Keratinocytes display normal proliferation, survival and differentiation in conditional β4-integrin knockout mice

Karine Raymond*, Maaike Kreft*, Hans Janssen, Jero Calafat and Arnoud Sonnenberg‡

Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

*These authors contributed equally to this work

‡Author for correspondence (e-mail: a.sonnenberg@nki.nl)

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Summary

The α6β4 integrin is located at the basal surface of keratinocytes, in hemidesmosomal structures that mediate stable adhesion of epidermal cells to the underlying basement membrane component laminin-5. The absence of α6β4 integrin causes junctional epidermolysis bullosa, a severe blistering disease of the skin leading to perinatal death, confirming its essential role in mediating strong keratinocyte adhesion. Several studies have suggested that α6β4 integrin can also regulate signaling cascades that control cell proliferation, survival and migration through a mechanism independent of its adhesive function. We have generated a conditional knockout mouse strain, in which the gene encoding the β4 integrin subunit (*Itgb4*) was inactivated only in small stretches of the skin. These mice were viable and permitted an accurate analysis of the consequences of the loss of β4 on various biological processes by comparing β4-positive and -negative parts of the skin in the same animal. Despite the complete loss of hemidesmosomes in regions lacking α6β4 integrin, the distribution of a range of adhesion receptors and basement membrane proteins was unaltered. Moreover, loss of α6β4 did not affect squamous differentiation, proliferation or survival, except for areas in which keratinocytes had detached from the basement membrane. These in vivo observations were confirmed in vitro by using immortalized keratinocytes – derived from β4-subunit conditional knockout mice – from which the gene encoding β4 had been deleted by Cre-mediated recombination. Consistent with the established role of α6β4 in adhesion strengthening, its loss from cells was found to increase their motility. Our findings clearly demonstrate that, after birth, epidermal differentiation, proliferation and survival all proceed normally in the absence of α6β4, provided that cell adhesion is not compromised.

Key words: Integrin, Epidermis, Knockout, Hemidesmosome, Adhesion, Proliferation

Introduction

The skin consists of an epidermal layer of stratified squamous keratinizing epithelium, and a dermal layer of connective tissue. These two layers are separated by a basement membrane (BM), which is a specialized extracellular matrix (ECM) rich in laminins and collagens. The basal layer of the epidermis is composed of keratinocytes that have proliferative capacities required for the renewal of the skin. When these cells become detached from the BM and migrate to the upper epidermal layers, they exit the cell cycle and start a program of squamous differentiation, in which cells progressively become keratinized and ultimately are sloughed off (Watt, 1989). Therefore, homeostasis of the skin requires coordinated regulation of cell proliferation, differentiation and survival. Integrin-mediated cell adhesion to the ECM is known to play an important role in the regulation of these processes (Adams and Watt, 1990).

In epidermal keratinocytes, the integrin repertoire is mainly restricted to α2β1-, α3β1-, α9β1- and α6β4-integrin (Watt, 2002). The α2β1 integrin binds to different types of collagen whereas α9β1 mediates the attachment to tenascin. α3β1 and α6β4 bind to laminin-5 (Ln-5), the major adhesive ligand for cells in the mature BM (Aumailley and Rousselle, 1999; Carter et al., 1991; Rousselle et al., 1991). The α3β1- and α6β4 receptors are recruited to different adhesion sites. α3β1 is present in focal contacts associated with the actin cytoskeleton. Gene targeting studies of the α3 subunit in mice indicated a limited contribution of α3β1 integrin to the maintenance of cell-substrate adhesion and this integrin is primarily involved in the proper organization of the epidermal BM (DiPersio et al., 1997). By contrast, the α6β4 is connected to the keratin-based intermediate filament system and is a major component of hemidesmosomes (HDs), structures that provide stable adhesion of the epidermis to the dermis (Jones et al., 1998; Borradori and Sonnenberg, 1999; Koster et al., 2004).

