β-Catenin is not required for proliferation and differentiation of epidermal mouse keratinocytes

Horst Posthaus[1], Lina Williamson[1], Dominique Baumann[1], Rolf Kemler[2], Reto Caldelari[1], Maja M. Suter[1], Heinz Schwarz[3] and Eliane Müller[1,*]

[1]Institute of Animal Pathology, University of Bern, Länggassstr. 122, 3012 Bern, Switzerland
[2]Max Planck Institute for Immunobiology, PO Box 1169, Stübeweg 51, 7800 Freiburg Zähringen, Germany
[3]Max Planck Institute for Developmental Biology, Spemannstr.35, 72076 Tübingen, Germany
*Author for correspondence (e-mail: eliane.mueller@itpa.unibe.ch)

Accepted 4 September 2002
Journal of Cell Science 115, 4587-4595 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00141

Summary

Despite the pivotal role of β-catenin in a variety of biological processes, conditional *β-catenin* gene ablation in the skin of transgenic mice failed to affect interfollicular epidermal morphogenesis. We elucidated the molecular mechanisms underlying this phenomenon. Long-term cultures of homozygous, heterozygous and β-catenin-null mutant keratinocytes were established to demonstrate that epidermal keratinocyte proliferation, cell cycle progression and cyclin D1 expression occur independently of β-catenin and correlate with repression of transcription from Tcf/Lef-responsive promoters. Moreover, during differentiation, β-catenin-null cells assemble normal intercellular adhesion junctions owing to the substitution of β-catenin with plakoglobin, whereas the expression of the other adhesion components remains unaffected. Taken together, our results demonstrate that epidermal proliferation and adhesion are independent of β-catenin.

Key words: Keratinocytes, Epidermal renewal, Plakoglobin, Wnt signaling

Introduction

β-Catenin is an ubiquitously distributed protein with multiple functions (Gumbiner, 1996; Willert and Nusse, 1998; Vleminckx and Kemler, 1999; Kuhl et al., 2000). Known first for its role in intercellular adhesion structures called adherens junctions, β-catenin was subsequently found to act as a signaling molecule in the Wnt/wingless pathway. In response to Wnt signals, it translocates to the nucleus and transactivates transcription of target genes together with members of the Tcf/Lef1 transcription factor family (Behrens et al., 1996; Huber et al., 1996). Owing to this bipartite function, β-catenin is crucially involved in a variety of biological processes, both during embryonic development and in the adult. Its essential role during development was emphasized by the finding that β-catenin-knockout mice fail to develop beyond embryonic day 6.5 (Haegel et al., 1995; Huelsken et al., 2000). In the adult, reduced levels of β-catenin have been implicated, for instance, in the programmed neuronal cell death described in Alzheimer's disease (Zhang et al., 1998). Alternatively, aberrantly activated β-catenin triggers uncontrolled proliferation, hyperplastic and delayed wound closure and finally tumorigenesis in many different tissues and cell types including keratinocytes (Chan et al., 1999; Bienz and Clevers, 2000; Barker et al., 2000; Cheon et al., 2002).

In epithelia, the role of β-catenin in cell adhesion and signaling has been widely investigated. In these tissues two distinct junctional complexes mediate intercellular adhesion: adherens junctions and desmosomes (Kowalczyk et al., 1999; Green and Gaudry, 2000). Both junctions consist of transmembrane cadherins, mainly E-cadherin in adherens junctions, and desmogleins and desmocollins in desmosomes. The cytoplasmic tails of these adhesion molecules link to the cytoskeleton via intracellular plaque proteins. β-Catenin is one of these plaque proteins, which is exclusively confined to adherens junctions. In epithelia, evidence exists that the cell adhesion and signaling roles of β-catenin are interdependent. In cultured epithelial cells it was demonstrated that intercellular adhesion components can interfere with the signaling role of β-catenin by sequestering it at the plasma membrane (Simcha et al., 1998; Sadot et al., 1998; Orsulic et al., 1999; Gottardi et al., 2001). Moreover, evidence has been provided that Wnt signaling promotes assembly of intercellular junctions (Bradley et al., 1993).

In the epidermis, an epithelial tissue of high complexity, Wnt signaling via β-catenin was recently implicated in important steps during morphogenesis, a process that is now widely believed to rely on differentiation of the epidermal stem cells along hair, sebaceous gland and epidermal lineages (Fuchs and Segre, 2000; Lavker and Sun, 2000; Watt, 2001; Huelsken and Birchmeier, 2001). First indications that β-catenin signaling can interfere with stem cell differentiation came from transgenic overexpression of a N-terminally stabilized mutant of β-catenin in mouse skin (Gat et al., 1998). The elevated levels of β-catenin resulted in de novo hair follicle morphogenesis, in addition to tumor formation. This further correlated with a high incidence of N-terminally stabilized β-catenin mutation in hair follicle tumors in man (Chan et al., 1999). Collectively, these findings indicated that β-catenin plays a major role during hair development. This hypothesis received key support by the finding that conditional ablation of the *β-catenin* gene in murine skin abrogated hair formation (Huelsken et al., 2001). This phenotype was further reproduced

by transgenic expression of a Lef1 mutant lacking the β-catenin-interacting domain (Merrill et al., 2001; Niemann et al., 2002). Most surprisingly, despite the important function attributed to β-catenin in the formation and renewal of skin appendages, β-catenin ablation did not appear to affect the formation of interfollicular epidermis (Huelsken et al., 2001). This was unexpected as c-myc, which can be a target of β-catenin (He et al., 1998), had been suggested to be essential in promoting epidermal stem cell differentiation (Gandarillas and Watt, 1997; Arnold and Watt, 2001). In addition, several Wnts, which can potentially activate β-catenin signaling, are expressed in interfollicular murine epidermis (Reddy et al., 2001). Nonetheless, misexpression of Tcf3 in transgenic mouse skin brought additional support to the hypothesis that β-catenin signaling via the Tcf/Lef family members is dispensable for the establishment of the epidermal phenotype (Merrill et al., 2001). Tcf3, which was suggested to inhibit β-catenin signaling in the epidermal context, also failed to disturb the epidermal architecture with the exception of an aberrant expression of some terminal differentiation markers (Merrill et al., 2001).

The collective results of *β-catenin* gene ablation in epidermis prompted two hypotheses with respect to β-catenin adhesion and signaling. Firstly, that plakoglobin, which binds to the same cytoplasmic domain in E-cadherin, substitutes for the deficiency of β-catenin in adherens junctions and enables the development of an apparently intact epidermal architecture (Haegel et al., 1995). Secondly, the apparently normal epidermis despite impaired β-catenin-mediated Wnt signaling suggested that signaling via β-catenin is, and must be, shut off to allow normal epidermal proliferation and differentiation to proceed (Huelsken et al., 2001; Merrill et al., 2001).

Here we wished to address these two hypotheses and to further elucidate the contribution of β-catenin to epidermal keratinocyte biology at the molecular level. The approach we chose was to establish long-term epidermal keratinocyte cultures (Caldelari et al., 2000) from the skin of conditional β-catenin knockout mice [using Cre/LoxP (Brault et al., 2001)]. The sequential deletion of the *β-catenin* gene and analysis of wild-type, heterozygous β-catenin and β-catenin-null keratinocytes allowed us to rule out the involvement of β-catenin- and Tcf-mediated transcriptional transactivation in proliferation, as well as in junction assembly and differentiation in epidermal keratinocytes.

