Dual Role of $\alpha 6\beta 4$ Integrin in Epidermal Tumor Growth: Tumor-suppressive Versus Tumor-promoting Function[□]

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An increased expression of the integrin $\alpha 6\beta 4$ is correlated with a poor prognosis in patients with squamous cell carcinomas. However, little is known about the role of $\alpha 6\beta 4$ in the early stages of tumor development. We have isolated cells from mouse skin (mouse tumor-initiating cells [mTICs]) that are deficient in both p53 and Smad4 and carry conditional alleles of the $\beta 4$ gene (*Itgb4*). The mTICs display many features of multipotent epidermal stem cells and produce well-differentiated tumors after subcutaneous injection into nude mice. Deletion of *Itgb4* led to enhanced tumor growth, indicating that $\alpha 6\beta 4$ mediates a tumor-suppressive effect. Reconstitution experiments with $\beta 4$ -chimeras showed that this effect is not dependent on ligation of $\alpha 6\beta 4$ to laminin-5, but on the recruitment by this integrin of the cytoskeletal linker protein plectin to the plasma membrane. Depletion of plectin, like that of $\beta 4$, led to increased tumor growth. In contrast, when mTICs had been further transformed with oncogenic Ras, $\alpha 6\beta 4$ stimulated tumor growth, as previously observed in human squamous neoplasms. Expression of different effector-loop mutants of Ras^{V12} suggests that this effect depends on a strong activation of the Erk pathway. Together, these data show that depending on the mutations involved, $\alpha 6\beta 4$ can either mediate an adhesion-independent tumor-suppressive effect or act as a tumor promotor.

INTRODUCTION

The skin consists of an epidermis and a dermis, separated by a specialized extracellular matrix (ECM), called the basement membrane (BM). The major adhesive component of a mature BM is laminin-5 (laminin-332), which is a ligand for two integrins, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ (Aumailley and Rousselle, 1999). Although $\alpha 3\beta 1$ is required for focal adhesion and BM assembly, linking the ECM to the actin cytoskeleton, $\alpha 6\beta 4$ connects the ECM to intermediate filaments (IF) and is essential for hemidesmosome (HD) formation (Litjens *et al.*, 2006). Genetic analyses in humans and mice have shown an essential role of HDs in maintaining firm adhesion of the epidermis to the dermis. Loss of $\alpha 6\beta 4$ leads to junctional epidermolysis bullosa, characterized by skin blistering caused by detachment of the epidermis (Borradori and Sonnenberg, 1999; Uitto and Pulkkinen, 2001). Based on their structural constituents, two subtypes of HDs have been de-

scribed. Type II HDs contain $\alpha 6\beta 4$ and plectin and are found in simple epithelia, e.g., the intestinal epithelium (Uematsu *et al.*, 1994; Orian-Rousseau *et al.*, 1996). They are believed to represent the first step in the assembly of type I HDs, which additionally contain the bullous pemphigoid (BP) antigens 180 and 230 (BP180 and BP230) and are found in complex and stratified epithelia such as the epidermis (Borradori and Sonnenberg, 1999).

Normal homeostasis of the skin requires a precise balance between proliferation, differentiation, and apoptosis of the transit-amplifying keratinocytes, which are replenished from stem cells (SCs). The skin SCs reside mainly in the bulge of the hair follicle (HF) and are able to self-renew but also to give rise to the three major epithelial lineages: interfollicular epidermis (IFE), HF, and sebaceous gland (Fuchs *et al.*, 2004; Ito *et al.*, 2005). Because SCs persist throughout the lifetime of the organism, multiple mutations may accumulate in them, thus increasing the probability of malignant transformation (Owens and Watt, 2003). Skin cancer comprises different tumor types, including basal cell carcinomas (BCCs), squamous cell carcinomas (SCCs), trichofolliculoma, pilomatricomas, and sebaceous adenomas (Owens and Watt, 2003). The characteristics of a differentiated tumor depend 1) on the nature of the founding cell, e.g., BCCs are believed to arise from keratinocytes of the outer root sheath (ORS), whereas SCCs have characteristics of the IFE, and 2) on the genetic lesions involved (Owens and Watt, 2003). p53 and Ras are of undisputed importance in the genesis of epidermal tumors. TP53 mutations are found in most human BCCs and SCCs, whereas Ras can contribute to the development of human SCCs. Other proteins are also believed to play a role. BCCs and trichofolliculoma are induced by constitutive activation of the Sonic Hedgehog (SHH) and β -catenin/Tcf signaling pathways, respectively (Owens and

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Abbreviations used: BCC, basal cell carcinoma; BM, basement membrane; BMP, bone morphogenic protein; BP, bullous pemphigoid; BrdU, 5-bromo-2-deoxyuridine; ECM, extracellular matrix; HD, hemidesmosome; HF, hair follicle; IF, intermediate filament; IFE, interfollicular epidermis; IRS, inner root sheath; K10, keratin-10; Ln-5, Laminin-5; mTIC, mouse tumor-initiating cell; ORS, outer root sheath; SC, stem cell; SCC, squamous cell carcinoma; TGF- β , transforming growth factor β .

Watt, 2003). Recently, the skin-specific deletion of the α -catenin gene was shown to cause precancerous lesions in keratinocytes, in which the nuclear factor κB (NF- κB) pathway is activated (Owens and Watt, 2003; Kobiela and Fuchs, 2006). Furthermore, disruption of the transforming growth factor β (TGF- β) and bone morphogenic protein (BMP) signaling pathways was reported to be a key event in the genesis of skin tumors. Targeted disruptions of BMPRIA or Smad4 both lead to skin tumor formation (Kobiela *et al.*, 2003; Andl *et al.*, 2004; Ming Kwan *et al.*, 2004; Qiao *et al.*, 2005; Yang *et al.*, 2005).

Several studies have implicated $\alpha 6\beta 4$ in tumor progression. Increased and suprabasal expression of $\alpha 6\beta 4$ is correlated with a poor prognosis in both mouse and human SCCs (Tennenbaum *et al.*, 1993; van Waes *et al.*, 1995). Through its influence on other receptors and key signaling pathways, $\alpha 6\beta 4$ promotes tumor progression by affecting invasion, survival of carcinoma cells and angiogenesis (Lipscomb and Mercurio, 2005; Wilhelmsen *et al.*, 2006). $\alpha 6\beta 4$ is mobilized from HDs to actin-rich protrusions in invasive carcinomas (Rabinovitz *et al.*, 1999) and $\alpha 6\beta 4$ -dependent invasiveness is phosphoinositide 3-kinase-dependent (Shaw *et al.*, 1997). In p53-deficient carcinoma cells, $\alpha 6\beta 4$ promotes survival by activating Akt/PKB, whereas, when p53 is intact, it induces apoptosis by stimulating the caspase-3-dependent cleavage of the Akt/PKB kinase (Bachelder *et al.*, 1999). Finally, human keratinocytes lacking $\alpha 6\beta 4$ are resistant to transformation induced by oncogenic Ras and blockade of NF- κB (Dajee *et al.*, 2003).

We have explored the role of $\alpha 6\beta 4$ in tumor development and growth using p53-deficient cells carrying conditional alleles of the $\beta 4$ gene (*Itgb4*). These studies provide novel information on the expression of $\alpha 6\beta 4$ in those skin cells that display many features of multipotent stem cells and also show for the first time that this integrin can mediate a tumor-suppressive effect, which is dependent on its ability to recruit the cytoskeletal linker protein plectin to the plasma membrane and on the genetic background of the cells.