The classical, or type I, HD is a multi-protein complex composed of at least six proteins, the two subunits of α6β4, the Bullous Pemphigoid (BP) antigen 180 and 230 (BP180 and BP230, respectively), CD151 and plectin. The cytoplasmic domain of the β4 subunit is unusually large and acts as scaffolding for the binding of the other hemidesmosomal components (Borradori et al., 1997; Schaapveld et al., 1998; Hopkinson and Jones, 2000; Koster et al., 2003). The

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cytoskeletal linker protein plectin and BP230 are located in the cytoplasm and mediate binding of α6β4 to the intermediate filament system (Geerts et al., 1999; Niessen et al., 1997; Rezniczek et al., 1998; Koster et al., 2003), whereas two other hemidesmosomal components, BP180 and the tetraspanin CD151, are transmembrane proteins (Koster et al., 2004; Sterk et al., 2000). Evidence for a physiological role of α6β4 in the formation of HDs was provided in studies on patients with a mutation in the genes for either the α6- or the β4-subunit (Vidal et al., 1995; Pulkkinen et al., 1997; Ruzzi et al., 1997) and also in gene-targeting studies in mice (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). In the absence of α6β4 the formation of HDs is compromised and the epidermis becomes detached from the dermis. Splitting occurs at the level of the BM but also within the cell, immediately above the sites of HDs where intermediate filaments are inserted. These defects cause a skin-blistering disease called pyloric atresia associated with junctional epidermolysis bullosa (PA-JEB) in humans and lead to perinatal death in both humans and mice.

In addition to the important role of maintaining a firm adhesion of the epidermis to the dermis, α6β4 has also been reported to influence a wide range of cellular functions such as migration, survival and proliferation (Giancotti, 1996; Nievers et al., 1999). The long cytoplasmic domain of its β4 subunit recruits a range of signalling molecules and thereby influences physiological and pathological conditions of the skin. α6β4 promotes carcinoma cell migration and invasion (Chao et al., 1996; O'Connor, 1998; Tozeren et al., 1994) in a phosphoinositide 3-kinase (PI3-kinase)-dependent manner (Shaw et al., 1997). Depending on the status of p53 in carcinoma cell lines, α6β4 promotes the survival of p53-deficient cells by activating Akt/PKB kinase (Bachelder et al., 1999b), whereas it induces apoptosis in a number of carcinoma cells (Clarke et al., 1995; Kim et al., 1997; Sun et al., 1998) by stimulating the caspase 3-dependent cleavage of Akt/PKB kinase in a p53-dependent manner (Bachelder et al., 1999a). Expression of the β1 subunit extends from the basal site of actively proliferating basal keratinocytes to the suprabasal layers of the epidermis. The restricted expression of α6β4 integrin, i.e. to the basal site of actively proliferating basal keratinocytes, however, suggests that α6β4-mediated anchorage to BM is the key regulator of proliferation versus differentiation (Hall and Watt, 1989). Similarly, culturing of keratinocytes without anchorage induces their exit from the cell cycle and results in their differentiation (Green, 1977). Biochemical studies showed that the cytoplasmic domain of the β4 subunit binds the adaptor-protein Shc and activates the Ras-MAPK pathway (Mainiero et al., 1997), providing thereby a strong molecular link between α6β4 and the control of proliferation. In pathological situations, squamous cell carcinomas with high proliferative potential often express α6β4 at high levels (Carey et al., 1992; Van Waes et al., 1991). These observations suggest that the α6β4 may provide epithelial cells with a signal, important for their survival and progression of the cell cycle. However, data obtained in vivo are somewhat conflicting. Deletion of the cytoplasmic domain of the β4 subunit results in a two-fold decrease of keratinocyte proliferation in embryos at embryonic day (ED) 18.5, compared with the one in control mice (Murgia et al., 1998). However, such a decrease was not observed in β4-null-embryos at ED 16.5 (DiPersio et al., 2000).