Materials and Methods

Establishment of keratinocyte cultures

Mice carrying a β-catenin allele in which two loxP sites flanked exons 2 to 6 (β-cat^{+loxP}) were used in this study (Brault et al., 2001). Keratinocytes cultures from eight individual E16.5 embryos generated by β-cat^{+} β-cat^{-} and β-cat^{+loxP} β-cat^{+loxP} parents were isolated and cultured according to our previously published protocol (Caldelari et al., 2000). Consistent with Mendelian distribution, cultures of the genotype β-cat$^{+/+}$ [β-cat^{+} β-cat^{+loxP}] or β-cat$^{+/-}$ [β-cat^{-} β-cat^{+loxP}] were obtained as identified by PCR (data not shown). Subcultures from four different embryos were infected with Adenovirus encoding Cre-recombinase (Saitou et al., 1995) at a multiplicity of infection (Moi) of 100 (a kind gift of I. Saitou, Tokyo, Japan). The morphology of infected cultures as seen by light microscopy and their ability to be passaged were the same as those for the corresponding uninfected cells (data not shown). To confirm β-catenin deletion from the entire culture, cellular DNA was extracted and analyzed by PCR (Brault et al., 2001). The primers used were RM41, RM42 and RM43, which yield amplification products of 221 bp for the β-cat^{+}, 324 bp for the β-cat^{+loxP} and 500 bp for the β-cat^{-} allele. In β-cat$^{-/-}$ cultures, complete loss of β-catenin protein was demonstrated by western blot analysis and on a single cell level by immunofluorescence (see below). These tests were repeated during subsequent passages. Cell cultures were expanded in low Ca^{2+} medium [0.07 mM $CaCl_2$, supplemented KSFM (Invitrogen)]. At confluency, they were either left in low Ca^{2+} or differentiation was induced by increasing the Ca^{2+} concentration in the medium to 1.2 mM (high Ca^{2+} medium).

Antibodies

Primary antibodies used were: E-cadherin (DECMA), β-catenin and plakoglobin (Transduction Laboratories, Heidelberg, Germany, Catalog No. C1922, C26220), α-catenin (Zymed, San Francisco, USA. Catalog No. 71-1200), plakophilin 1 (kind gift of P. Wheelock, Nebraska Medical Center, Omaha, NE), plakophilin 3 and desmoglein1/2 (Progen, Heidelberg, Germany, Catalog No. 651113, 61002), filaggrin and loricrin (Covance, Richmond, USA, Catalog No. PRB-417P, PRB-145P), cyclin D1 (Pharmingen, Heidelberg, Germany, Catalog No. 556470), involucrin (kind gift of F. Watt; Cancer Research UK, London, UK) and desmoglein3 (kind gift of. J. Stanley, Philadelphia, PA).

Proliferation assay and cell cycle analysis

2.5×10^5 cells were seeded into 8.8 cm^2 culture dishes in low Ca^{2+} medium. At 24 hour intervals triplicates were trypsinized and counted. 96 hours after seeding, cultures were also evaluated for cyclin D1 expression by western blot analysis of whole cell lysates and for DNA content by flow cytometry. For the latter analysis, cells were fixed in 75% ethanol and stained with propidium iodide (0.1% NP40, 3.4 mM Tris pH 7.4, 0.2 mg/ml RNaseA, 20 μg/ml propidium iodide).

Reporter gene assay

Cells were seeded in duplicate in 10 cm^2 wells and low Ca^{2+} medium. One day later, they were transfected using the lipid-based SuperFect reagent (Qiagen, Basel, Switzerland) with either 1.875 μg of pTOP-flash (containing triple Tcf/Lef1 binding sites, the basic thymidine kinase promoter and firefly luciferase reporter gene) or pFOP-flash (containing mutated Tcf/Lef1 binding sites). When indicated, cells were co-transfected with 1.875 μg stabilized β-catenin or Lef or with the same amount of vector. Plasmids containing Lef1 (Huber et al., 1996), N-terminally mutated stabilized β-catenin [a kind gift from E. R. Frearon, Ann Arbor, MI (Caca et al., 1999)], and pTOP-flash or pFOP-flash reporter constructs have been described previously [a kind gift of N. Barker and H. Clevers, Utrecht, Belgium (Molenaar et al., 1996)]. All samples were normalized by transfecting 37.5 ng pRL-tk (renilla luciferase reporter under the control of a constitutively active thymidine kinase promoter; Promega, Wallisellen, Switzerland; a kind gift of J-M. Zingg and A. Azzi, University of Berne, Switzerland). 34 hours after transfection, cells were lysed and firefly and renilla luciferase activity measured in the same sample with the DualLuciferase™ Reporter Assay System (Promega, Wallisellen, Switzerland). This experiment was repeated three times.

RT-PCR

Using the RNeasy kit (Quiagen, Catalog No. 74104), total RNA was isolated from cultures grown in low or high Ca^{2+} medium for 30 hours. Random-primed cDNA was prepared using standard techniques. Specific primers for Tcf/Lef family members were adapted from those published for the human sequences (Brantjes et al., 2001): Tcf3: gaaatccccagttacggtg (sense), caggttgggtagagctgc

(antisense); Tcf4: gcaccctccagatatatc (sense), tggagtcctgatgctttg (antisense); Lef1: ctccacccatcccgagg (sense), gaggcttcacgtgcattag (antisense). Amplification of GAPDH from the same cDNA was done using gctcctttctgctgatgcccc (sense) and gggtggcagtgatggcatgg (antisense) primers. 30 amplification cycles were performed for Lef1 and Tcf4, whereas 20, 25 and 30 cycles were done for Tcf3 (shown are 25 cycles) and 15 for GAPDH to obtain semi-quantitative results. Control reactions were carried out on either cloned full-length cDNA of mouse Tcf3, human Tcf4 (kind gifts from N. Barker and H. Clevers, University Medical Center Utrecht) and mouse Lef1 (Huber et al., 1996) as well as on a mix of genomic DNA isolated from these cells or water.

Protein extraction and co-precipitation

Confluent cell cultures were incubated in KSFM containing 1.2 mM $CaCl_2$ for 30 hours, washed with PBS and incubated with cytoplasmic extraction buffer (0.015% digitonin, 10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA, 1 mM PMSF, complete EDTA-free protease inhibitor cocktail [Roche Diagnostics]) for 10 minutes. Cytoplasmic extracts were removed and cells were scraped into membrane extraction buffer (0.5% TritonX-100, 10 mM PIPES pH 6.8, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 10 mM $Na_4P_2O_7$, 20 mM NaF, 1 mM Na_3Vo_4, 0.1mg/ml RNaseA, 0.1 mg/ml DNase 1, 1 mM PMSF and protease inhibitor cocktail) according to Pasdar et al. (Pasdar et al., 1995) with some modifications (Caldelari et al., 2001). The TritonX-100-insoluble fraction was pelleted (20 minutes, 20,000 ***g***) and dissolved by sonication and boiling in SDS-PAGE buffer. For co-precipitation, 500 μg total protein of the TritonX-100-soluble fraction were incubated with 2 μg plakoglobin antibodies, 5 μg rat E-cadherin antibodies or 5 μl Dsg3 polyclonal serum and processed according to standard protocols. For total lysates, cells were directly scraped into SDS-PAGE buffer.