MATERIALS AND METHODS

Establishment of the Mouse Tumor-initiating Cells, Culture Conditions, Viral Transduction, and Stable Transfection

To obtain immortalized keratinocytes carrying $\beta 4$ conditional knockout alleles, we used the $p53^{\Delta 2-10}/\Delta 2-10$ and $\beta 4^{flox/flox}$ mouse strains (Jonkers *et al.*, 2001; Raymond *et al.*, 2005) to generate $p53^{\Delta 2-10}/\Delta 2-10$; $\beta 4^{flox/flox}$ mice. Keratinocytes were prepared from neonatal mice as previously described (Raymond *et al.*, 2005). After several days, a mixed population of cells called mouse tumor-initiating cells (mTICs), appeared to be immortalized and could be maintained in DMEM supplemented with 10% fetal calf serum (Invitrogen-BRL, Carlsbad, CA) and 100 U/ml penicillin and streptomycin. The mouse squamous cell carcinoma line 1 (mSCC1) has been isolated from an SSC induced by the two-stage chemical carcinogenesis protocol and contains an amplified Ras^{L61} allele (K. Raymond, unpublished results). The Rac-11P cell line was originally isolated from a mouse mammary tumor (Sonnenberg *et al.*, 1993). Retroviral constructs used in this study were H-Ras^{V12}, Smad 4, human (h) $\beta 4$, $\beta 4$ -EGFP, $\beta 4^*$ -EGFP, and EGFP- $\beta 4$ (enhanced GFP [EGFP]); Geuijen and Sonnenberg, 2002) cloned in pLZRS-MS-IRES-Zeo and H-Ras^{V12,S35}, H-Ras^{V12,C40} and H-Ras^{V12,G37} cloned in pLZRS-MS-IRES-Neo/pBR (Rodriguez-Viciana *et al.*, 1997). Retroviral transductions were performed as previously described (Stern *et al.*, 2000). Cre-mediated recombination of $\beta 4^{flox/flox}$ alleles was induced using adenovirus expressing Cre enzyme under the control of cytomegalovirus immediate-early promoter essentially as described previously (Raymond *et al.*, 2005). The $\beta 4$ -positive and -negative cells were subsequently sorted by flow cytometry. The procedure for preparing constructs coding for siRNA has been described previously (Brummelkamp *et al.*, 2002). The target sequence in plectin was 5'-GAAGCTACAGGAGACGTTA-3' and a standard luciferase (luc) target was used as a control. mTICs cells were electroporated using the Cell Line Optimization Nucleofector kit from Amax Biosystems (Gaithersburg, MD). The cells were maintained under G-418 selection (400 $\mu g/ml$) and sorted for green fluores-

cent protein (GFP) expression. A mTIC($\beta 4^+$, plect⁺) bulk population, expressing a short hairpin RNA (shRNA) against luciferase, and two mTIC($\beta 4^+$, plect⁻) clones, selected for efficient and stable down-regulation of plectin, were used in further experiments.

Antibodies

Mouse monoclonal antibodies (mAbs) used in this study were as follows: 121 against plectin from Dr. K. Owaribe (University of Nagoya, Nagoya, Japan), LL001 against keratin-14 from Dr. B. Lane (University of Dundee, Dundee, United Kingdom), AE13 (Lynch *et al.*, 1986), and AE15 (O'Guin *et al.*, 1992) from Dr. T-T. Sun (New York University Medical School, New York, NY); antibodies against E-cadherin, N-cadherin, β -catenin, Ras, and Erk2 were from BD Transduction Laboratories (Lexington, KY), against p53 (Sc-100), Smad4 (Sc-7966), and plectin (clone 10F6, Sc-33649) from Santa Cruz Biotechnology (Santa Cruz, CA); against Erk1/2, phospho-Erk1/2, Akt, and phospho-Akt from Cell Signaling Technology (Beverly, MA); against bromodeoxyuridine (BrdU) from DakoCytomation (Fort Collins, CO); against hemagglutinin (HA; 12CA5) from Abcam (Cambridge, MA); and against α -tubulin (clone B-5-1-2) from Sigma (St. Louis, MO). Rat mAbs were against CD31 (MEC 13.3 from BD Biosciences, San Jose, CA) and the following integrin subunits: R1-2 against $\alpha 4$ (PharMingen, San Diego, CA), GoH3 against $\alpha 6$, 346-11A against $\beta 4$ (PharMingen), and BMA5 against $\alpha 5$ and MB1.2 against $\beta 1$, both from Dr. B.M.C. Chan (University of Ontario, ON, Canada). Rabbit polyclonal antibodies against BP180 (mo-NC16a) were from Dr. L. Bruckner-Tuderman (University of Freiburg, Freiburg, Germany), Ln-5 from Dr. T. Sasaki (Max-Planck Institute, Munich, Germany), keratin-17 (K17; McGowan and Coulombe, 1998) from Dr. P. Coulombe (John Hopkins University School of Medicine, Baltimore, MD), keratins 5, 6, 10 and 14 from BabCO (Berkely, CA), vimentin (K36) from Dr. F. Ramaekers (University of Maastricht, Maastricht, The Netherlands), and cleaved caspase-3 (Asp 175) from Cell Signaling Technology. Guinea pig antibody used was hHb5 (Langbein *et al.*, 2001) from Dr. L. Langbein (German Cancer Research Centre, Heidelberg, Germany). Human mAb 5E against BP230 was from Dr. T. Hashimoto (Keio University, Tokyo, Japan). Texas red and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Molecular Probes (Eugene, OR).

Ultrastructural Analysis

To determine the presence of HDs in the different mTIC lines, the cells were grown to confluence on Thermanox Plastic coverslips (Nunc, Rochester, NY), fixed in 2.5% glutaraldehyde, postfixed in 1% OsO₄, stained en bloc with uranylacetate, and flat-embedded. The samples were examined with a FEI Tecnai 12 electron microscope (Beaverton, OR).

Immunofluorescence Microscopy and Flow Cytometry

Cryosections of the tumors and cells grown on glass coverslips were prepared, fixed, and blocked as previously described (Raymond *et al.*, 2005). Samples were examined using a confocal microscope TCS-NT (Leica, Mannheim, Germany). For flow cytometry and cell sorting, cells were processed, analyzed, and sorted as described previously (Stern *et al.*, 2000). For analysis of the intracellular antigen Smad4, the cells were successively fixed 10 min with 1% paraformaldehyde and permeabilized 5 min with 0.2% Triton X-100, before the addition of the first antibody.

Immunohistochemistry

Paraffin-embedded sections (5 μm) were deparaffinized in xylene and rehydrated in phosphate-buffered saline (PBS). The retrieval of masked antigens was either not necessary (for keratin-10 [K10] staining) or accomplished in a solution containing 0.25 mg/ml trypsin, 9 mM CaCl₂, 50 mM Tris-HCl, pH 7.8, for 20 min at RT (for CD31 staining) or by heating the samples in 0.1 M citrate buffer, pH 6, in a microwave oven for 20 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 10 min. The sections were then blocked with 1% BSA (for CD31) or 5% normal goat serum for 1 h at RT. The first antibodies were then incubated overnight at 4°C. When rabbit primary antibody was used, the secondary immunohistochemical reaction was visualized using the peroxidase labeled polymer conjugated to goat anti-rabbit IgG from the DakoCytomation EnVision + System, peroxidase (DakoCytomation) and diaminobenzidine. For mouse primary antibodies, a goat anti-mouse biotin-labeled antibody, the avidin-biotin complex (ABC) technique (DakoCytomation) and diaminobenzidine were used. To detect CD31, sections were blocked in Tris sodium buffer with 0.5% blocking reagent (TNB), supplied with Tyramide Signal Amplification (TSA) kit (NEN Life Sciences, Boston, MA) for 30 min and then incubated 30 min with biotinylated rat anti-mouse antibody, followed by streptavidin peroxidase. These two latter incubations were repeated after a step of 10-min incubation with tyramide (1:50) in amplification reagent supplied with the TSA kit. Finally, diaminobenzidine was used. All sections were counterstained with hematoxylin and eosin (H&E). Quantifications of the immunohistochemistry slides were made using the Olympus Comedia camera coupled with the Soft imaginga System analySIS software (Melville, NY).

Figure 1. Establishment of tumor cells (mTICs) deficient for p53 and Smad4 and carrying conditional alleles of *Itgb4*. (A) Phase-contrast image of subconfluent mTICs. (B) Immunoblot analysis of the expression levels of p53 and different epithelial and mesenchymal markers in mTICs compared with those in NMK-1 cells. Rac-11P and mouse embryonic endothelial cells (MEEC) were used as control cell lines. (C) The mTICs do not respond to TGF- β stimulation in a (CAGA)₁₂ MLP-luciferase reporter assay, unlike the control line Rac-11P. Coexpression of a constitutively active type I TGF- β receptor (Alk5^{ΔD}) does not restore the luciferase activity, unlike coexpression of Smad4. Asterisk denotes statistically significant data with $p \leq 0.05$. (D) Retroviral transduction of mTICs with Smad4 leads to a bulk population of cells that respond to TGF- β in a luciferase assay. Asterisk denotes statistically significant data with $p \leq 0.05$. Immunoblotting shows that Smad4 is expressed after retroviral transduction of

mTICs. (E) Culture of the mTICs-Smad4 bulk population of cells in high-calcium medium selected for Smad4-deficient cells, indicating that the restoration of Smad4 expression induces calcium-dependent differentiation *in vitro*. (F) FACS analysis of the mTICs-Smad4 clonal population obtained by subcloning of the bulk population. Restoration of Smad4 expression abrogates the tumorigenic potential of the cells, *in vivo* (0 vs. 14 tumors initiated from 16 injections of mTICs-Smad4 clonal population and mTIC, respectively; $n = 3$ independent experiments).