To study the role of α6β4 integrin in adult mice and to avoid any effects that might be owing to differences between adults and embryos, and individual embryos, we used a gene-targeting approach and the Cre-*loxP* recombination system of the bacteriophage P1 to generate mice with a mosaic expression of the β4 subunit in the skin. We postulated that the lack of α6β4 from only a small proportion of the skin will lead to viable animals and that areas of epithelium that are positive or negative for β4 can be compared in regard to defects in adhesion and also in cell signalling. Such a mosaic pattern of α6β4 expression was seen in mice homozygous for the conditional β4 allele and expressing the Keratin-14–Cre transgene. These mice are viable but frequently show abnormalities of the skin on the ear during adulthood. There was no evidence that the loss of adhesion-function of α6β4 in the epidermis is compensated by other laminin- or cell-matrix receptors. Moreover, as already shown in the developing epidermis, the presence of α6β4 integrin seems to be essential for the formation and stability of HDs but not for the assembly of the BM or for the differentiation of the epidermis. The absence of the α6β4 integrin increases cell motility in wound healing assays. Importantly, we found no evidence for a role of α6β4 in controlling cell proliferation and survival that is independent of its function as an adhesion receptor.

Materials and Methods

Engineering β4-subunit conditional knockout mice

An 18.3 kb genomic fragment encompassing exons 1a to 11 of *Itgb4* (hereafter referred to as the β4 gene) was isolated from a 129/Sv library and subcloned into plasmid vector pGEM5 (van der Neut et al., 1996). After restriction enzyme mapping of that fragment, a single *loxP* site and a *loxP*-PGK*neo*^{r}-PGK*tk*-*loxP* sequence (floxed *neo*/*tk* cassette) were inserted into intron 1a (unique *Sac*II site) and intron 5 (unique *Kpn*I site), respectively. The targeting construct (excised from the plasmid with *Not*I) was electroporated into 129/Ola-derived embryonic stem (ES) cells. Colonies resistant to geneticin (G418) were screened for the desired homologous recombination by Southern blotting. The presence of the first *loxP* site in intron 1a was detected by PCR amplification with primers P1 (5′-CTCACTGTA-TTAAGCGGAC-3′) and P2 (5′-AAGGGCTGCGGCTCAACC-3′) specific for exon 1a and intron 1b, respectively. The floxed *neo-tk* cassette was deleted by transient transfection of a Cre-expression plasmid pOG231 (O'Gorman and Wahl, 1997). One recombinant ES cell clone (12-15 cells) harboring the conditional allele of the β4 gene was injected into mouse C57Bl/6 blastocysts, which were transferred to mothers of the same strain. The chimeric male offspring was then mated with FVB/N females. Agouti-coat-colored offspring was screened for the presence of the conditional allele of the β4 gene by PCR analysis of tail DNA, with primers P3 (5′-GCCTCTA-TGGACACCAGG-3′) and P4 (5′-GACGCTGACTTTGTCCACAA-ACTTTCC-3′) specific for intron 5 and exon 6, respectively. Heterozygous mice were intercrossed and homozygous mice were used to generate animals that were transgenic for the K14-Cre recombinase and carried the conditional alleles of the β4 gene. The K14-Cre transgene was detected by PCR amplification with the primers K14-cre3 (5′-CGATGCAACGAGTGATGAGGTTC-3′) and K14-cre5 (5′-GCACGTTCACCGGCATCAAC-3′). The removal of exon 1b to exon 5 by Cre-mediated recombination was confirmed by PCR analysis using primers P1 and P4. All animal experiments were carried out with approval from the relevant institutional animal ethics committees.