Immunofluorescence microscopy and electron microscopy

Keratinocytes were grown to confluency on coverslips (Lab-Tek™ II, Nunc, Roskilde, Denmark, Catalog No. 154534) and incubated in high Ca^{2+} medium for 30 hours. For immunofluorescence analyses, cells were fixed in 1% paraformaldehyde for 20 minutes, permeabilized with 0.5% TritonX-100 for 10 minutes prior to incubation with primary and conjugated secondary antibodies. For electron microscopy, cells were fixed with 4% formaldehyde, 0.2% glutaraldehyde in 200 mM HEPES pH 7.2 for 20 minutes at room temperature and overnight at 4°C, postfixed with 1% osmium tetroxide in PBS for 1 hour on ice, washed with H_2O, treated with 1% aqueous uranyl acetate for 1 hour at 4°C, then dehydrated through a graded series of ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a Philips CM10 electron microscope at 60 kV using a 30 μm objective aperture.

Adhesion assay

Cells seeded in quadruplicates were switched to high Ca^{2+} medium at confluency for 30 hours (cell density $2.5\times10^5/cm^2$) and intercellular adhesiveness assessed by a modified protocol (Caldelari et al., 2001) originally described by Calautti and colleagues (Calautti et al., 1998).

Results

Long-term keratinocyte cultures and conditional inactivation of the *β-catenin* gene

Long-term cultures were established according to our previous protocol (Caldelari et al., 2000) from the skin of transgenic mouse embryos engineered to delete one β-catenin allele at will [using CreLox (Brault et al., 2001)]. The results obtained with cultures from three different embryos, in which the transgenic β-catenin allele (β-cat^{+loxP}) has been inactivated, are presented in this study (designated 1-3). Uninfected and infected culture 1 [β-cat$^+$ β-cat^{+loxP} and β-cat$^+$ β-cat$^–$] served as a control for cultures 2 and 3 that were either heterozygous (uninfected) or null (infected) for β-catenin [β-cat$^–$ β-cat^{+loxP} and β-cat$^–$ β-cat$^–$]. Correct and complete recombination was evaluated by PCR and the absence of β-catenin protein assessed by western blot analysis (Fig. 1) and immunofluorescence studies (Fig. 3 shows culture 2) and reassessed at random during subsequent experimentation.

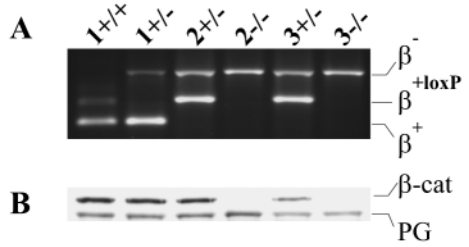

Fig. 1. Geno- and phenotyping of selected keratinocyte cultures before and after conditional *β-catenin* gene ablation by Adenovirus-encoded Cre-recombinase. (A) PCR performed on genomic DNA of cultured mouse keratinocytes depicts complete recombination of β-cat^{+loxP} into β-cat$^–$ alleles in cultures 1–3. (B) Western blot analysis of whole cell lysates was used to confirm lack of β-catenin expression in conditional null mutant keratinocytes. Plakoglobin was assessed on the same blot and used as a loading control. β$^–$, deleted β-catenin allele; β^{+loxP}, transgenic allele carrying two loxP sites; β$^+$, wild-type allele; PG, plakoglobin.

Consequences of *β-catenin* gene inactivation on epidermal keratinocyte proliferation

Elevated cytosolic β-catenin levels were recently demonstrated to induce hyperproliferation of cultured epidermal keratinocytes via β-catenin/Tcf-mediated upregulation of cyclin D1 and altered cell cycle progression (Xia et al., 2001). It was thus conceivable that the inverse situation, namely lack of β-catenin, would impede these essential biological processes in proliferating keratinocytes. Accordingly we compared the overall proliferative capacity of the homozygous, heterozygous and β-catenin-null mutant keratinocytes. Proliferation curves were established from cultures ranging from low cell density (proliferative) to high cell density (contact inhibited) (Fig. 2A). Cells were harvested at 24 hour intervals and counted. Interestingly, β-catenin deletion did not affect the growth rate within each individual culture, nor did it interfere with contact-induced growth arrest. Culture 3 in general had a slower growth rate than culture 1 and 2. This effect was, however, independent of the presence of β-catenin. In the proliferative phase (96 hours, Fig. 2A) the cells were also stained with propidium iodide and subjected to FACS analysis (Table 1). The proportions of cells in G1, S or G2/M phase within the corresponding keratinocyte cultures did not differ. Normal cell cycle progression further correlated with unaltered cyclin D1 expression and was independent of inactivation of one or both β-catenin alleles (Fig. 2B).

To further assess whether Tcf/Lef1-mediated transcriptional activity was reduced in the absence of β-catenin in proliferating