Immunoblotting and Ras Pulldown Assay

Cells were lysed and proteins analyzed as previously described (Raymond *et al.*, 2005). The relative amount of Ras-GTP was determined according to a method described by van Triest *et al.* (2001) using the Ras binding domain of Raf fused to glutathione *S*-transferase (a gift from Dr. L. Bos, University of Utrecht, Utrecht, The Netherlands) and mAb against Ras.

Transfection and Reporter Assays

Transient transfections were performed using lipofection (Lipofectamine, Plus Reagent, Invitrogen) according to the manufacturer's instructions. For reporter assays, 1.5×10^5 cells were transfected with 400 ng total DNA, containing 100 ng of reporter plasmid (TOP/FOP) from Dr. H. Clevers (Hubrecht Laboratory, Utrecht, The Netherlands; Korinek *et al.*, 1997) and (CAGA)₁₂ MLP-Luc from Dr. J. M. Gauthier (Laboratoire Glaxo Wellcome, Les Ulis, France; Dennler *et al.*, 1998), and 10 ng pCMV-Renilla-luciferase control vector (Promega, Madison, WI). Thirty-six hours after transfection and 18 h after TGF- β 1 (5 ng/ml) addition for (CAGA)₁₂ MLP-Luc, the cells were lysed, and the Firefly and Renilla activities were measured on a Victor Wallace luminometer, according to the manufacturer (Dual-Reporter Luciferase Activity, Promega). The experiments were performed in triplicate at least three times; representative experiment is shown.

Cell Proliferation and Transformation Assays

The growth curves were determined and the growth was measured in semi-solid medium (Raymond *et al.*, 2005). Subcutaneous cell injections into nude mice were performed as previously described (Raymond *et al.*, 2005). A total of 5×10^5 mTICs and 1×10^5 mTICs-Ras^{V12} in 0.15 ml PBS was injected. Groups of five mice (10 injections) were analyzed in at least two independent experiments. Mice were examined weekly and killed when any single animal had lost 20% of its body weight (study end point, which was about 70 and 15 d, for mTICs and mTICs-Ras^{V12}, respectively). For the scatter plots presented, tumor size was measured in three directions (L, length; W, width; and D, depth) on killed animals. Cells from which no tumors initiated are not represented in these plots. For the growth curves, tumor size was measured in two directions (L, length and W, width) and the volumes are presented by $(L \times W \times ((L + W)/2))$. For cell proliferation *in vivo*, the mice were injected intraperitoneally with BrdU (Sigma) at 50 mg/kg, 2 and 5 h before sacrifice for mTIC and mTICs-Ras^{V12} variants, respectively. For cell injections under the capsule of the kidney, 4-wk-old female athymic nu/nu (BALB/c) mice were anesthetized by intraperitoneal injection of 7 μ l Hypnorm/Dormicum/water (1:1:2) per gram body weight (Hypnorm, Janssen Pharmaceutica, Beerse, Belgium; Dormicum, Roche Pharmaceuticals, Basel, Switzerland), followed by injection of 1×10^6 cells in 50 μ l of PBS under the capsule of the left kidney. PBS was used as a negative control. Animals were weighed and subjected to palpation once a week and killed when a 20% loss of body weight was observed. At the study end point, all animals were subjected to macroscopic observation and all tumor-like lesions were weighted and examined micro-

scopically. All animal husbandry and experimental procedures were conducted with approval from the relevant institutional animal ethics committees.

Statistics

Statistical significance was evaluated using Mann-Whitney *U* test for comparisons between two groups. A value of $p < 0.05$ or $p < 0.01$ denoted statistical significance.

RESULTS

Isolation of mTICs Carrying β 4 Conditional Knockout Alleles

To study the effect of α 6 β 4 on tumor growth, we generated mice of the *p53*^{Δ2-10/Δ2-10}; *β4*^{lox/lox} genotype and isolated cells from the skin of newborns. The loss of p53 is known to facilitate immortalization of keratinocytes as well as to enable cells to resist apoptosis induced by DNA damage, allowing further genetic changes to occur. We obtained a population of immortalized keratinocytes (mTICs) that grew in epithelial islands with a homogeneous morphology (Figure 1A). Immunoblotting revealed that mTICs are true epithelial cells, expressing the type I hemidesmosomal components BP180 and BP230, and the keratins 5, 6, and 14 at levels comparable to those in NMK-1 cells (Raymond *et al.*, 2005). The levels of the adherens junction proteins, E-cadherin and β -catenin, are also similar in the two cell types, whereas N-cadherin and the mesenchymal marker vimentin are undetectable in mTICs. Consistent with their genotype, mTICs do not express p53 (Figure 1B).

Molecular Defects Associated with Transformation of mTICs

mTICs are resistant to differentiation induced by calcium, which is a hallmark of cell transformation (Kulesz-Martin *et al.*, 1983), suggesting that, in addition to the absence of p53, mTICs have acquired other mutation(s). Indeed, using a (CAGA)₁₂ MLP-luciferase reporter assay, we found that the response to stimulation by TGF- β is impaired in mTICs, in contrast to that in Rac-11P control cells (Figure 1C). Further-

Figure 2. The mTIC cells show features of epidermal SCs. (A–E) Paraffin-embedded sections of a tumor sample stained with H&E (A–C) and keratin-10 (D), and -14 (E), showing the presence of squamous (A, D, and E), basaloid (B, D, and E), and sebaceous (C) differentiation within a single tumor sample. The nuclei of the basaloid cells are hyperchromatic, whereas the cells are arranged in a trabecular manner, and some of them form pseudoductular structures. Bar, 1000 μ m. (F) Cryosections of a tumor sample stained with Nile Red confirms the presence of clusters of sebocytes (S). Bar, 500 μ m. (G–I) Indirect immunofluorescence on cryosections of tumor samples indicates that some cornified squamous nests express the IFE-specific marker, K10 (G, arrows), and others the hair-matrix keratin hHb5 (H) and the ORS-specific marker, K17 (I). Bar, 500 μ m. (J–M) Indirect immunofluorescence indicates the presence of a network of K17 in mTICs (K), whereas NMK-1 lack K17 (J). The skin SC marker CD34 is expressed by mTICs (M) but not by NMK-1 cells (L). Bar, 10 μ m. (N) Immunohistochemistry using the antibody AE15 shows differentiation toward IRS in HF of the normal skin but not in the tumor delimited by the dotted line. E, epidermis; HF, hair follicle; IRS, inner root sheath; T, tumor.

more, the luciferase activity was not significantly increased by coexpression of a constitutively active type I TGF- β receptor (Alk5^{QD}), whereas the response to stimulation by TGF- β was restored by coexpression of Smad4, indicating that Smad4 function is deficient in mTICs. Subsequent immunoblotting confirmed that Smad4 is absent from mTICs (Figure 1C). Because we found no evidence for mutations in other oncogenes or tumor suppressors in mTICs (e.g., Ras, Wnt/ β -catenin, α -catenin, NF- κ B, and PTEN; Supplementary Figure 1, A–C, and data not shown), we assume that the loss of Smad4- and p53-functions are the only two molecular events responsible for the tumorigenicity of mTICs. This is supported by the finding that re-expression of Smad4 by retroviral transduction into mTICs renders the cells sensitive to the differentiation signals induced by calcium and abrogates their tumorigenic potential. No tumors were formed after subcutaneous injection of a clonal population of mTIC-Smad4 cells into nude mice and, when shifted to high-calcium-containing medium, only cells that still lacked Smad4 were able to grow (Figure 1, E and F). As expected, the Smad4-positive mTICs did respond to stimulation by TGF- β in the (CAGA)₁₂ MLP-luciferase reported assay (Figure 1D).

mTICs Have Multipotent Differentiation Capacity, Suggesting That They Originate from SCs

The tumors produced after subcutaneous injection of mTICs into nude mice showed features of both basal and squamous cell carcinomas with areas in which the cells had differentiated into sebocytes (Figure 2, A–I). In the same tumor samples, cornified squamous nests reacted positively with antibodies against ORS-, hair matrix-, or squamous cell-specific keratins: K17 (Stark *et al.*, 1987), hHb5 (Rogers *et al.*, 1997), and K10, respectively, suggesting that mTICs originated from the ORS of the HF and can differentiate *in vivo* into hair matrix cells and along IFE lineages (Figure 2, G–I).

Furthermore, cells of all the clones generated from mTICs possessed this differentiation potential (not shown). Thus, mTICs seem to have characteristics of the multipotent SCs present in the permanent portion of the HF. In addition, mTICs express K17 in culture and CD34, an established marker of epithelial SCs of the skin (Tumbar *et al.*, 2004; Morris *et al.*, 2004) (Figure 2, J–M). Moreover, because of the lack of Smad4, the hHb5-positive matrix cells are unable to differentiate toward inner root sheath (IRS) and hair shaft, processes known to be dependent on BMP signaling (Kobielak *et al.*, 2003), as illustrated by the lack of staining with the AE15 and AE13 antibodies, respectively (Figure 2N, not shown). These data support the hypothesis that mTICs have originated from SCs or from transit-amplifying progenitor cells that have regained stem cell-like properties.