Establishment of the NMK-1 cell line and its culture conditions

To generate an immortalized cell line from β4 conditional knockout mice, primary keratinoctes were prepared from neonatal mice. Briefly, the epidermis was separated from the dermis by incubation with 0.25% trypsin overnight at 4°C. Next day, the epidermis was peeled off from the dermis and the two tissues were incubated separately in keratinocyte serum-free medium (Keratinocyte-SFM, Life Technologies-BRL, Rockville, MD) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 100 U/ml penicillin and 100 U/ml streptomycin (complete Keratinocyte-SFM medium), and gently shaken for 30 minutes at 4°C to release the separated cells into the medium. The cells were then filtered through a cell strainer (70 μm) and centrifuged, and the two cell types were plated together on tissue-culture dishes in complete Keratinocyte-SFM medium at 37°C in the presence of 5% CO_2. After several weeks, one clone appeared to be spontaneously immortalized and was called normal mouse keratinocyte-1 (NMK-1). To stimulate HD formation before confocal microscopy analysis, NMK cells were grown for 24 hours in calcium-rich medium, consisting of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies-BRL) and HAM-F12 Nutrient Mixture (Life Technologies-BRL) in a ratio of 3:1.

Antibodies

Mouse monoclonal antibodies (mAbs) used in this study were: 121 against plectin from K. Owaribe (University of Nagoya, Nagoya, Japan), LLOO1 against keratin 14 from B. Lane (University of Dundee, Dundee, UK), against E-cadherin, N-cadherin and β-catenin from Transduction Laboratory (Lexington, KY), against p53 (Sc-100) from Santa Cruz Biotechnology (Santa Cruz, CA), against phospho-Erk1/2 from Cell Signaling Technology (Beverely, MA), against 5-bromo-2-deoxyuridine (BrdU) from DAKO Corp. (Carpinteria, CA). Rat mAbs against the following integrin subunits were: R1-2 from PharMingen (San Diego, CA) against α4, GoH3 against the α6 (Sonnenberg et al., 1996), 346-11A against β4 from S. J. Kennel (Oak Ridge Laboratories, Oak Ridge, TN), BMA5 against α5 and MB1.2 against β1, both from B.M.C. Chan (University of Ontario, Ontario, Canada). Other rat mAbs were 33A10 (Sonnenberg et al., 1986), CD9 (H6) from M. Schachner (Swiss Federal Inst. of Technology, Zurich, Switzerland), CD44 (1M7.8.1) from PharMingen. Rabbit polyclonal antibodies were directed against the cytoplasmic domain of the following integrin subunits α3A from M. DiPersio (DiPersio et al., 1995), α6A (U21E, affinity purified) from U. Mayer (University of Manchester, Manchester, UK), αv from G. Tarone (University of Torino, Torino, Italy) (Hirsch et al., 1994), β4 (H101) from Santa Cruz Biotechnology, β5 (5HK2) and β6 (5HK1) from H. Kemperman (Sánchez-Aparicio et al., 1997), and against mouse fibronectin (A117) from Gibco-BRL (Gaithersburg, MD), β-dystroglycan (affinity purified, AP38) from K. P. Campbell (University of Iowa, Iowa City, IA), BP180 (J17) from J. C. R. Jones (Northwestern University, Chicago, IL), BP180 (mo-NC16a) from L. Bruckner-Tuderman (University of Freiburg, Freiburg, Germany), Ln-5 and nidogen from T. Sasaki (Max-Planck Institute, Munich, Germany), collagen IV from E. Engvall (The Burnham Inst., La Jolla, CA), Erk1/2 from Cell Signaling Technology, keratins 1, 5 and 6, and involucrin from BabCO (Berkely, CA), and vimentin (K36) from F. Ramaekers (University of Maastricht, Maastricht, The Netherlands). Human mAbs 5E and 10D against BP230 were from T. Hashimoto (Keio University, Tokyo, Japan). Hamster mAbs against the integrin subunits α1 (Ha31/8), α2 (HMα2) and αv (H9.2B8) from PharMingen. Texas Red and fluorescein isothiocyanate (FITC) conjugated secondary antibodies were from Molecular Probes (Eugene, OR).