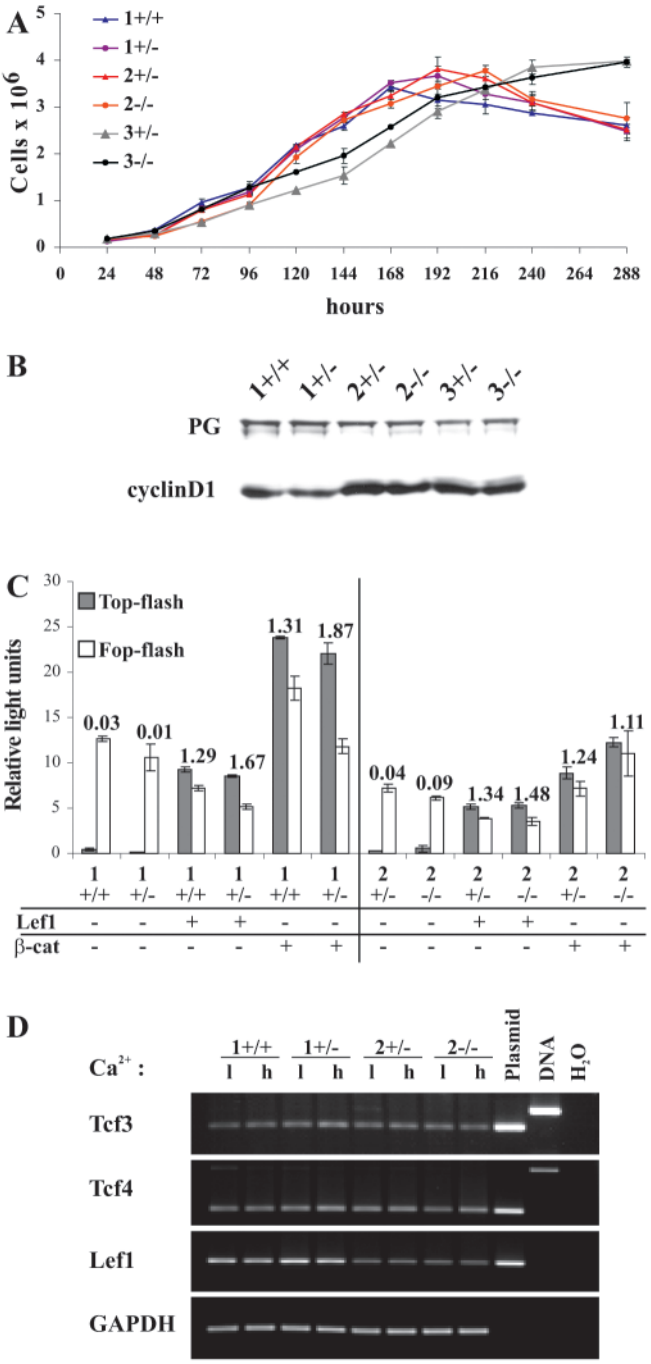

Fig. 2. Proliferation capacity and transcriptional transactivation from Tfc/Lef1 promoters in homozygous, heterozygous and β-catenin-null mutant keratinocytes. (A) Proliferation curves were established under low Ca^{2+} conditions for the indicated keratinocyte cultures. Error bars represent standard deviation of the mean of triplicate samples within one out of two experiments. (B) During the proliferative phase (in A, 96 hours), cyclin D1 expression was simultaneously assessed by western blot analysis. Plakoglobin expression examined on the same blot served as a control for equal loading. (C) Keratinocytes were transfected with pTOP-flash (gray bars) or the same amount of pFOP-flash (open bars) reporter along with Lef1 or β-catenin as indicated. Relative light units (firefly luciferase over renilla luciferase) are indicated. Numbers are the ratios of normalized TOP-flash over FOP-flash activity. Bars represent minimum and maximum values of duplicate samples of one experiment. This experiment was repeated three times. (D) Expression of Tcf/Lef family members assessed by RT-PCR. Total RNA was isolated from confluent cell cultures grown under low (l) or high (h) Ca^{2+} conditions. Plasmids, full-length cDNA as positive control; DNA, genomic DNA used as negative control; H_2O, PCR performed without template. For Tcf-3 and GAPDH, the number of cycles was reduced to 25 and 15, respectively, to allow semi-quantitative analysis.

Table 1. Percentage of cells in G1, S or G2/M phase 96 hours after seeding determined by FACS analysis of propidium-iodide-stained cells

	G1	S	G2/M
1 +/+	47	29	24
1 +/–	45	30	23
2 +/–	38	24	38
2 –/–	38	28	33
3 +/–	37	22	34
3 –/–	38	20	34

Cell cycle progression.

keratinocytes, we transfected cultures 1 (β-cat$^{+/+}$ and β-cat$^{+/–}$) and 2 (β-cat$^{+/–}$ and β-cat$^{–/–}$) with TOP-flash and FOP-flash reporter constructs (Molenaar et al., 1996), the latter containing mutated Tcf/Lef1 binding motives. Strikingly, transfection of TOP-flash reporter plasmid alone (without addition of β-catenin or Lef1) resulted in a markedly lower activation of the TOP-flash promoter as compared with FOP-flash, and this was independent of the genotype (Fig. 2C). This situation was inverted when a N-terminal stabilizing mutant of β-catenin or Lef1 were co-transfected, with the TOP-flash activity exceeding that of FOP-flash by up to 1.87-fold. The finding that ectopic expression of Lef1 conferred transcriptional transactivation in the absence of β-catenin was unexpected and suggests the presence of alternate transactivators in epidermal keratinocytes as further discussed below. Taken together these results demonstrated that epidermal keratinocyte proliferation in vitro does not require β-catenin, and further revealed that this correlated with β-catenin-independent inhibition of Tcf/Lef promoters, an effect that could be overridden by overexpressed β-catenin or Lef1.

In epidermal stem cells, β-catenin-independent inhibition from Tcf/Lef promoters was suggested to be conveyed by Tcf3 (Merill et al., 2001). To address such a possibility in our cultured keratinocytes, we assessed expression of Tcf3 via semi-quantitative RT-PCR along with that of Lef1 and Tcf4 (Fig. 2D). Expression of Lef1 has previously been reported in primary epidermal keratinocyte cultures (Zhou et al., 1995), and Tcf4 transcription can readily be detected in epidermal extracts (H.P., L.W., D.B., R.K. et al., unpublished). In all cultures Tcf3, Tcf4 and Lef1 were present and persisted during early Ca^{2+}-induced differentiation (Fig. 2D). The fact that Tcf3 is expressed in these cultures whereas transcriptional transactivation from Tcf/Lef promoters is inhibited could thus provide a conceivable explanation for the latter phenomenon.

Consequences of *β-catenin* gene deletion for functional assembly of intercellular adhesion structures