Inactivation of *Itgb4* in mTICs Causes the Loss of Hemidesmosomes

We generated $\beta 4$ -negative mTICs by inactivating *Itgb4* using adenoviral-mediated delivery of Cre-recombinase into the $\beta 4$ -positive mTICs (mTICs($\beta 4^{+}$)). A pure $\beta 4$ -negative cell population (mTICs($\beta 4^{-}$)) was obtained by FACS sorting (Figure 3A). The morphology of the cells was not obviously affected by the absence of $\beta 4$ (not shown). The level of the $\alpha 6$ subunit was only slightly lower in mTICs($\beta 4^{-}$), suggesting that, because of the absence of $\beta 4$, more $\alpha 6 \beta 1$ was formed. Indeed, the $\beta 1$ levels were slightly higher in mTICs($\beta 4^{-}$). No $\alpha 1$, αV , or $\alpha 4$ subunits were detectable by fluorescence-activated cell sorting (FACS) in any of the mTIC variants, whereas $\alpha 2$ and $\alpha 5$ were expressed at similar levels (not shown). Expression of $\alpha 3$ in the various mTIC lines was assessed by immunoblotting, and no differences were observed (not shown). Expression of $\beta 4$ was restored in the mTICs($\beta 4^{-}$) by retroviral transduction of human $\beta 4$ cDNA (mTICs(h $\beta 4$); Figure 3A). Confocal microscopy revealed partial colocalization of $\beta 4$ with Ln-5, plectin, and BP230, indi-

Figure 3. Inactivation of $\beta 4$ in mTICs causes the loss of hemidesmosomes. (A) FACS analyses of the levels of mouse (m) and human (h) $\beta 4$, and of mouse $\alpha 6$ and $\beta 1$ in the different mTIC variants compared with those in the controls, in which only secondary antibody was used. (B) Indirect immunofluorescence of the localization of hemidesmosomal proteins in the various mTICs, visualized by confocal microscopy. The partial colocalization of the different hemidesmosomal components indicates that these cells form type I HDs in culture. Bar, 100 μm . (C) Ultrastructural analysis of the various mTICs further confirmed the presence of type I HDs in mTICs($\beta 4+$) and mTICs(h $\beta 4$) but not in mTICs($\beta 4-$).

cating that as in mTICs($\beta 4+$), type I and type II HDs were formed in mTICs(h $\beta 4$) (Figure 3B). This was further confirmed by electron microscopy, showing HDs with a tripartite structure, consisting of an inner and outer plaque, separated by an electro-lucent zone (Figure 3C). In contrast, no HDs were detected in mTICs($\beta 4-$), in which BP230 was diffusely distributed throughout the cytoplasm (Figure 3, B and C). Ln-5 was deposited by all three cell variants in similar amounts and distribution. Plectin was mostly diffusely distributed throughout the cytoplasm although it was somewhat concentrated at the periphery of mTICs($\beta 4-$) in structures resembling focal contacts (Figure 3B). However, they did not appear to contain α -actinin or F-actin (not shown).

Loss of $\alpha 6\beta 4$ Increases the Frequency of Tumor Formation and the Size of Tumors

To determine whether $\alpha 6\beta 4$ plays a role in tumor development, the mTIC($\beta 4+$) and mTICs($\beta 4-$) were subcutaneously injected into nude mice. mTICs($\beta 4-$) formed tumors with a lag-phase similar to that of the parental mTIC($\beta 4+$) cells. However, the frequency of mTIC($\beta 4-$) tumor formation was increased from 76 to 100%, and the tumors were approximately four times larger than mTIC($\beta 4+$) tumors. To confirm the tumor-suppressive effect by $\alpha 6\beta 4$, we re-expressed $\beta 4$ and this reduced the frequency of tumor forma-

tion and the size of the tumors (Figure 4, A and B; Supplementary Figure 1D). Its distribution was determined by immunostaining of tumor sections. In the squamous and basaloid regions of the mTIC($\beta 4+$)- and mTIC(h $\beta 4$)-induced tumors, $\beta 4$ was colocalized with Ln-5. There was no reaction with anti- $\beta 4$ in the corresponding regions in the mTIC($\beta 4-$) tumors, but staining was observed in the blood vessels and the peripheral nerves of the nude mice (Figure 4C and not shown). These results show that $\alpha 6\beta 4$ mediates a suppressive effect on tumor growth.

Because the stromal microenvironment also plays an important role in tumor development and progression, we transplanted cells of the mTIC variants under the kidney capsule of nude mice. Tumor growth at this site was comparable to that observed in skin (not shown). Furthermore, the differentiation characteristics of these tumors were largely similar to those of the skin tumors, with areas of basal-squamous differentiation and the presence of clusters of sebocytes (Figure 4D). However, in some foci the tumors were less differentiated than those in the skin. These data indicate that the characteristics of the tumor were only marginally influenced by the microenvironment.

Loss of $\alpha 6\beta 4$ Increases the Proliferative Index In Vivo

The absence of $\alpha 6\beta 4$ led to about a fourfold increased tumor volume, suggesting that the proliferative capacity of the cells

Figure 4. $\alpha 6 \beta 4$ mediates a suppressive effect on tumor growth. (A and B) Graphs representing the percentage of tumors initiated by subcutaneously injecting cells of the various mTIC variants into nude mice (A) and the volume of the tumors at the time the mice were killed (B). n represents the number of independent experiments each of 6 injections (A) or the total number of injections (B). The bars in B represent the median tumor size. (C) Indirect immunofluorescence on cryosections of tumor samples indicate that $\beta 4$ expression is lost in the mTIC($\beta 4^{-}$)-induced tumors. Bar, 50 μ m. (D) Paraffin-embedded sections stained with H&E showing the presence of basaloid (B) and squamous (Sq) differentiation in a tumor formed under the kidney capsule. Bar, 250 μ m.

is enhanced. To investigate this, tumor-carrying mice were injected intraperitoneally with BrdU, 5 h before they were killed. This revealed a more than twofold higher percentage of BrdU-positive cells in the absence of $\alpha 6 \beta 4$ (Figure 5A, top panels). No significant differences were detected in the number of apoptotic cells (Figure 5A, middle panels) or in the density of blood vessels (Figure 5A, bottom panels). The differentiation capacities of the $\beta 4$ -positive and -negative tumors also appeared to be similar, as illustrated by the percentage of cornified versus solid tumor areas as well as of K10-positive cornified squamous nests (Figure 5, B and C). As shown by Qiao *et al.* (2005), tumorigenesis induced by the loss of *Smad4* is accompanied by the activation of the kinase Akt, the inactivation of the phosphatase PTEN and the nuclear accumulation of Cyclin D1. We observed a broad and focal activation of Akt in the basaloid and squamous part of tumors, respectively, which correlated with the localization of Cyclin D1 in the nucleus, but not with a deletion of *PTEN*. However, $\beta 4$ did not appear to play a role in the regulation of these events (data not shown). Surprisingly, *in vitro* we did not see an effect of $\beta 4$ on proliferation at all serum concentrations and cell densities tested (Figure 5D). Erk1 and Erk2 were similarly activated by growth factors in the mTIC variants (Figure 5E). We conclude that reduced proliferation is the main reason for the suppressive effect by $\beta 4$ on tumor growth *in vivo*.

The Tumor-suppressive Effect by $\alpha 6 \beta 4$ Does Not Require Its Binding to Ln-5

To further investigate by which mechanism $\alpha 6 \beta 4$ influences tumor growth, we first tested $\alpha 6 \beta 4$ -mediated adhesion. We used a $\beta 4$ chimera construct in which the extracellular ligand-binding domain of $\beta 4$ was replaced by EGFP (EGFP- $\beta 4$, Figure 6; Geuijen and Sonnenberg, 2002). We compared the tumor-forming capacity of mTICs($\beta 4^{-}$) reconstituted with this construct with that of mTICs($\beta 4^{-}$) that express a control construct in which EGFP was fused to the carboxy-terminal end of the $\beta 4$ cytoplasmic domain ($\beta 4$ -EGFP, Figure 6). Both constructs induced the formation of type II HDs (colocalization of $\beta 4$ with plectin), but surprisingly, not of type I HDs (i.e., BP230

and $\beta 4$ were not colocalized; Figure 6B). The two fusion proteins were detected at the cell surface of the cells by FACS analysis with $\beta 4$ or EGFP antibodies (Figure 6C). The mTICs/EGFP- $\beta 4$ and mTICs/ $\beta 4$ -EGFP cells produced tumors of similar size as the parental mTICs($\beta 4^{+}$), indicating that the $\beta 4$ effect is not mediated by its extracellular domain and therefore not by ligation of $\alpha 6 \beta 4$ to Ln-5 (compare Figures 4B and 6D). Additionally, this approach indicates that type I HDs are not required for the effect. We conclude that the suppressive effect by $\alpha 6 \beta 4$ on tumor growth is independent of its adhesion to ligand.