Immunofluorescence microscopy and flow cytometry

Two-day-old mice and tissues of adult mice were collected and embedded in cryoprotectant (Tissue-Tek® O.C.T., Sakura Finetek Europe, Zoeterwoude, The Netherlands). Cryosections were prepared, fixed in ice-cold acetone and blocked with 1% BSA. Cells grown on glass coverslips were fixed with 1% paraformaldehyde. Subsequently, PBS containing 0.2% Triton X-100 and 1% BSA were used for permeabilization and blocking. Samples were then subjected to immunofluorescence analysis by successive 1-hour long incubations with primary and secondary antibodies. 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 (Sterk et al., 2000).

AdenoCre, obtained from F. Graham (McMaster University, Ontario, Canada), expresses Cre under the control of the cytomegalovirus immediate-early promoter (Anton and Graham, 1995) and was amplified in 293T cells following standard protocols. Because the efficiency of the conditional deletion of the β4 allele results from the combination of the efficiencies of the adenoviral infection and the recombination event, the minimum quantity of virus necessary to induce more than 95% of recombination of the $\beta4^{flox}$ allele, was empirically determined with FACS analysis of the β4 integrin levels.

Ultrastructural analysis

Immunogold labeling of β4 integrin for electron microscopy of skin sections was performed as previously described (Sonnenberg et al., 1991). Briefly, mouse skin was fixed for 2 hours in a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), and ultra-thin frozen sections were prepared and incubated at room temperature with antibodies against the β4 subunit followed by incubation with 10 nm gold conjugates. To analyze the presence of HDs in NMK-1 cells, the cells were grown to confluence on Thermanox Plastic coverslips (Nunc, Rochester, NY), fixed in 2.5% glutaraldehyde, post-fixed in 1% OsO_4, stained en bloc with uranylacetate and flat embedded. The samples were examined with a FEI Tecnai 12 electron microscope.

Immunoblotting

Cells were lysed in Triton X-100 lysis buffer [1% (vol/vol) Triton X-100, 50 mM Tris-HCl, pH 7.6, 4 mM EDTA, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate] containing a cocktail of protease inhibitors (Sigma-Aldrich), supplemented with 1% SDS when keratins were to be extracted. Lysates were clarified by centrifugation at 20,000 ***g*** for 15 minutes at 4°C. Protein concentrations were determined with the Pierce BCA Protein Assay reagent (Pierce, Rockford, IL). Immunoblot analysis was performed with 30-80 μg of protein run on 4-20% precast polyacrylamide gels (Life Technology) that were transferred to Immobilon-PVDF membranes (Millipore Corp., Bedford, MA). Secondary antibodies coupled to horseradish peroxidase (HRP) were purchased from Jackson ImmunoResearch Laboratories and used at 1:5000. Detection was performed by chemiluminescence.

Immunoprecipitation of ^{125}I-labeled cells

Cells were surface-labeled with ^{125}I by using the lactoperxidase/hydrogen peroxide method as previously described (Sonnenberg et al., 1993), washed and solubilized in lysis buffer containing 1% (vol/vol) Nonidet P-40, 20 mM Tris-HCl, pH 7.6, 4 mM EDTA, 100 mM NaCl, supplemented with a cocktail of protease inhibitors (Sigma-Aldrich). Lysates were clarified at 20,000 ***g*** and precleared with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc., Uppsala Sweden). Samples of the precleared lysates were immunoprecipitated with antibodies previously bound to Protein A-Sepharose or to Protein A-Sepharose to which rabbit-anti-rat IgG was bound. After incubation for 1 hour at room temperature, the beads carrying the immune complexes were washed and treated with SDS-sample buffer.

Precipitated proteins were analyzed on a 5% polyacrylamide gel at non-reducing conditions.