It has been suggested that plakoglobin substitutes for β-catenin

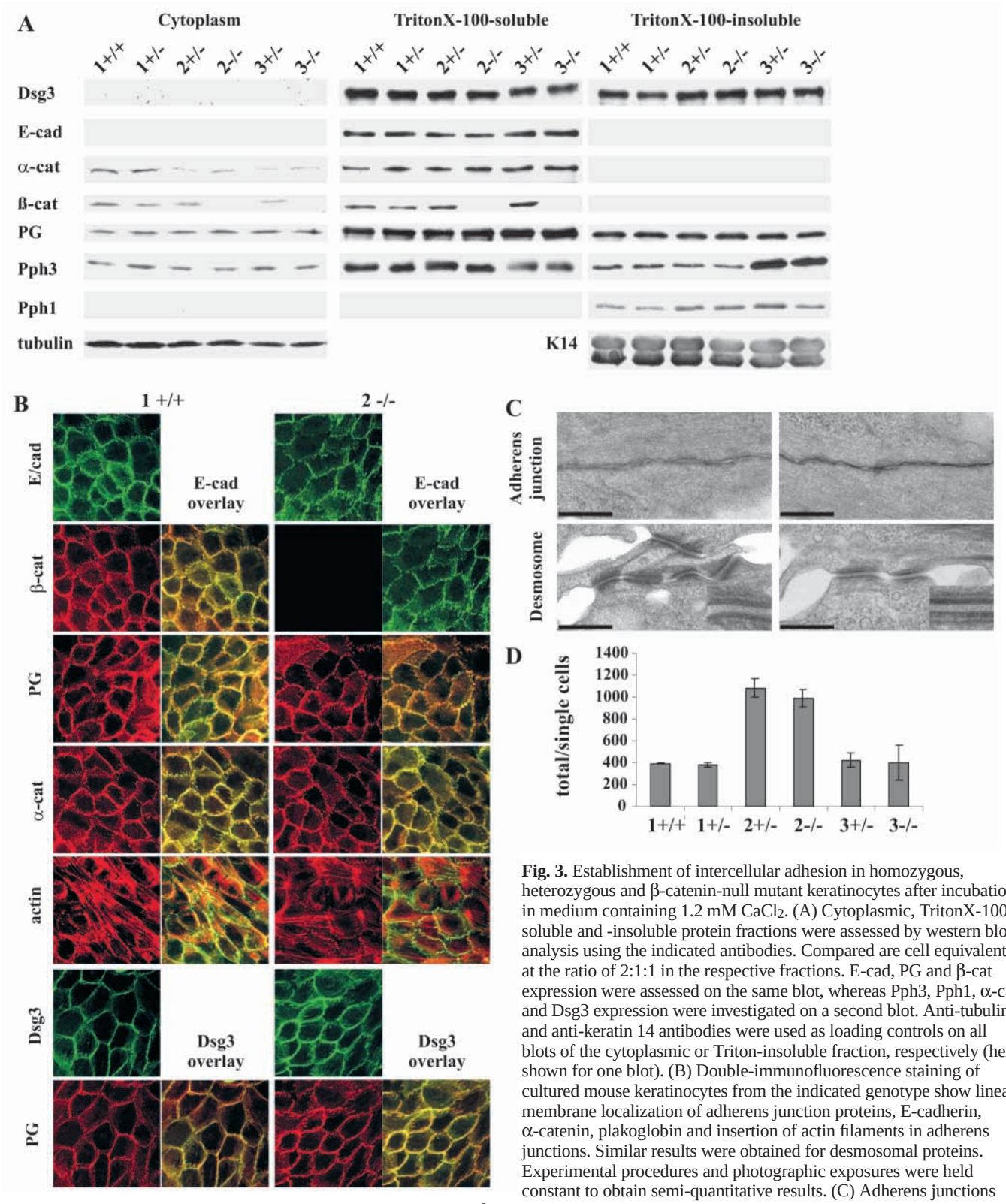

Fig. 3. Establishment of intercellular adhesion in homozygous, heterozygous and β-catenin-null mutant keratinocytes after incubation in medium containing 1.2 mM $CaCl_2$. (A) Cytoplasmic, TritonX-100-soluble and -insoluble protein fractions were assessed by western blot analysis using the indicated antibodies. Compared are cell equivalents at the ratio of 2:1:1 in the respective fractions. E-cad, PG and β-cat expression were assessed on the same blot, whereas Pph3, Pph1, α-cat and Dsg3 expression were investigated on a second blot. Anti-tubulin and anti-keratin 14 antibodies were used as loading controls on all blots of the cytoplasmic or Triton-insoluble fraction, respectively (here shown for one blot). (B) Double-immunofluorescence staining of cultured mouse keratinocytes from the indicated genotype show linear membrane localization of adherens junction proteins, E-cadherin, α-catenin, plakoglobin and insertion of actin filaments in adherens junctions. Similar results were obtained for desmosomal proteins. Experimental procedures and photographic exposures were held constant to obtain semi-quantitative results. (C) Adherens junctions and desmosomes demonstrate identical ultrastructure in wild-type and β-catenin-null mutant keratinocytes (Scale bars, 435 nm). (D) Intercellular adhesiveness was quantified. Values are means of n=4; bars indicate standard deviations. Note that the differences were not significant. Dsg3, desmoglein3; E-cad, E-cadherin; α-cat, α-catenin; β-cat, β-catenin; PG, plakoglobin; Pph, plakophilin.

in adherens junctions of β-catenin knockout cells and that this may result in a higher amount of plakoglobin at the plasma membrane (Haegel et al., 1995; Huelsken et al., 2000; Huelsken et al., 2001). We therefore addressed the effect of β-catenin depletion on the level and localization of plakoglobin and also on the functionality of both adherens junctions and desmosomes.

Differentiation-dependent assembly of adherens junctions and desmosomes was induced by incubating keratinocytes in high Ca^{2+} medium for 30 hours (Hennings et al., 1980; Caldelari et al., 2000). Interestingly, no increase in the steady-state level of plakoglobin was observed in whole cell lysates of heterozygous or β-catenin-null mutant keratinocytes (Fig. 1). We thus investigated a possible effect of β-catenin deletion on the subcellular distribution and relative expression level of plakoglobin and other junctional components. Cellular lysates were fractionated in cytoplasmic, membrane-bound TritonX-100-soluble and TritonX-100-insoluble proteins (Fig. 3A) as described previously (Pasdar et al., 1995). Using this fractionation procedure, the TritonX-100-soluble fraction contains most of the adherens junction components and desmosomal proteins not yet assembled into desmosomes, whereas the Triton-insoluble fraction mainly harbors fully assembled desmosomes (Pasdar et al., 1995). Interestingly, there was no substantial alteration in the steady-state level and distribution of plakoglobin and the major adherens junction and desmosomal proteins in case of β-catenin deletion. Consistent with this finding, semi-quantitative double-labeling immunofluorescence studies revealed comparable and linear membrane staining of E-cadherin, plakoglobin or α-catenin, and a similarly organized actin filament network in β-catenin-null keratinocytes (Fig. 3B). β-Catenin deletion did also not alter the membrane distribution of desmosomal proteins. Furthermore, ultrastructurally desmosomes and adherens junctions of β-catenin-null keratinocytes showed the typical epidermal morphology (Fig. 3C). Also no difference in the number of adherens junctions or desmosomes per cell was observed between the different genotypes by counting electron microscopic sections (data not shown). Thus, in the absence of β-catenin, cultured keratinocytes appeared to possess normal numbers of correctly assembled adherens junctions and desmosomes. To address whether these intercellular junctions were functional in the absence of β-catenin, we performed an adhesion assay, which is based on resistance to application of mechanical stress (Calautti et al., 1998; Caldelari et al., 2001). Overall we found intercellular adhesive strength to be unchanged between the individual cultures, despite deletion of the *β-catenin* gene (Fig. 3D). The more substantial adhesive strength observed in culture 2, which is independent of β-catenin, will be discussed below.

We further assessed whether plakoglobin could substitute for the lack of β-catenin in adherens junctions, thus explaining the observed undisturbed intercellular adhesiveness. Co-precipitation studies were performed of the TritonX-100-soluble protein fractions {which contain most of the adherens junction components [(Brault et al., 2001), see Fig. 3a]} using either E-cadherin or plakoglobin antibodies (Fig. 4). Clearly more plakoglobin was associated with E-cadherin in β-catenin-null keratinocytes than in those expressing β-catenin (Fig. 4, IP:E-cadherin). Consistently more E-cadherin was co-precipitated with a given amount of plakoglobin in these cells (Fig. 4, IP:PG). Conversely, slightly less TritonX-100-soluble desmoglein3 was seen in the precipitates in the absence of β-catenin. The subsequent co-precipitation of the same protein fraction with desmoglein3 antibodies revealed comparable amounts of co-precipitating plakoglobin (Fig. 4, IP:Dsg3). This indicated that the difference was not due to less plakoglobin associating with desmoglein3 but was probably due to an altered ratio of E-cadherin versus desmoglein3 in the immunoprecipitates of β-catenin-null cells.