The Tumor-suppressive Effect by $\alpha 6 \beta 4$ Requires the Recruitment of Plectin

We next determined the possible role of the $\beta 4$ -binding partner, plectin. The $\beta 4$ -deficient mTICs were reconstituted with a $\beta 4$ chimera, in which the $\beta 4$ cytoplasmic domain, carrying a R1218W mutation, is fused to EGFP. The R1218W mutation abrogates the interaction with plectin ($\beta 4^{*}$ -EGFP, Figure 6A; Koster *et al.*, 2001). The mTICs/ $\beta 4^{*}$ -EGFP completely lacked HDs, as shown by the absence of colocalization of $\beta 4$ with plectin or BP230 (Figure 6B). The level of the $\beta 4^{*}$ -EGFP chimera at the surface was similar to that of the $\beta 4$ -EGFP chimera (Figure 6C). The tumors induced by mTICs/ $\beta 4^{*}$ -EGFP were significantly larger than the mTICs/ $\beta 4$ -EGFP tumors and similar to those induced by mTICs($\beta 4^{-}$). This strongly indicates that the tumor suppressive-effect of $\beta 4$ requires the recruitment of plectin to the plasma membrane (compare Figures 4B and 6D).

shRNA-mediated Depletion of Plectin Leads to Increased Tumor Growth

To further study the role of plectin, we generated several mTIC($\beta 4^{+}$, plect $^{-}$) clones in which the endogenous expression of plectin was virtually eliminated by the expression of an shRNA against plectin (Figure 7, A and B). In these clones, the K5/K14 network appeared to be more loosely organized, and the cells seemingly had deposited less Ln-5 (Figure 7A). Furthermore, $\alpha 6 \beta 4$ was more diffusely distributed at the basal cell surface of the plectin-deficient clones.

Figure 5. The suppressive effect by $\alpha 6\beta 4$ on tumor growth is attributable to a reduction of the proliferative capacity of the cells in vivo. (A) Immunohistochemistry of tumor samples using antibodies against BrdU (top panels), cleaved caspase-3 (middle panels) and CD31 (bottom panels) to measure the proliferative index, apoptotic index, and the number of blood vessels, respectively. Note that the tumor size is correlated with the proliferative index. Histograms represent a quantitative analysis of the different stainings. The number of tumor samples analyzed is indicated (n) and the surface of the areas analyzed is indicated between parentheses. The p values are indicated when the difference is statistically significant. (B and C) Histograms, representing the percentage of the tumor samples showing cornified nests (B) and K10 immunoreactivity (C). Note that the percentages are not statistically different in the various mTIC variants. (D) mTICs($\beta 4+$) and ($\beta 4-$) do not form colonies in semisolid medium (left panels) and the differences in growth capacity are not statistically significant under the various conditions tested (right). (E) Erk1/2 phosphorylation in EGF-stimulated mTIC variants. Cells were cultured for 16 h in serum-free DMEM and then stimulated with EGF (20 ng/ml) for the time periods indicated. Lysates were blotted with antibodies against phospho-Erk and total Erk as control.

Small differences in the distribution of the actin cytoskeleton could also be observed with more actin stress fibers traversing the bodies of the plectin-deficient cells. The distribution of the $\beta 1$ subunit in the plectin-deficient and -positive mTICs was comparable. The levels of $\alpha 6\beta 4$ in the two plectin-deficient clones were similar to those in the parental mTICs and in a clone of mTICs that no longer express the shRNA construct against plectin (Figure 7B). Cells from the two plectin-deficient clones formed significantly larger tumors (about fourfold) than control mTICs($\beta 4+$), expressing an shRNA against luciferase (Figure 7C). In 70% of the tumors (n = 10), ~30% of the cells re-expressed plectin (not shown). Thus, down-regulation of plectin was not fully stable in vivo and although the loss of plectin gave a growth advantage, there was apparently no selective pressure to maintain plectin at low levels in vivo. These data support the notion that the recruitment of plectin to the plasma membrane is essential for the tumor-suppressive effect that is mediated by $\beta 4$.

Expression of Oncogenic Ras Results in a Further Transformation of mTICs

Next, we retrovirally transduced oncogenic H-Ras^{V12} (Ras^{V12}) and selected a clonal population of cells expressing

Ras^{V12} at the plasma membrane (mTICs-Ras^{V12}($\beta 4+$); Figure 8A). In most of these cells, $\beta 4$ was colocalized with plectin, indicating the formation of type II HDs (Figure 8B). The morphology remained mainly epithelial, although some cells seemed to escape the epithelial islands (not shown). Compared with mTICs, the Ras^{V12}-transformed cells form many more lamellipodia (Figure 8A). Moreover, they showed anchorage-independent growth, indicating a more advanced stage of transformation (Figure 8A, soft agar). Interestingly, although the K5/K14 and K6/K16 IF networks were not obviously affected by oncogenic Ras (not shown), the K7/K17 network was completely remodeled (Figure 8C).

The Tumor-suppressive Effect by $\beta 4$ Is Abrogated by Oncogenic Ras

Previously, $\alpha 6\beta 4$ was shown to cooperate with oncogenic Ras in the transformation of human keratinocytes (Dajee *et al.*, 2003). In accord with this observation, we found that the tumors arising from mTICs-Ras^{V12}($\beta 4+$) were significantly and reproducibly larger than those formed from mTICs-Ras^{V12}($\beta 4-$). Re-expression of human $\beta 4$ also led to larger tumors, confirming the tumor-promoting effect of $\alpha 6\beta 4$ (Figure 8D and Supplementary Figure 1E). Furthermore, the

Figure 6. The suppressive effect by $\alpha 6 \beta 4$ on tumor growth is dependent on the recruitment of plectin at the plasma membrane. (A) Diagrams depicting the $\beta 4$ -EGFP, $\beta 4^*$ -EGFP, and EGFP- $\beta 4$ chimeric constructs are shown. (B) Indirect immunofluorescence shows a complete absence of HDs in mTICs($\beta 4^-$) reconstituted with $\beta 4^*$ -EGFP, because there is no colocalization of $\beta 4$ and plectin, or of $\beta 4$ and BP230. In mTICs($\beta 4^-$) reconstituted with EGFP- $\beta 4$ and $\beta 4^*$ -EGFP, the presence of type II HDs is indicated by the colocalization of $\beta 4$ and plectin; however, only few type I HDs were formed, as illustrated by the absence of colocalization of $\beta 4$ and BP230. All three mTIC variants have deposited Ln-5 underneath their basal cell surface. Bar, 10 μ m. (C) FACS analyses showing the levels of GFP signals by the different chimeras and their expression at the cell surface either with antibodies against the $\beta 4$ subunit (when extracellular domain of $\beta 4$ is present) or against EGFP (for mTIC-EGFP- $\beta 4$). Note that $\beta 4$ -EGFP and $\beta 4^*$ -EGFP are expressed at similar levels (human, h $\beta 4$, and EGFP Ab). (D) The suppressive effect of $\alpha 6 \beta 4$ on tumor growth also occurred when mTICs($\beta 4^-$) were reconstituted with EGFP- $\beta 4$ but not when reconstituted with $\beta 4^*$ -EGFP, suggesting that the effect is mediated by type II HDs. n represents the number of injections. The bars represent the median of tumor size.

latency in tumor formation of the mTIC-Ras^{V12} variants was similar, but much shorter than that of the corresponding mTIC variants. After injection of 1×10^5 mTICs-Ras^{V12}, the mice had to be killed after ~ 14 versus ~ 70 d for 5×10^5 mTICs (Figure 8D). The size of the tumors correlated with proliferation, as shown by BrdU incorporation (Figure 8E).

