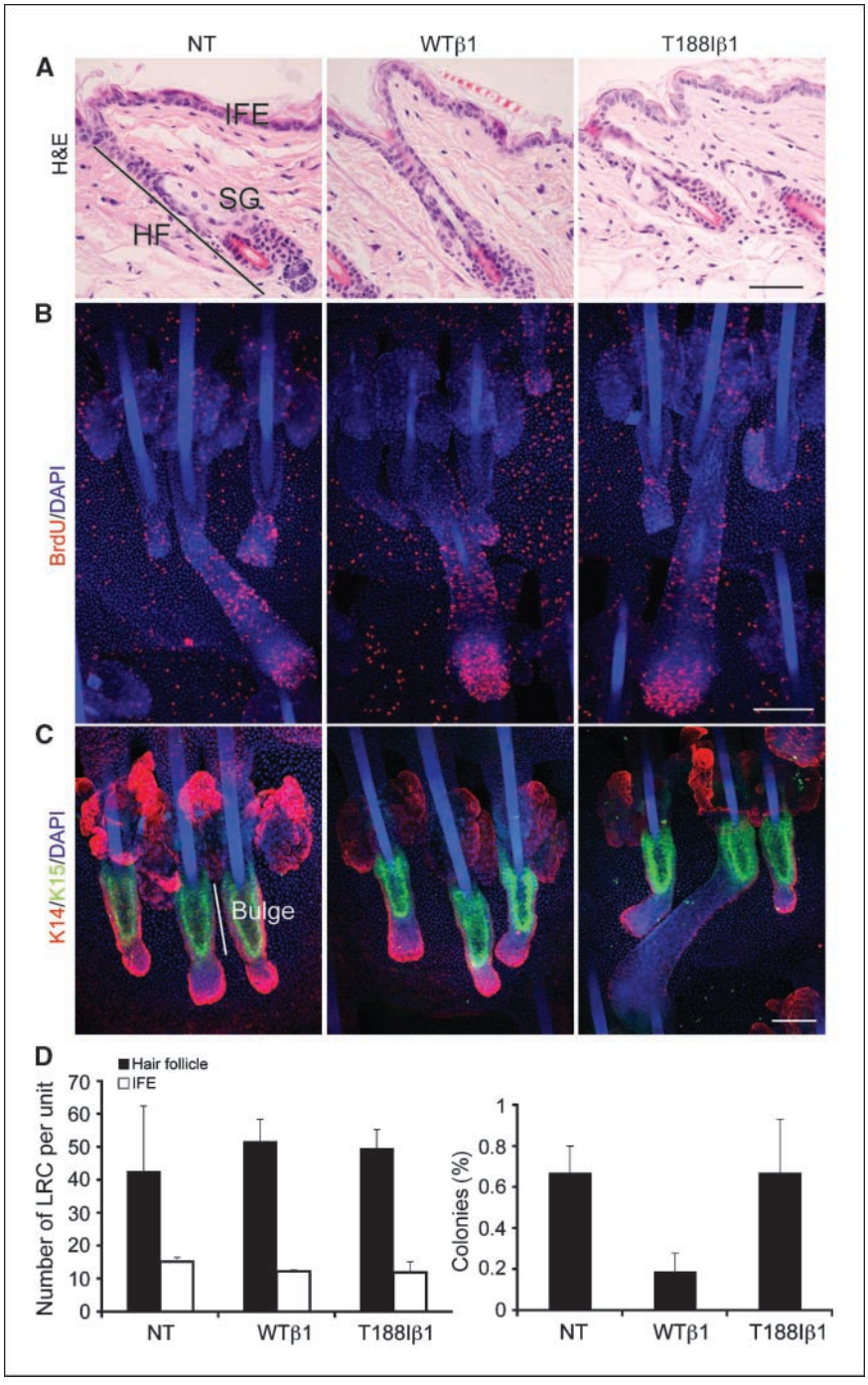

Figure 3. Transgene expression does not affect epidermal proliferation or result in expansion of the stem cell compartment. *A,* H&E-stained sections of adult mouse dorsal skin. *IFE,* interfollicular epidermis; *SG,* sebaceous gland; *HF,* hair follicle. *B* and *C,* adult tail epidermal whole mounts labeled with anti-BrdUrd to detect S phase cells (*red; B*) or K15 (*green; C*) with DAPI nuclear counterstain (*blue*). Double labeling for K14 (*red; C*) reveals epidermal morphology. *Bars,* 100 μm (*A–C*). *D,* quantitation of DNA label-retaining cells (*LRC*) per epidermal unit (ref. 28; *left*) and percentage of colony-forming cells (*right*). Label-retaining cells in hair follicles and interfollicular epidermis (*IFE*) are shown separately. *Columns,* average number of label-retaining cells in five epidermal units per mouse