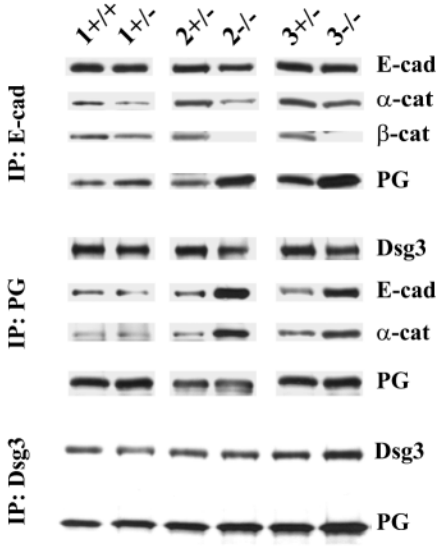

Fig. 4. Substitution of β-catenin by plakoglobin in β-catenin-null mutant keratinocytes in the TritonX-100-soluble fraction (same as in Fig. 3). Co-precipitation was done with E-cadherin, plakoglobin or desmoglein3 antibodies. The same western blots were sequentially developed with the indicated antibodies. Note that clearly more plakoglobin associated with E-cadherin in β-catenin$^{-/-}$ cells than in β-catenin$^{+/-}$ or β-catenin$^{+/+}$ cells. Dsg3, desmoglein3; E-cad, E-cadherin; α-cat, α-catenin; β-cat, β-catenin; PG, plakoglobin; Pph, plakophilin.

Collectively these data confirm the hypothesis that plakoglobin substitutes for the lack of β-catenin in adherens junctions (Haegel et al., 1995; Huelsken et al., 2000; Huelsken et al., 2001). They further demonstrate that this substitution does not visibly affect the steady-state level and compartment distribution of plakoglobin, or its association partners, in adherens junctions and desmosomes in epidermal keratinocytes (see also Fig. 5). This finding is consistent with the functionally and ultrastructurally intact intercellular adhesion structures demonstrated herein.

Consequences of *β-catenin* gene deletion on terminal differentiation

To define the involvement of β-catenin in the terminal differentiation capacity of these keratinocytes, expression of several constituents of the cornified envelope such as involucrin, loricrin and filaggrin were evaluated during Ca^{2+}-induced differentiation (Fig. 5). We also addressed the steady-state level of desmoglein1. Western blot analyses of total extracts from differentiating keratinocytes (high Ca^{2+} medium for 130 hours) showed no obvious differences in the expression levels of desmoglein1 and involucrin, both markers of early terminal differentiation. Independent of the presence or absence of β-catenin, culture 2 showed higher levels of desmoglein1 expression at that time point compatible with its stronger adhesion observed in the adhesion assay at 30 hours (see Fig. 3D). However, differences were apparent in the steady-state levels of filaggrin and loricrin, two markers of late terminal differentiation. Both β-catenin-null cultures (2$^{-/-}$ and

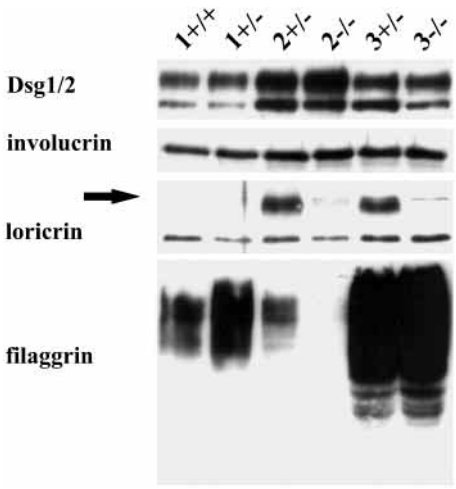

Fig. 5. Expression of early and late terminal differentiation markers in homozygous, heterozygous and β-catenin-null mutant keratinocytes. Western blot analysis of whole cell lysates of cultures 130 hours in KSFM high Ca^{2+} were developed with the indicated antibodies. The arrow indicates the partially crosslinked form of loricrin. Dsg1/2: desmoglein1/2.

$3^{-/-}$) showed lower levels of a partially cross-linked form of loricrin (Fig. 5, arrow) compared with their heterozygous counterparts, although this form was not yet detected at that time point in culture 1. Moreover, filaggrin expression was inconsistent overall. Taken together, these analyses indicated that expression of early terminal differentiation markers was similar in all cell cultures, whereas that of loricrin was inconsistent in the absence of β-catenin.

Discussion

In adherens junctions, β-catenin and plakoglobin both bind to the same site on E-cadherin's cytoplasmic tail and fulfill similar adhesive functions (Stappert and Kemler, 1994). It was therefore hypothesized that the increased lateral membrane staining for plakoglobin in the trophectoderm of β-catenin$^{-/-}$ mouse embryos emanates from a compensatory mechanism in which plakoglobin functionally substitutes for β-catenin in the cadherin-catenin complex (Haegel et al., 1995; Huelsken et al., 2000). The observation that conditional *β-catenin* gene ablation in transgenic mice failed to disrupt the interfollicular epidermal architecture further supported this hypothesis (Huelsken et al., 2001). Our results now provide the molecular evidence for this assumption.

Our biochemical, ultrastructural and functional studies using β-catenin-null keratinocytes demonstrate that plakoglobin compensates for the lack of β-catenin by binding to E-cadherin. Interestingly, although this compensation results in a visible change of the amount of plakoglobin at the plasma membrane in trophectorderm (Haegel et al., 1995; Huelsken et al., 2000), this is not the case in the epidermal keratinocytes. The most likely reason for this phenomenon is the relatively large amount of plakoglobin in epidermal keratinocytes. This is mainly due to the abundant desmosomes, which are characteristic of tissues that experience substantial mechanical stress (Green and Gaudry, 2000). Accordingly, the additional plakoglobin, which binds to E-cadherin in the β-catenin-null cells, appears to be too small to notably change the already substantial plakoglobin pool at the plasma membrane. This notion is consistent with our finding that levels of plaque and adhesion molecules in these cellular fractions remained unchanged and that the stability, assembly and adhesiveness of the respective intercellular junctions was not altered detectably. Together with our finding that the expression of early differentiation markers also remained unchanged, these results demonstrate that β-catenin is dispensable during early terminal differentiation. Consequently, this further rules out any compulsory adhesion or signaling requirement for β-catenin during this process.

It has already been discussed that β-catenin signaling must be silenced to allow differentiation of stem cells into interfollicular epidermal keratinocytes (DasGupta and Fuchs, 1999; Merrill et al., 2001; Huelsken et al., 2001; Niemann et al., 2002). This was suggested to occur by binding of inhibitory Tcf3 to the Tcf/Lef promoter (Merrill et al., 2001). Our finding that Tcf3 is transcribed in cultured keratinocytes whereas Tcf/Lef promoters are repressed, supports such an inhibitory role by Tcf3. That the repression persists in the cultured keratinocytes in spite of Tcf4 and Lef1 transcription may have several reasons. The level of the endogenously expressed Tcf4, Lef1 protein may be too low. This is probably the case, as evidenced by the finding that ectopically expressed Lef1 reversed inhibition and supported transcription in the same cells. A complementary mechanism for Tcf/Lef promoter silencing would comprise inhibition of the basic transcription machinery by Tcf-binding proteins. This possibility is suggested by our results: transcriptional activity from the pTOP-flash was lower than from the pFOP-flash promoter (having mutated Tcf binding motives). The previously described crosstalk between β-catenin and basic transcription could occur via the TATA-box-binding protein (Hecht et al., 1999). Even though this mechanisms is interesting, inhibition of basic transcription from Tcf/Lef-responsive promoters has so far not been observed in cultured murine epidermal keratinocytes. Typically however, in the absence of exogenous factors (for example stabilized β-catenin), activation was found to be very low (e.g. Merill et al., 2001; Xia et al., 2001). Absence of inhibition of the basic transcription machinery in the former studies is probably due to the use of alternate promoters in conjunction with the Tcf/Lef-binding motifs [c-Fos (Merill et al., 2001; Xia et al., 2001), thymidine kinase in this study]. Weak promoters, like the one used in this study, may be completely repressed by inhibitory proteins binding to the Tcf-motifs, whereas stronger promoters will still show basic transcriptional activity. Despite the discrepancy between the former and our results, promoter analyses consistently showed enhanced transcriptional transactivation when stabilized β-catenin was co-expressed. In adult transgenic mice, the same transgene can promote tumor formation in the epidermis (Gat et al., 1998), although it drives differentiating cultured epidermal keratinocytes back into the putative stem cell compartment (Zhu and Watt, 1999). These findings emphasize the necessity of a tight control of β-catenin signaling during epidermal keratinocyte proliferation and differentiation. To fully understand this control and in particular β-catenin silencing in epidermal keratinocytes, it will be necessary to address the highly orchestrated interplay