In humans, tumorigenesis by oncogenic Ras requires the blockade of the NF- κ B pathway (Dajee *et al.*, 2003). In the mTIC-induced tumors, the NF- κ B pathway was not activated, because phospho-I κ B was absent and p50NF- κ B did

not accumulate in the nucleus (data not shown). Additionally, Akt was more activated than in the parental mTICs, and this was not due to PTEN deletion. However, no effect of $\beta 4$ could be established (data not shown). The mTIC-Ras^{V12} tumors were mainly SCCs with areas of differentiation toward sebocytes (Figure 8F). Compared with the mTIC tumors, they were much more solid, with hardly any nests of cornified material and they were, on the whole, less differentiated. There was no obvious $\beta 4$ -dependent effect on the histological differentiation characteristics of these tumors

Figure 7. Depletion of plectin from mTICs leads to increased tumor growth. (A) Indirect immunofluorescence showing subtle differences in the organization of the actin cytoskeleton and K5/K14 networks in two plectin-deficient clones compared with the parental mTICs and mTICs expressing an shRNA construct against luciferase. Note that the two plectin-deficient clones seemingly had deposited less Ln-5 and $\alpha 6 \beta 4$ was less clustered in them. The distribution of $\beta 1$ was similar between cells lacking or still expressing plectin. (B) Immunoblot analysis of the expression levels of $\beta 4$, plectin, and GFP in the plectin-deficient clones compared with the parental mTICs and a clone of mTICs, no longer expressing the shRNA construct against plectin. The level of the $\beta 4$ integrin in each clone was similar. (C) After subcutaneous injection of the plectin-deficient clones (5×10^5 cells) into nude mice the size of the tumors was about fourfold that of those induced by control cells. n represents the number of injections. The bars represent the median of tumor size.

Figure 8. The suppressive effect of $\alpha 6\beta 4$ on tumor growth is lost when the cells are further transformed by oncogenic Ras. (A–F) Characterization of mTICs-Ras^{V12}. (A) Compared with the parental cells, the constitutive activation of Ras results in extensive lamellipodia formation, illustrated by staining of F-actin with phalloidin (left panels) and in colony formation in semisolid medium (right panels). Indirect immunofluorescence reveals that Ras is expressed at the plasma membrane in mTICs-Ras^{V12} (middle panels). (B) Indirect immunofluorescence of the hemidesmosomal components indicates that type II HDs are still formed in mTICs-Ras^{V12}($\beta 4+$). (C) The network, as well as the level of expression of K17 present in mTICs, are modified by oncogenic Ras. (D) The subcutaneous injection of mTIC-Ras^{V12} variants (1×10^5 cells) into nude mice revealed that the suppressive effect by $\alpha 6\beta 4$ on tumor growth does not occur in the presence of oncogenic Ras. *n* represents the number of injections. The bars represent the median of tumor size. (E) The size of the tumors induced by the mTICs-Ras^{V12} is correlated with their mitotic index as shown by incorporation of BrdU. (F) H&E staining revealed that the mTIC-Ras^{V12}-induced tumors are solid SCCs (Sq) with sebaceous (S) differentiation.

(not shown). These data show that the tumor-suppressive effect by $\alpha 6\beta 4$ is abrogated after transformation of the cells by oncogenic Ras. Instead, and similarly to what has been reported previously in human cells, we find a cooperative effect of $\alpha 6\beta 4$ on Ras-mediated transformation in mTICs, although much weaker than in human cells.

Role of Erk Activation

To investigate the role of Ras effectors, we expressed in mTICs, through retroviral transduction, three different HA tagged effector-loop point mutants of Ras^{V12}, each of which activate a different Ras effector pathway. Ras^{V12,S35} binds Raf-1 but much more weakly than Ras^{V12}, and Ras^{V12,C40} binds and activates the PI3K p110 α subunit, whereas Ras^{V12,G37} binds RalGDS (Rodriguez-Viciano *et al.*, 1997). Of

each Ras mutant, a clonal population of cells was selected in which the mutant protein was expressed at the plasma membrane (Figure 9A). In all these cells, $\beta 4$ was colocalized with plectin. By transient expression of a Cre-recombinase, $\beta 4$ was deleted. The levels at which the different Ras^{V12} effector-loop mutants were expressed were comparable and lower than that of Ras^{V12}, but much higher than that of endogenous Ras (Figure 9B). As expected, Ras^{V12,S35} induced a modest activation of the Erk pathway; Ras^{V12,C40} activated PKB/Akt pathway, whereas Ras^{V12,G37} did not activate either of these pathways. Activation of PKB/Akt by Ras^{V12,C40} was stronger than by Ras^{V12}, suggesting that in mTICs oncogenic Ras signals preferentially toward the Erk pathway. Constitutive activation of the Erk pathway by either Ras^{V12} or Ras^{V12,S35} was slightly reduced in the ab-

Figure 9. The cooperative effect of $\beta 4$ on oncogenic Ras-mediated transformation appears to require strong Erk pathway activation. (A and B) Characterization of mTICs-Ras^{V12} effector-loop mutants. (A) Indirect immunofluorescence shows that in mTICs($\beta 4+$) expressing the various HA-tagged Ras-mutants, the mutant Ras proteins are localized at the plasma membrane and that $\alpha 6\beta 4$ is colocalized with plectin. (B) Immunoblot analysis of the expression levels of $\beta 4$, plectin and Ras in the mTICs, expressing different Ras effector-loop mutants. The activation of the downstream pathways of Ras, Erk, and PI3K/Akt was evaluated in the transformed cell lines, grown in the presence of serum.

sence of $\alpha 6\beta 4$. This may point to a role of $\alpha 6\beta 4$ in enhancing Erk signaling downstream of oncogenic Ras.

None of the Ras^{V12} effector-loop mutants had the tumorigenic potential of Ras^{V12}. Instead, the mutants induced a relatively long and variable tumor latency period (>4–8 wk when 1×10^5 cells were injected) and a low tumor frequency (0–50%). No effect of $\beta 4$ was observed (not shown). The histopathological characteristics of the tumors were similar to those of the parental mTICs, i.e., there were numerous nests of cornified material. These data suggest that the increased transformation induced by Ras^{V12} requires the strong activation of the Erk pathway, which could be essential in the cooperative effect of $\alpha 6\beta 4$ and Ras^{V12} on tumor growth.

DISCUSSION

To study whether $\alpha 6\beta 4$ influences tumor growth, we have generated p53-deficient mTICs carrying $\beta 4$ conditional alleles. We found that the tumor suppressor Smad4 is not expressed in these mTICs, indicating that both TGF- β and BMP signaling pathways are impaired. By removing *Itgb4* we showed that in these cells $\alpha 6\beta 4$ mediates the suppression of tumor growth. This effect is due to decreased proliferation and depends on $\beta 4$ -mediated recruitment of plectin to the plasma membrane. In contrast, in mTICs, expressing oncogenic Ras, $\alpha 6\beta 4$, instead, cooperated with Ras in stimulating tumor growth.

Although most of the proliferating cells in the skin differentiate and die after a few weeks, SCs persist throughout life. Hence, mutations can accumulate in these cells, leading to an increased risk of malignant transformation (Owens and Watt, 2003). The finding that mTICs can differentiate toward the different cell lineages of the skin suggests that these tumor cells have originated from SCs or from committed progenitors that have regained SC properties and supports the idea that the tumor-initiating cells arise from SCs (Bjerkvig *et al.*, 2005). Moreover, their multipotent capacity and the fact that they generate well-differentiated, slowly growing, noninvasive tumors indicate that mTICs are in an early stage of transformation. This notion is in line with their *in vitro* properties: they grow in epithelial islands, are phenotypically stable, and do not grow without anchorage or serum.

The loss of p53 function is a well-documented event in the genesis of SCCs (Owens and Watt, 2003). In addition, we found that mTICs do not express the tumor suppressor Smad4. Smad4 is the mediator of the TGF- β and BMP pathways, and both these pathways are involved in the homeostasis of the epidermis. TGF- β inhibits growth and differentiation of keratinocytes and induces apoptosis in HF (Wang *et al.*, 1997; Foitzik *et al.*, 1999, 2000), whereas signaling by BMP plays a role in the induction and differentiation of the HF (Botchkarev *et al.*, 1999), and its inhibition is essential for the transition from quiescent bulge SCs to proliferating progeny (Kobiela *et al.*, 2007). Therefore, it is not surprising that deregulation of these pathways leads to the formation of tumors in the skin (Kobiela *et al.*, 2003; Andl *et al.*, 2004; Ming Kwan *et al.*, 2004; Qiao *et al.*, 2005; Yang *et al.*, 2005). Our data further support these findings. The loss of Smad4 expression in multipotent cells renders the daughter cells of the IFE insensitive to TGF- β -induced growth inhibition. It also renders the daughter cells of the HF (the hHb5-positive matrix cells) resistant to TGF- β -induced apoptosis as well as to BMP-induced differentiation of HF, events that both might contribute to a hyperplastic phenotype. The fact that after re-expression of Smad4, the mTICs

become sensitive to calcium-induced differentiation suggests that the mTICs-Smad4 cells are not tumorigenic. Indeed, restoration of Smad4 expression completely abrogated their tumorigenic potential. Because we found no evidence for other genetic lesions in mTICs, the loss of p53 and Smad4 seem to be the sole molecular events responsible for their tumorigenicity. However, we cannot completely exclude the possibility that undetected genetic events have contributed to the progression of these cells into a fully neoplastic state. Nevertheless, we think that the above results allow the generation of a well-defined model of early tumorigenesis.