from three mice per genotype; *bars,* SD. Colony-forming efficiency is the average of six wells per mouse, four mice per genotype ± SD. *NT,* nontransgenic; *WTβ1,* K14WTβ1 transgenic; *T188Iβ1,* K14T188Iβ1 transgenic.

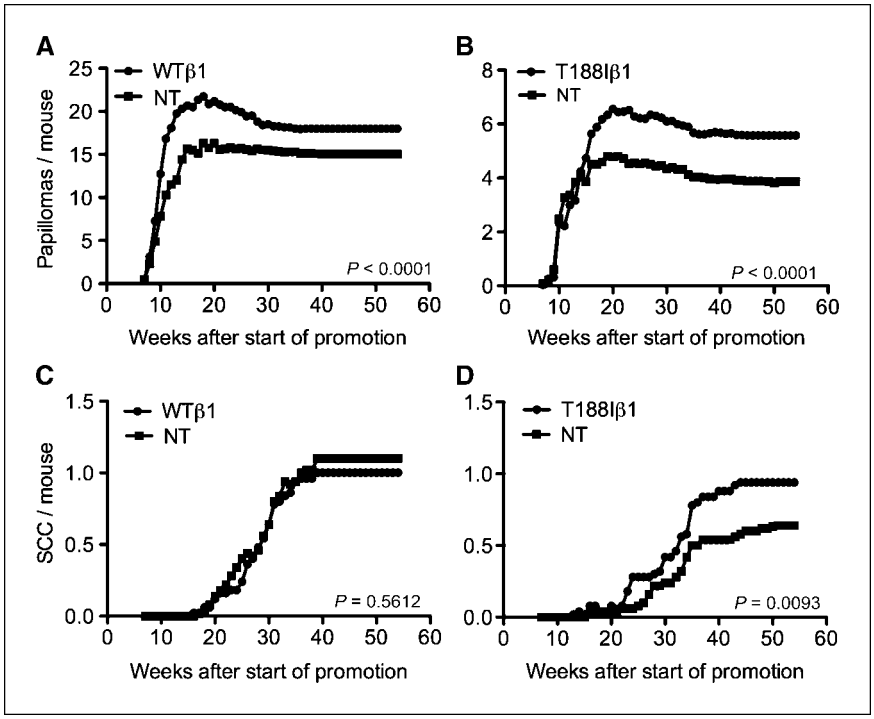

Figure 4. Papillomas and SCCs induced by chemical carcinogenesis. Number of papillomas (*A* and *B*) or malignant tumors (*SCC*; *C* and *D*) per mouse is shown relative to the start of promotion with TPA. *A* and *C*, K14WTβ1 transgenic mice (*WTβ1*) and transgene-negative littermate controls (*NT*). *B* and *D*, K14T188Iβ1 transgenic mice (*T188Iβ1*) and transgene-negative littermate controls (*NT*). Data pooled from two replicate experiments, each with a starting cohort of 25 mice per group. *P* values (unpaired two-tailed Student's *t* test) show the difference between transgenic and nontransgenic mice.