between transcription factors, transactivators and inhibitors in addition to repressor proteins like Groucho (Barker et al., 2000) or CtBP (Chinnadurai, 2002).

As already mentioned, silencing of β-catenin signaling is required to allow epidermal differentiation (DasGupta and Fuchs, 1999; Huelsken and Birchmeier, 2001; Merrill et al., 2001). The opposite appears to occur in skin appendages, where β-catenin was found to be operative and required for lineage differentiation (Merrill et al., 2001). Silencing of β-catenin signaling in one case but not in the other, provides a likely explanation for how the many Wnts that are expressed in neonatal skin (Reddy et al., 2001) are selective for either tissue compartment. The observation that conditional gene ablation of β-catenin failed to affect interfollicular epidermal morphogenesis (Huelsken et al., 2001) could even suggest that Wnt signals are not required to drive differentiation of stem cells along the interfollicular epidermal lineage. The expression of different Wnts in this tissue nevertheless raises the possibility that these potent activators do signal, but through alternative pathways that do not involve β-catenin stabilization (Kuhl et al., 2000; Huelsken and Birchmeier, 2001; Malbon et al., 2001). Nonetheless, this possibility fails to explain activation of the c-myc promoter. This promoter was found to be crucial during epidermal differentiation (Gandarillas and Watt, 1997; Arnold and Watt, 2001) and can be modulated by Tcf/Lef enhancers (He et al., 1998). This could suggest that other factors are more potent than the 'silenced' β-catenin in trans-activating these specific promoters. In this respect it is interesting to mention that plakoglobin was suggested to be a stronger transactivator of the c-myc promoter than β-catenin (Kolligs et al., 2000). Even though signaling via plakoglobin awaits more in-depth analyses, it is noteworthy that ectopically expressed Lef1 was found in this study to override transcriptional inhibition of Tcf/Lef promoters in wild-type but also in the β-catenin-null cells. As complete depletion of *β-catenin* gene and protein had been confirmed in the knockout cells, this activation strongly points towards the presence of alternate transactivators than β-catenin in epidermal keratinocytes. The hypothesis that plakoglobin fulfills this role in the epidermis certainly awaits further proof. So far only indirect evidence has been provided that this protein exerts the signaling function in the epidermis (Charpentier et al., 2000), which has been demonstrated in yeast expression (Hecht et al., 2000).

Interestingly, although β-catenin signaling appears to be dispensable during proliferation and early terminal differentiation, its role in late terminal differentiation of epidermal keratinocytes remains puzzling. We observed inconsistent expression of late differentiation markers such as filaggrin and loricrin in homozygous, heterozygous and null mutant keratinocytes. It is presently unclear what the reason underlying this inconsistency might be. Most interestingly however, transgenic mouse epidermis with misexpression of Tcf3, which as discussed was postulated to inhibit β-catenin-mediated Wnt signaling, had a similar epidermal phenotype (Merrill et al., 2001).

In summary, our results provide the molecular proof that proliferation of cultured epidermal keratinocytes and the establishment of the epidermal phenotype in vitro do not require β-catenin. Evidence obtained herein further points towards the possibility that β-catenin signaling is inhibited in these cells by Tcf3 at the level of the Tcf/Lef promoters. This inhibition might prevent inadequate transactivation by β-catenin, which could trigger proliferation at the expense of differentiation, hamper the exit from the putative stem cell compartment and ultimately lead to tumorigenesis.

We thank the many researchers who provided us with antibodies used in this study. We are indebted to P. Girling for proofreading of the manuscript. Our special thanks goes to those who supported this work, that is, the Swiss National Science Foundation grants # 31-59456.99 and #31-63146.00, and the Martha Stiftung Zürich (Reto Caldelari).

References

Arnold, I. and Watt, F. M. (2001). c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. *Curr. Biol.* **11**, 558-568.

Barker, N., Morin, P. J. and Clevers, H. (2000). The Yin-Yang of TCF/β-catenin signaling. *Adv. Cancer Res.* **77**, 1-24.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of β-catenin with the transcription factor LEF-1. *Nature* **382**, 638-642.

Bienz, M. and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. *Cell* **103**, 311-320.

Bradley, R. S., Cowin, P. and Brown, A. M. C. (1993). Expression of *Wnt-1* in PC12 cells results in modulation of plakoglobin and E-cadherin and increased cellular adhesion. *J. Cell Biol.* **123**, 1857-1865.

Brantjes, H., Roose, J., van de Wetering, M. and Clevers, H. (2001). All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Res.* **29**, 1410-1419.

Brault, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D. H., McMahon, A. P., Sommer, L., Boussadia, O. and Kemler, R. (2001). Inactivation of the β-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* **128**, 1253-1264.

Caca, K., Kolligs, F. T., Ji, X., Hayes, M., Qian, J., Yahanda, A., Rimm, D. L., Costa, J. and Fearon, E. R. (1999). Beta- and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. *Cell Growth Differ.* **10**, 369-376.

Calautti, E., Cabodi, S., Stein, P. L., Hatzfeld, M., Kedersha, N. and Dotto, G. P. (1998). Tyrosine phosphorylation and Src-family kinases control keratinocyte cell-cell adhesion. *J. Cell Biol.* **141**, 1449-1465.

Caldelari, R., Suter, M. M., Baumann, D., de Bruin, A. and Müller, E. (2000). Long-term culture of murine epidermal keratinocytes. *J. Invest. Dermatol.* **114**, 1064-1065.

Caldelari, R., de Bruin, A., Baumann, D., Suter, M. M., Bierkamp, C., Balmer, V. and Müller, E. (2001). A central role for the armadillo protein plakoglobin in the autoimmune disease pemphigus vulgaris. *J. Cell Biol.* **153**, 823-834.

Chan, E. F., Gat, U., McNiff, J. M. and Fuchs, E. (1999). A common human skin tumour is caused by activating mutations in β-catenin. *Nat. Genet.* **21**, 410-413.

Charpentier, E., Lavker, R. M., Acquista, E. and Cowin, P. (2000). Plakoglobin suppresses epithelial proliferation and hair growth in vivo. *J. Cell Biol.* **149**, 503-520.