RT-PCR

Total RNA from immortalized NMK-1 keratinocytes was isolated using RNA-Bee (Tel-test, Inc., Friendswood, TX). Concentration in the samples was measured in a UV spectrophotometer and integrity of the RNA samples was verified by agarose gel electrophoresis. First strand cDNA was prepared from 1.5 μg of RNA in a 20 μl reaction volume, containing 200 units of SuperScript™ II RNase H^- reverse transcriptase, 1× first strand buffer, 25 ng/μl of oligonucleotide (dT), 0.5 mM dNTP (Invitrogen Corp., Carlsbad, CA). One μl of cDNA was used as a template in PCR reactions. The primer sequences of the oligonucleotides used for PCR were as follows:

Ln-5 γ2: E7-F and E10-9 described by (Meng et al., 2003); α6: P9 (5′-GAAGACCAGTGGATGGGAG-3′) and P10 (5′-CACTGTGA-TTGGCTCTTGGGA-3′); β4: P11 (5′-GACGCTGACTTTGTCCA-CAAACTTTCC-3′) and P12 (5′-ATGGCAGGGCCCTGTTGCAGC-CCATGGGTGAAGC-3′). The primers for envoplakin (Maatta et al., 2001) and the different desmogleins (Runswick et al., 2001) have been previously described. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Meng et al., 2003) were used as a control. In all PCR reactions, 30 cycles of amplification were performed, except for the amplification of GAPDH where 20 cycles were used to be in the linear range of detection.

Cell proliferation and transformation assays

All experiments were repeated at least three times.

Growth curves

Cells were seeded into 96-well plates (2.5×10^3 cells per well) in triplicates. The growth capacities of the cells were measured at different time points (1 to 96 hours) by using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega, Madison, WI) according to the manufacturer's recommendations. Formation of formazan was detected at 490 nm by using a Model 550 plate reader (Bio-Rad Laboratories, Hercules, CA).

Clonogenic growth potential

Cells were plated under sparse conditions (500 cells in a 10 cm dish) and cultured for up to 2 weeks. Medium was changed every 3 days. Colonies were visualized after fixation and staining of the cells with 70% ethanol and 0.1% Crystal Violet.

Growth in semi-solid medium

For colony formation in semi-solid medium, 5×10^4 cells were plated in complete Kera-SFM medium and 0.35% low-melting point agarose (LMP) (Gibco-BRL) into six-well plates coated with 0.6% LMP.

Cell-cycle analysis

For cell-cycle analysis, exponentially growing cells were collected by trypsinization, fixed with 70% ethanol and stained with propidium iodide (5 μg/ml) in PBS containing 0.2% Triton X-100, and 500 μg/ml RNase A. Samples were then analyzed by flow cytometry.

Injection into nude mice

Cells were harvested, washed and resuspended in PBS (10^7 cells in 0.15 ml) and then inoculated subcutaneously into the left and right flanks of 4-weeks-old female athymic nu/nu (Balb/c) mice, which were housed in barrier environments with food and water provided ad libitum. Animals were kept for 3 months to observe whether tumors would arise.

BrdU incorporation

Cells were labeled with 10 μM BrdU for 6 hours, fixed in PBS containing 2% paraformaldehyde for 10 minutes, followed by permeabilization with 0.5% Triton X-100 in PBS for 5 minutes. Thereafter, 2 M HCl was added to the fixed cells, incubated for 1 hour at room temperature and then neutralized with 0.1 M $Na_2B_4O_7$. Non-specific binding was blocked by incubation with 5% normal goat serum in PBS for 20 minutes. Slides were then incubated with anti-BrdU antibody and subjected to indirect immunofluorescence. Nuclei were counterstained with 2 μg/ml Hoechst 33258 (Molecular Probes).

Cell proliferation in skin

Newborn mice were injected intraperitoneally with BrdU (Sigma) at 50 mg/kg and sacrificed 1 hour later. Cryosections were subsequently co-stained with anti-BrdU antibody, anti-β4 antibody and a marker of BM (nidogen), dermis (fibronectin), or nuclei (TOPRO-3 from Molecular Probes) to detect the β4-positive and -negative areas of skin and to verify that proliferation is restricted to the basal compartment of the epidermis. BrdU-labeled nuclei in the interfollicular regions were counted in each field and the total number of nuclei was determined. The number of BrdU labeled cells was divided by the total number of cells and multiplied by 100 to estimate the proliferation index.