40 weeks (Fig. 4*C*). There was no significant difference in the number of SCCs in K14WTβ1 transgenic and transgene-negative mice (Student's *t* test, $P = 0.5612$). In addition, the wild-type transgene did not increase SCC incidence (29 of 50 for WTβ1 and 36 of 50 for nontransgenic mice).

K14T188Iβ1 transgenic mice started to develop SCC 3 weeks earlier than nontransgenic and K14WTβ1 transgenic mice, exhibiting malignancies by week 16 after the start of TPA promotion (Fig. 4*D*). Whereas both transgenes stimulated papilloma formation (Fig. 4*A* and *B*), only K14T188Iβ1 transgenic mice developed more SCC than littermate controls (Student's *t* test, $P = 0.0093$). The incidence of SCC was also higher in K14T188Iβ1 mice than in the nontransgenic controls (32 of 50 for T188Iβ1 and 26 of 50 for nontransgenic mice).

Metastasis is increased in transgenic mice. The reproductive tract, liver, lung, spleen, and lymph nodes of mice from the chemical carcinogenesis experiments were screened histologically for the presence of tumor cells. Both transgenes stimulated lymph node metastasis. In the K14T188Iβ1 group, 14 of 50 mice developed metastases, whereas 8 of 50 littermate controls developed metastases. Thirteen of 50 K14WTβ1 mice developed metastases compared with 8 of 50 controls.

T188Iβ1 papillomas are less highly differentiated than WTβ1 papillomas. Papillomas were scored as well or poorly differentiated on the basis of whether or not they contained a high proportion of cornified cells (Fig. 5*A* and *B*). In well differentiated papillomas, the boundary between the basal layer and the adjacent stroma was clearly defined, whereas in poorly differentiated papillomas, there were often regions in which the basal cell layer invaded the stroma (Fig. 5*A*). The basement membrane was intact in both categories of papillomas, but laminin deposition was more diffuse in the regions of basal layer disturbance (Fig. 5*A*). At 20 to 30 weeks after DMBA treatment, the time when conversion to SCC occurs (Fig. 4*C* and *D*), the proportion of well differentiated papillomas was significantly lower in K14T188Iβ1 mice (total number of papillomas examined: $n = 42$) than in K14WTβ1 mice ($n = 44$) and transgene-negative littermates ($n = 110$, Pearson's χ^2 test; Fig. 4*B*).

In cultured keratinocytes, integrin signaling through Erk/MAPK suppresses terminal differentiation (5, 34). To investigate whether Erk/MAPK signaling was influenced by the T188I mutant *in vivo*, we immunostained sections with an antibody to phosphorylated Erk (Fig. 5*C* and *D*). Between 60% and 90% of the papillomas that developed in nontransgenic (total number of papillomas examined: $n = 152$) and K14WTβ1 transgenic mice ($n = 68$) were negative for phosphorylated Erk, whereas ~90% ($n = 43$ papillomas examined in total) of the T188Iβ1 papillomas were positive (Pearson's χ^2 test; Fig. 5*D*).

K14T188Iβ1 transgenic mice are more likely to develop poorly differentiated SCCs than K14WTβ1 and nontransgenic mice. All the SCCs from every tumor-bearing mouse in the chemical carcinogenesis experiments were assigned to one of four categories (Fig. 6*A*) based on Broders' classification (17). Undifferentiated tumors (spindle cell carcinomas) contained cells of fibroblastic morphology (Broders' grade 4). Well (grade 1), moderately (grade 2), and poorly (grade 3) differentiated tumors had a decreasing proportion of differentiated epithelial cells (Fig. 6*A*). In undifferentiated tumors, there were proliferating, Ki67-positive cells throughout the tumor mass (Fig. 6*B*). There was also widespread proliferation in the poorly differentiated tumors (Fig. 6*B*), whereas in the moderately and well differentiated tumors, proliferation was confined to the cell layers closest to the tumor stroma (Fig. 6*B*).