Cheon, S. S., Cheah, A. Y., Turley, S., Nadesan, P., Poon, R., Clevers, H. and Alman, B. A. (2002). Beta-catenin stabilization dysregulates mesenchymal cell proliferation, motility, and invasiveness and causes aggressive fibromatosis and hyperplastic cutaneous wounds. *Proc. Natl. Acad. Sci. USA* **99**, 6973-6978.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol. Cell* **9**, 213-224.

DasGupta, R. and Fuchs, E. (1999). Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. *Development* **126**, 4557-4568.

Fuchs, E. and Segre, J. A. (2000). Stem cells: a new lease on life. *Cell* **100**, 143-155.

Gandarillas, A. and Watt, F. M. (1997). c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev.* **11**, 2869-2882.

Gat, U., Das Gupta, R., Degenstein, L. and Fuchs, E. (1998). De novo hair

follicle morphogenesis and hair tumors in mice expressing a truncated β-catenin in skin. *Cell* **95**, 605-614.

Gottardi, C. J., Wong, E. and Gumbiner, B. M. (2001). E-cadherin suppresses cellular transformation by inhibiting β-catenin signaling in an adhesion-independent manner *J. Cell Biol.* **153**, 1049-1060.

Green, K. J. and Gaudry, C. A. (2000). Are desmosomes more than tethers for intermediate filaments? *Nat. Rev. Mol. Cell. Biol.* **1**, 208-216.

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345-357.

Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K. and Kemler, R. (1995). Lack of β-catenin affects mouse development at gastrulation. *Development* **121**, 3529-3537.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-1512.

Hecht, A., Litterst, C. M., Huber, O. and Kemler, R. (1999). Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. *J. Biol. Chem.* **274**, 18017-18025.

Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. and Yuspa, S. H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**, 245-254.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G. and Kemler, R. (1996). Nuclear localization of β-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* **59**, 3-10.

Huelsken, J. and Birchmeier, W. (2001). New aspects of Wnt signaling pathways in higher vertebrates. *Curr. Opin. Genet. Dev.* **11**, 547-553.

Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C. and Birchmeier, W. (2000). Requirement for β-catenin in anterior-posterior axis formation in mice. *J. Cell Biol.* **148**, 567-578.

Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G. and Birchmeier, W. (2001). β-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* **105**, 533-545.

Kolligs, F. T., Kolligs, B., Hajra, K. M., Hu, G., Tani, M., Cho, K. R. and Fearon, E. R. (2000). Gamma-Catenin is regulated by the APC tumor suppressor and its oncogenic activity is distinct from that of beta-catenin. *Genes Dev.* **14**, 1319-1331.

Kowalczyk, A. P., Bornslaeger, E. A., Norvell, S. M., Palka, H. L. and Green, K. J. (1999). Desmosomes: intercellular adhesive junctions specialized for attachment of intermediate filaments. *Int. Rev. Cytol.* **185**, 237-302.

Kuhl, M., Sheldahl, L. C., Park, M., Miller, J. R. and Moon, R. T. (2000). The Wnt/Ca^{2+} pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet.* **16**, 279-283.

Lavker, R. M. and Sun, T. T. (2000). Epidermal stem cells: properties, markers, and location. *Proc. Natl. Acad. Sci. USA* **97**, 13473-13475.

Malbon, C. C., Wang, H. and Moon, R. T. (2001). Wnt signaling and heterotrimeric G-proteins: strange bedfellows or a classic romance? *Biochem. Biophys. Res. Commun.* **287**, 589-593.

Merrill, B. J., Gat, U., DasGupta, R. and Fuchs, E. (2001). Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. *Genes Dev.* **15**, 1688-1705.

Molenaar, M., van de Wetering, M., Ousterwegel, M., Peterson-Manduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. *Cell* **86**, 391-399.

Niemann, C., Owens, D. M., Hulsken, J., Birchmeier, W. and Watt, F. M. (2002). Expression of DeltaNLef1 in mouse epidermis results in differentiation of hair follicles into squamous epidermal cysts and formation of skin tumours. *Development* **129**, 95-109.

Orsulic, S., Huber, O., Aberle, H., Arnold, S. and Kemler, R. (1999). E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. *J. Cell. Sci.* **112,** 1237-1245.

Pasdar, M., Li, Z. and Chlumecky, V. (1995). Plakoglobin: kinetics of synthesis, phosphorylation, stability, and interactions with desmoglein and E-cadherin. *Cell Motil. Cytoskeleton* **32**, 258-272.

Reddy, S., Andl, T., Bagasra, A., Lu, M. M., Epstein, D. J., Morrisey, E. E. and Millar, S. E. (2001). Characterization of Wnt gene expression in developing and postnatal hair follicles and identification of Wnt5a as a target of Sonic hedgehog in hair follicle morphogenesis. *Mech. Dev.* **107**, 69-82.

Sadot, E., Simcha, I., Shtutman, M., Ben-Ze'ev, A. and Geiger, B. (1998). Inhibition of beta-catenin-mediated transactivation by cadherin derivatives. *Proc. Natl. Acad. Sci. USA* **95**, 15339-15344.

Saitou, M., Sugai, S., Tanaka, T., Shimouchi, K., Fuchs, E., Narumiya, S. and Kakizuka, A. (1995). Inhibition of skin development by targeted expression of a dominant-negative retinoic acid receptor. *Nature* **374**, 159-162.

Simcha, I., Shtutman, M., Salomon, D., Zhurinsky, J., Sadot, E., Geiger, B. and Ben-Ze'ev, A. (1989). Differential nuclear translocation and transactivation potential of beta-catenin and plakoglobin. *J. Cell Biol.* **141**, 1433-1448.

Stappert, J. and Kemler, R. (1994). A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes. Commun.* **2**, 319-327.

Vleminckx, K. and Kemler, R. (1999). Cadherins and tissue formation: integrating adhesion and signaling. *Bioessays* **21**, 211-220.

Watt, F. M. (2001). Stem cell fate and patterning in mammalian epidermis. *Curr. Opin. Genet. Dev.* **11**, 410-417.

Willert, K. and Nusse, R. (1998). β-Catenin: a key mediator of Wnt signaling. *Curr. Opin. Genet. Dev.* **8**, 95-102.

Xia, X., Qian, S., Soriano, S., Wu, Y., Fletcher, A. M., Wang, X. J., Koo, E. H., Wu, X. and Zheng, H. (2001). Loss of presenilin 1 is associated with enhanced β-catenin signaling and skin tumorigenesis. *Proc. Natl. Acad. Sci. USA* **98**, 10863-10868.

Zhang, Z. H., Hartmann, H., Do, V. M., Abramowski, D., Sturchler-Pierrat, C., Staufenbiel, M., Sommer, B., van de Wetering, M., Clevers, H., Saftig, P. et al. (1998). Destabilization of β-catenin by mutations in presenilin-1 potentiates neuronal apoptosis. *Nature* **395**, 698-702.

Zhou, P., Byrne, C., Jacobs, J. and Fuchs, E. (1995). Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. *Genes Dev.* **9**, 700-713.

Zhu, A. J. and Watt, F. M. (1999). β-Catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. *Development* **126**, 2285-2298.