We found that the size of tumors produced by these mTICs is clearly reduced in the presence of $\alpha 6\beta 4$, revealing for the first time a suppressive effect mediated by this integrin on tumor growth. *In vivo*, this effect was not correlated with a change in the differentiation capacity of the cells but was directly related to the mitotic index, indicating that the main influence by $\alpha 6\beta 4$ is on the proliferative capacity of the tumor cells. This growth-suppressive effect by $\alpha 6\beta 4$ was also observed in a different stromal environment (kidney capsule) and therefore is not dependent on specific conditions in the skin. Reconstitution of the $\beta 4$ -deficient cells with various $\beta 4$ -chimeras revealed that the growth-suppressive effect by $\alpha 6\beta 4$ does not depend on its interaction with its ligand but on the recruitment of plectin by $\beta 4$ to the plasma membrane. The fact that plectin-deficient cells also induce tumors with an increased size suggests that the tumor-suppressive effect by $\beta 4$ is not exerted by this molecule itself but by plectin, which has this effect when present at the plasma membrane. The observed loss of the Ln-5-dependent promitotic effect of $\alpha 6\beta 4$ previously reported in nontransformed keratinocytes might be dependent on an entirely different mechanism, i.e., becoming independent of environmental cues (Nikolopoulos *et al.*, 2005).

The molecular events initiated downstream of the $\alpha 6\beta 4$ /plectin complex and critical for tumor growth suppression of plectin, when it is located at the plasma membrane, remain unknown. However, plectin has multiple functions, in addition to linking the actin cytoskeleton to the IF system. First, it binds to the nonreceptor tyrosine kinase Fer (Lunter and Wiche, 2002) and regulates protein kinase C activity, by sequestering the receptor for activated C kinase 1 (RACK1) in the cytoskeleton (Osmanagic-Myers and Wiche, 2004). Furthermore, plectin regulates the dynamics of the actin cytoskeleton by influencing the activity of Rho family GTPases and energy homeostasis via the binding of the regulatory $\gamma 1$ subunit of AMP-activated protein kinase, in mouse myotubes (Andra *et al.*, 1998; Gregor *et al.*, 2006). Thus, by acting as a molecular scaffold for proteins involved in cellular signaling, plectin can control different signal transduction pathways, which when plectin is not recruited to the plasma membrane by $\beta 4$ remain inactive. On the other hand, tumor development might be affected because the normal anchorage of the IFs at the plasma membrane is, in that case, also prevented, which in turn can affect membrane dynamics during mitosis.

We could not reproduce the effect of $\alpha 6\beta 4$ on the proliferation of mTIC-derived tumors in monolayer cell cultures. Surprisingly, the mTICs-Ras^{V12} grow as fast as mTICs *in vitro*, whereas *in vivo* the tumor growth rate is dramatically increased by the oncogene. Thus, suppression of growth by $\alpha 6\beta 4$ occurs only *in vivo*, which renders further investigation of the molecular mechanisms involved more difficult. Discrepancies between *in vitro* and *in vivo* findings have frequently been reported in the literature and underscore the necessity of developing suitable three-dimensional *in vitro* models to better mimic the *in vivo* situation.

When oncogenic Ras is expressed in mTICs, $\alpha 6\beta 4$ no longer suppresses growth but instead cooperates with Ras to stimulate tumor growth. This is in agreement with results of Dajee *et al.* (2003) showing that $\alpha 6\beta 4$ is essential for the tumorigenic effect of Ras on human keratinocytes. In our study, tumors did form in the absence of $\beta 4$. This might be due to the different genetic backgrounds as well as to the higher susceptibility to transformation of rodent compared with human cells (Hahn and Weinberg, 2002). Also, the results with Ras effector mutants suggest that the further state of cell transformation induced by oncogenic Ras^{V12} requires a strong activation of the Erk pathway. The slight decrease in Erk activation observed in the absence of $\alpha 6\beta 4$ might point to a role of $\alpha 6\beta 4$ in enhancing Erk signaling downstream of oncogenic Ras. It is also conceivable that the potent oncogene Ras interferes with a signal transduction pathway downstream of the integrin $\alpha 6\beta 4$ /plectin complex that controls growth.

To conclude, our studies reveal, for the first time, that $\alpha 6\beta 4$, by its ability to recruit the cytoskeletal scaffolding protein plectin to the plasma membrane, induces a suppressive effect on the growth of skin tumors, in the early stages of tumor development. This study also shows that, depending on the genetic background (presence or absence of oncogenic Ras), $\alpha 6\beta 4$ can either mediate a suppressive effect or have a promoting effect on tumor growth.

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REFERENCES

- Andl, T. *et al.* (2004). Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. *Development* 131, 2257–2268.
- Andra, K., Nikolic, B., Stocher, M., Drenckhahn, D., and Wiche G. (1998). Not just scaffolding: plectin regulates actin dynamics in cultured cells. *Genes Dev.* 12, 3442–3451.
- Aumailley, M., and Rousselle, P. (1999). Laminins of the dermo-epidermal junction. *Matrix Biol.* 18, 19–28.
- Bachelder, R. E., Ribick, M. J., Marchetti, A., Falcioni, R., Soddu, S., Davis, K. R., and Mercurio, A. M. (1999). p53 inhibits $\alpha 6\beta 4$ integrin survival signaling by promoting the caspase 3-dependent cleavage of AKT/PKB. *J. Cell Biol.* 147, 1063–1072.
- Bjerkvig, R., Tysness, B. B., Aboody, K. S., Najbauer, J., and Terzis, A. J. (2005). Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat. Rev. Cancer* 5, 899–904.
- Borradori, L., and Sonnenberg, A. (1999). Structure and function of hemidesmosomes: more than simple adhesion complexes. *J. Invest. Dermatol.* 112, 411–418.
- Botchkarev, V. A. *et al.* (1999). Noggin is a mesenchymally derived stimulator of hair follicle induction. *Nat. Cell Biol.* 1, 158–164.
- Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550–553.
- Dajee, M. *et al.* (2003). NF- κ B blockade and oncogenic Ras trigger invasive human epidermal neoplasia. *Nature* 421, 639–643.
- Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998). Direct binding of Smad3 and Smad4 to critical TGF β -inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.* 17, 3091–3100.
- Foitzik, K., Paus, R., Doetschman, T., and Dotto, G. P. (1999). The TGF- β 2 isoform is both a required and sufficient inducer of murine hair follicle morphogenesis. *Dev. Biol.* 212, 278–289.
- Foitzik, K. *et al.* (2000). Control of murine hair follicle regression (catagen) by TGF- β 1 in vivo. *FASEB J.* 14, 752–760.
- Fuchs, E., Tumber, T., and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. *Cell* 116, 769–778.
- Geuijen, C. A., and Sonnenberg, A. (2002). Dynamics of the $\alpha 6\beta 4$ integrin in keratinocytes. *Mol. Biol. Cell* 13, 3845–3858.
- Gregor, M., Zeöld, A., Oehler, S., Marobela, K. A., Fuchs, P., Weigel, G., Hardie, D. G., and Wiche, G. (2006). Plectin scaffolds recruit energy-controlling AMP-activated protein kinase (AMPK) in differentiated myofibres. *J. Cell Sci.* 119, 1864–1875.
- Hahn, W. C., and Weinberg, R. A. (2002). Modelling the molecular circuitry of cancer. *Nat. Rev. Cancer* 2, 331–341.
- Ito, M., Liu, Y., Yang, Z., Nguyen, J., Liang, F., Morris, R. J., and Cotsarelis, G. (2005). Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat. Med.* 11, 1351–1354.
- Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. (2001). Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nat. Genet.* 29, 418–425.
- Kobiela, A., and Fuchs, E. (2006). Links between α -catenin, NF- κ B and squamous cell carcinomas in skin. *Proc. Natl. Acad. Sci. USA* 103, 2322–2327.
- Kobiela, K., Pasolli, H. A., Alonso, L., Polak, L., and Fuchs, E. (2003). Defining BMP functions in the hair follicle by conditional ablation of BMP receptor IA. *J. Cell Biol.* 163, 609–623.
- Kobiela, K., Stokes, N., de la Cruz, J., Polak, L., and Fuchs, E. (2007). Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. *Proc. Natl. Acad. Sci. USA* 104, 10063–10068.
- Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a β -catenin-Tcf complex in APC-/- colon carcinoma. *Science* 275, 1784–1787.
- Koster, J., Kuikman, I., Kreft, M., and Sonnenberg, A. (2001). Two different mutations in the cytoplasmic domain of the integrin $\beta 4$ subunit in nonlethal forms of epidermolysis bullosa prevent interaction of $\beta 4$ with plectin. *J. Invest. Dermatol.* 117, 1405–1411.
- Kulesz-Martin, M., Kilkenny, A. E., Holbrook, K. A., Digernes, V., and Yuspa, S. H. (1983). Properties of carcinogen altered mouse epidermal cells resistant to calcium-induced terminal differentiation. *Carcinogenesis* 4, 1367–1377.
- Langbein, L., Rogers, M. A., Winter, H., Praetzel, S., and Schweizer, J. (2001). The catalog of human hair keratins. II. Expression of the six type II members in the hair follicle and the combined catalog of human type I and II keratins. *J. Biol. Chem.* 276, 35123–35132.
- Lipscomb, E. A., and Mercurio, A. M. (2005). Mobilization and activation of a signaling competent $\alpha 6\beta 4$ integrin underlies its contribution to carcinoma progression. *Cancer Metastasis Rev.* 24, 413–423.
- Litjens, S. H. M., de Pereda, J. M., and Sonnenberg, A. (2006). Current insights into the formation and breakdown of hemidesmosomes. *Trends Cell Biol.* 16, 376–383.
- Lunter, P. C., and Wiche, G. (2002). Direct binding of plectin to Fer kinase and negative regulation of its catalytic activity. *Biochem. Biophys. Res. Commun.* 296, 904–910.
- Lynch, M. H., O'Guin, W. M., Hardy, C., Mak, L., and Sun, T. T. (1986). Acidic and basic hair/nail ("hard") keratins: their colocalization in upper cortical and cuticle cells of the human hair follicle and their relationship to "soft" keratins. *J. Cell Biol.* 103, 2593–2606.
- McGowan, K. M., and Coulombe, P. A. (1998). Onset of keratin 17 expression coincides with the definition of major epithelial lineages during skin development. *J. Cell Biol.* 143, 469–486.
- Ming Kwan, K., Li, A. G., Wang, X. J., Wurst, W., and Behringer, R. R. (2004). Essential roles of BMP1-IA signaling in differentiation and growth of hair follicles and in skin tumorigenesis. *Genesis* 39, 10–25.
- Morris, R. J., Liu, Y., Marles, L., Yang, Z., Trempus, C., Li, S., Lin, J. S., Sawicki, J. A., and Cotsarelis, G. (2004). Capturing and profiling adult hair follicle stem cells. *Nat. Biotechnol.* 22, 411–417.
- Nikolopoulos, S. N., Blaikie, P., Yoshioka, T., Guo, W., Puri, C., Tacchetti, C., and Giancotti, F. G. (2005). Targeted deletion of the integrin $\beta 4$ signaling domain suppresses laminin-5-dependent nuclear entry of mitogen-activated protein kinases and NF- κ B, causing defects in epidermal growth and migration. *Mol. Cell. Biol.* 25, 6090–6102 [Erratum in: *Mol. Cell. Biol.* 25, 7926].