The frequency of undifferentiated (spindle) tumors was higher in K14WTβ1 transgenic (total number of SCC analyzed: $n = 24$) and nontransgenic mice ($n = 50$) than in K14T188Iβ1 transgenic mice ($n = 35$; Pearson's χ^2 test; Fig. 6*C*). In contrast, the frequency of poorly differentiated tumors was significantly higher in K14T188Iβ1 transgenic mice (Fig. 6*C*).

Although the distribution of proliferating cells differed between the different tumor types (Fig. 6*B*), the proportion of proliferating cells within the Ki67-positive regions (demarcated in Fig. 6*B*) did not differ significantly between tumor types nor between transgenic and transgene-negative mice (Fig. 6*D*). This suggests that the effect of the T188I mutation is primarily on tumor differentiation rather than on proliferation.

Discussion

We have developed an *in vivo* model to examine the effect of a tumor-associated integrin. Expression of the T188I mutation did not affect normal skin architecture or homeostasis, but did increase the susceptibility of the epidermis to developing malignant tumors and also reduced tumor differentiation.

The transgenic human β1 integrins were coexpressed with endogenous mouse β1 at the cell surface, were functional in mediating extracellular matrix adhesion, and had the same turnover time as the endogenous mouse β1 integrins. The T188I mutation enhanced Erk/MAPK signaling and actin cytoskeletal assembly, and promoted cell spreading on low fibronectin concentrations to a greater extent than the wild-type subunit (20).

In comparison with transgene-negative mice, there was no change in proliferation and no evidence for any disruption of normal skin architecture or epidermal homeostasis in K14WTβ1 and K14T188Iβ1 transgenic mice. This was unexpected because high integrin expression is an epidermal stem cell marker (2–4), and integrins regulate keratinocyte growth and differentiation (5, 20, 35–38). Thus, whereas integrin-mediated adhesion is required for the maintenance of the epidermal stem cell niche, β1 integrin activation is not sufficient to expand the stem cell compartment.

Expression of either the wild-type or mutant β1 transgene resulted in increased development of papillomas and increased lymph node metastases. This is consistent with reports that β1 integrin deletion impairs tumor initiation and maintenance (39). The positive effect of the transgenic integrins on papilloma development does not reflect simple overexpression of β1 integrins at the cell surface (Fig. 1; ref. 30). Instead, it is likely to reflect the fact that the K14 promoter is not subject to the same negative regulation as the endogenous mouse β1 promoter (10, 24).

Three effects were selectively attributable to the T188I mutant integrin: more rapid conversion of papillomas to SCCs, a higher conversion frequency, and a higher proportion of poorly differentiated tumors. Expression of T188Iβ1 reduced differentiation in both the papillomas and SCCs, without affecting proliferation in the SCCs. This is consistent with the observation that the T188I mutation was found in a poorly differentiated SCC and that it has an impaired ability to trigger the initiation of terminal differentiation of keratinocytes in culture (20).

One surprising effect of the T188I mutation was that it reduced the number of spindle cell tumors. These tumors are believed to arise from SCCs by a process of epithelial-mesenchymal transition (40). It is possible that this requires the down-regulation of extracellular matrix adhesion, which would be inhibited by the T188Iβ1 integrin transgene. The T188I mutation may prevent the changes in actin cytoskeletal assembly associated with spindle cell tumor formation (41). Alternatively, expression of the mutant integrin may decrease responsiveness to transforming growth factor-β (18, 19, 42, 43) or negatively regulate metalloproteinase expression (44), both of which are implicated in the formation of spindle cell tumors.