- O'Guin, W. M., Sun, T. T., and Manabe, M. (1992). Interaction of trichohyalin with intermediate filaments: three immunologically defined stages of trichohyalin maturation. *J. Invest. Dermatol.* 98, 24–32.
- Orian-Rousseau, V., Aberdam, D., Fontao, L., Chevalier, L., Meneguzzi, G., Keding, M., and Simon-Assmann, P. (1996). Developmental expression of laminin-5 and HD1 in the intestine: epithelial to mesenchymal shift for the laminin $\gamma 2$ chain subunit deposition. *Dev. Dyn.* 206, 12–23.
- Osmanagic-Myers, S., and Wiche, G. (2004). Plectin-RACK1 (receptor for activated C kinase 1) scaffolding: a novel mechanism to regulate protein kinase C activity. *J. Biol. Chem.* 279, 18701–18710.
- Owens, D. M., and Watt, F. M. (2003). Contribution of stem cells and differentiated cells to epidermal tumors. *Nat. Rev. Cancer* 3, 444–451.
- Qiao, W., Li, A. G., Owens, P., Xu, X., Wang, X. J., and Deng, C. X. (2005). Hair follicle defects and squamous cell carcinoma formation in Smad4 conditional knockout mouse skin. *Oncogene* 25, 207–217.
- Rabinovitz, I., Toker, A., and Mercurio, A. M. (1999). Protein kinase C-dependent mobilization of the $\alpha 6\beta 4$ integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. *J. Cell Biol.* 146, 1147–1160.
- Raymond, K., Kreft, M., Janssen, H., Calafat, J., and Sonnenberg, A. (2005). Keratinocytes display normal proliferation, survival and differentiation in conditional $\beta 4$ -integrin knockout mice. *J. Cell Sci.* 118, 1045–1060.
- Rodriguez-Viciano, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* 89, 457–467.
- Rogers, M. A., Langbein, L., Praetzel, S., Moll, I., Krieg, T., Winter, H., and Schweizer, J. (1997). Sequences and differential expression of three novel human type-II hair keratins. *Differentiation* 61, 187–194.
- Shaw, L. M., Rabinovitz, I., Wang, H. H., Toker, A., and Mercurio, A. M. (1997). Activation of phosphoinositide 3-OH kinase by the $\alpha 6\beta 4$ integrin promotes carcinoma invasion. *Cell* 91, 49–60.
- Sonnenberg, A., de Melker, A. A., Martinez de Velasco, A. M., Janssen, H., Calafat, J., and Niessen, C. M. (1993). Formation of hemidesmosomes in cells of a transformed murine mammary tumor cell line and mechanisms involved in adherence of these cells to laminin and kalinin. *J. Cell Sci.* 106, 1083–1102.
- Stark, H. J., Breitkreutz, D., Limat, A., Bowden, P., and Fusenig, N. E. (1987). Keratins of the human hair follicle: "hyperproliferative" keratins consistently expressed in outer root sheath cells in vivo and in vitro. *Differentiation* 35, 236–248.
- Sterk, L. M., Geuijen, C. A., Oomen, L. C., Calafat, J., Janssen, H., and Sonnenberg, A. (2000). The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin $\alpha 6\beta 4$ and may regulate the spatial organization of hemidesmosomes. *J. Cell Biol.* 149, 969–982.
- Tennenbaum, T., Weiner, A. K., Belanger, A. J., Glick, A. B., Hennings, H., and Yuspa, S. H. (1993). The suprabasal expression of $\alpha 6\beta 4$ integrin is associated with a high risk for malignant progression in mouse skin carcinogenesis. *Cancer Res.* 53, 4803–4810.
- Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. *Science* 303, 359–363.
- Uematsu, J., Nishizawa, Y., Sonnenberg, A., and Owaribe, K. (1994). Demonstration of type II hemidesmosomes in a mammary gland epithelial cell line, BMGE-H. *J. Biochem.* 115, 469–476.
- Uitto, J., and Pulkkinen, L. (2001). Molecular genetics of heritable blistering disorders. *Arch. Dermatol.* 137, 1458–1461.
- van Triest, M., de Rooij, J., and Bos, J. L. (2001). Measurement of GTP-bound Ras-like GTPases by activation-specific probes. *Methods Enzymol.* 33, 343–348.
- van Waes, C., Surh, D. M., Chen, Z., Kirby, M., Rhim, J. S., Brager, R., Sessions, R. B., Poore, J., Wolf, G. T., and Carey, T. E. (1995). Increase in suprabasilar integrin adhesion molecule expression in human epidermal neoplasms accompanies increased proliferation occurring with immortalization and tumor progression. *Cancer Res.* 55, 5434–5444.
- Wang, X. J., Greenhalgh, D. A., Bickenbach, J. R., Jiang, A., Bundman, D. S., Krieg, T., Derynck, R., and Roop, D. R. (1997). Expression of a dominant-negative type II transforming growth factor β (TGF- β) receptor in the epidermis of transgenic mice blocks TGF- β -mediated growth inhibition. *Proc. Natl. Acad. Sci. USA* 94, 2386–2391.
- Wilhelmsen, K., Litjens, S.H.M., and Sonnenberg, A. (2006). Multiple functions of the integrin $\alpha 6\beta 4$ in epidermal homeostasis and tumorigenesis. *Mol. Cell. Biol.* 26, 2877–2886.
- Yang, L., Mao, C., Teng, Y., Li, W., Zhang, J., Cheng, X., Han, H., Xia Z., Deng, H., and Yang, X. (2005). Targeted disruption of Smad4 in mouse epidermis results in failure of hair follicle cycling and formation of skin tumors. *Cancer Res.* 65, 8671–8678.