K14T188Iβ1 papillomas had high levels of phosphorylated Erk (Fig. 5), which is likely to contribute to their reduced differentiation, and expression of T188Iβ1 enhanced Erk/MAPK signaling *in vitro* (Fig. 2*B*; refs. 5, 20). β1 Integrin signaling through Erk/MAPK is associated with the maintenance of the stem cell

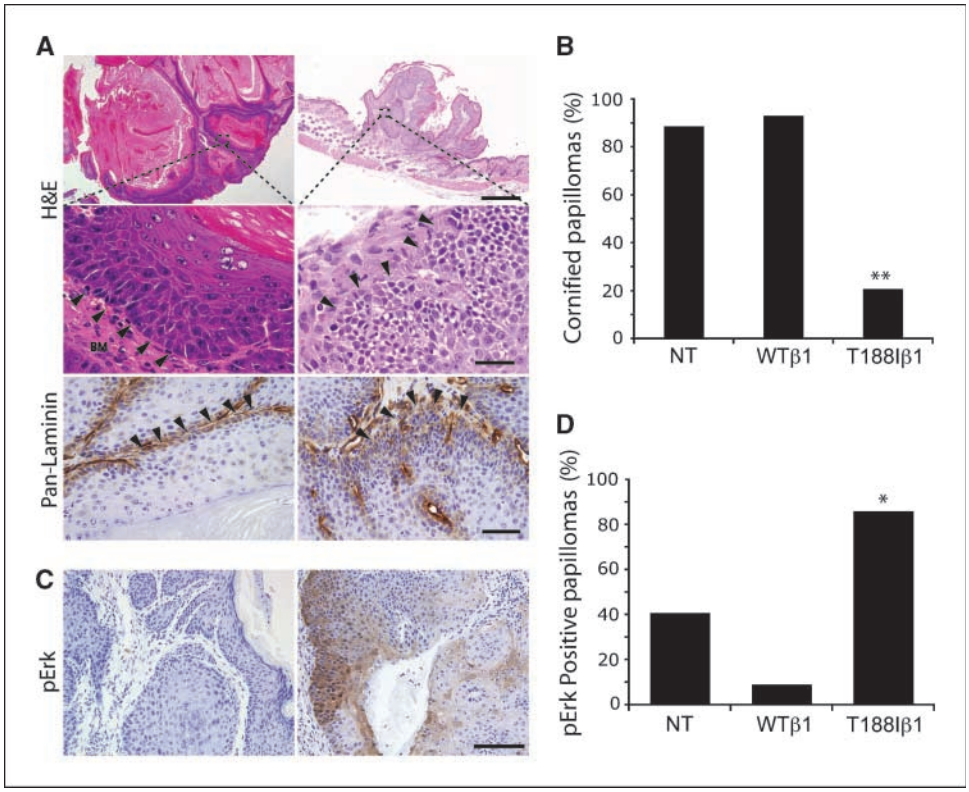

Figure 5. Papilloma differentiation. *A* and *C,* sections of papillomas stained with H&E (*A, top* and *middle*), anti-laminin (*brown; A, bottom*), or anti-phosphorylated Erk (*brown; C*) with hematoxylin counterstain. *Middle row,* higher magnification views of regions from the top row. *Left,* well differentiated papillomas; *right,* poorly differentiated papillomas. *Arrowheads,* boundary between epithelium and stroma. *Bars,* 200 μm (*A*), 100 μm (*C*). *B,* quantitation of percentage of well-differentiated papillomas 20 to 30 weeks after the start of promotion [number of papillomas examined: 110 (*NT*), 44 (*WTβ1*), and 42 (*T188Iβ1*)]. *D,* percentage of phosphorylated Erk–positive papillomas [total examined: 152 (*NT*), 68 (*WTβ1*), and 43 (*T188Iβ1*)]. *NT,* nontransgenic; *WTβ1,* K14WTβ1 transgenic; *T188Iβ1,* K14T188Iβ1 transgenic. Statistical analysis was performed using Pearson's χ^2 test (*, $P < 0.01$; **, $P < 0.001$).

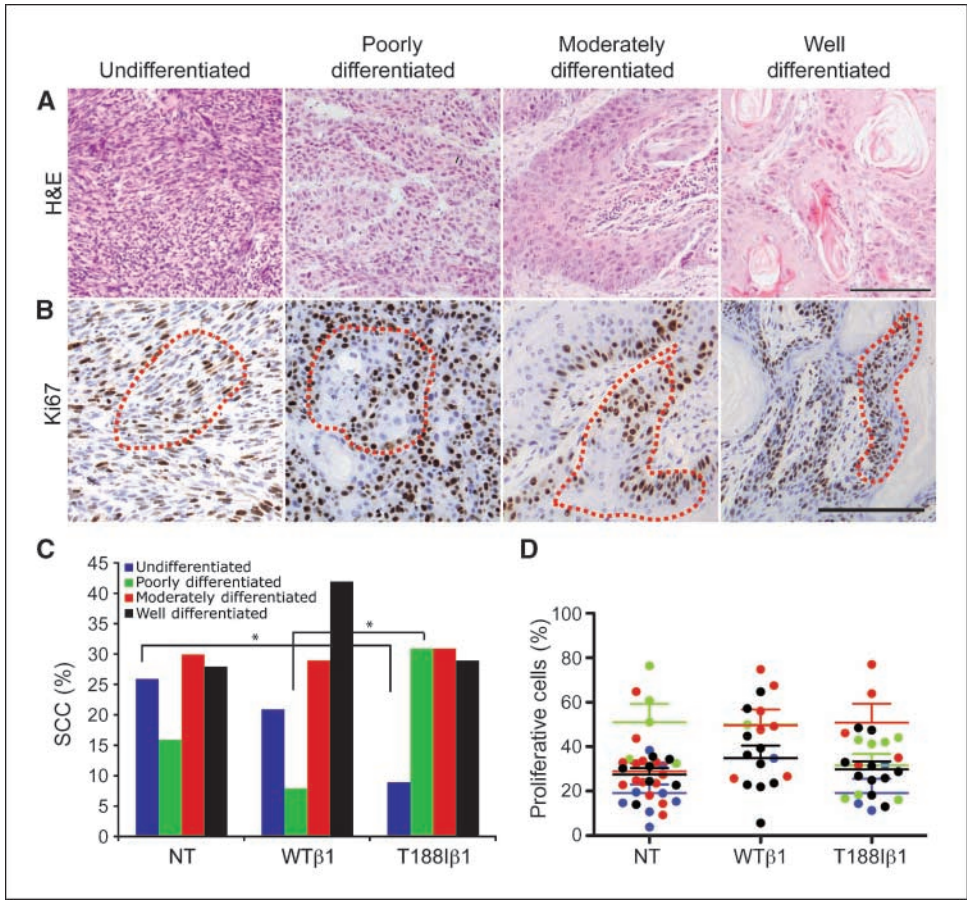

Figure 6. Different types of malignant tumors. Sections of malignant tumors labeled with H&E (*A*) or anti-Ki67 (*brown*) with hematoxylin counterstain (*B*). Red dotted lines in *B* demarcate proliferative regions quantitated in *D*. *Bars,* 100 μm. *C* and *D,* quantitation of proportion of each tumor type (*C*) and percentage of Ki67-positive cells within the proliferative regions of each tumor type (*D*). *NT,* nontransgenic; *WTβ1,* K14WTβ1 transgenic; *T188Iβ1,* K14T188Iβ1 transgenic (*, $P < 0.05$; Pearson's χ^2 test).

compartment in cultured human epidermis (5, 34), and is linked to papilloma development in transgenic mice (16, 45). It is possible that activation of other signals, such as focal adhesion kinase (46), also contribute to the effect of T188I, although in cultured keratinocytes we have not observed a correlation between β1 integrin–mediated adhesion and focal adhesion kinase activity (5).

Our data show that tumor-associated integrin mutations and polymorphisms have the potential to influence cancer susceptibility and disease outcome. Although the tumor-associated integrin mutations are relatively rare, common integrin polymorphisms are associated with a range of other diseases, such as systemic lupus erythematosus (47, 48). Our studies indicate that it will be worthwhile to develop mouse models of additional integrin polymorphisms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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