Analysis of apoptosis in the skin

Apoptosis was evaluated on skin cryosections by Tdt-mediated dUTP nick-end (TUNEL) labeling using an apoptosis detection system (Promega, Madison, WI), which measures the fragmented DNA of apoptotic nuclei by incorporating fluorescein-12-dUTP at the 3′-OH DNA ends using terminal deoxynucleotidyl transferase.

Analysis of keratinocyte adhesion and motility

For adhesion assays, subconfluent keratinocytes were trypsinized, washed twice with Iscove's modified Dulbecco's medium (IMDM, Life Technologies-BRL) containing 2% BSA, seeded in 96-well flat-bottom plates (10^5 cells per well) previously coated with Ln-5 (a gift from M. Aumailley, University of Cologne, Cologne, Germany) or fibronectin (Sigma) for 16 hours at 4°C and blocked for 1 hour with RPMI medium containing 2% BSA. After incubation for 30 minutes at 37°C, the cells were washed three times with PBS, lysed in 0.5% Triton-X-100 and incubated with 50 μl of p-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma) in 0.1 M Na-citrate buffer (pH 5.0) for 16 hours. The reaction was stopped by adding 75 μl 50 mM glycine (pH 10.4), 5 mM EDTA. Cell adhesion was determined by measuring the OD at 405 nm using a Model 550 plate-reader (Bio-Rad Laboratories). Results are expressed as percent of adhesion: (OD of the sample – OD of unspecific adhesion) ÷ (OD of the total number of cells) ×100.

For detachment assay, cells (1.5×10^4 cells /cm^2) were grown in 96-well plates for 5 days to reach 80% confluency. The monolayers were then treated with a solution of trypsin/EDTA in PBS for several time-ranges (0-10 minutes). Plates were washed three times with PBS and the percentage of bound cells was determined as described above for the adhesion assay.

For the centrifugal-force-based assay, the strength of adhesion of NMK-1(+) and NMK-1(–) cells to the substratum was compared in an assay adapted from Lotz et al. (Lotz et al., 1989). Adhesion was led to occur during 10 minutes, on a matrix of Ln-5 deposited by RAC-11P cells, as previously described (Delwel et al., 1993). The wells of the plate were then completely filled with Iscove medium containing 0.35% BSA, sealed with Thermowell sealing tape (Costar,

United Kingdom) and inverted before centrifugation in a tabletop, refrigerated centrifuge (Rotanta 46RS, Heltich, Zentrifugen) for 8 minutes at in three steps at increasing forces (500, 750 and 1000 ***g***). Cells that remained attached to the wells were fixed in 100% ethanol for 5 minutes and stained with 0.4% Crystal Violet in methanol for 5 minutes. Cell adhesion was determined by measuring OD_{540} after the cells had been solubilized in 1% SDS. The percentage of cells bound was calculated as [(OD after centrifugation ÷ OD after initial adhesion) ×100]. The background (binding to BSA alone) was identical at the different centrifugal forces and in the replicates, and was therefore not taken into account. The results are expressed as the mean of four replicates ±s.d.

Wound healing assays were performed as previously described (Geuijen and Sonnenberg, 2002). Phase-contrast images were recorded at the time of wounding (0 hours) and 4 and 8 hours thereafter.

Results

Generation of mice conditionally targeted for inactivation of the β4 gene in parts of the skin

We have previously shown that inactivation of the β4 gene in the mouse germ line results in perinatal death because of severe blistering of the skin (van der Neut et al., 1996). To investigate whether the α6β4 integrin is involved in adhesion-independent processes in the skin, we used a gene-targeting approach by homologous recombination and the Cre-*loxP* recombination system of the bacteriophage P1 to create mice with a mosaic expression of the β4 gene in the skin. We postulated the viability of such animals, allowing us to compare β4-positive and -negative areas of epithelium in the same animal. Therefore, we engineered a targeting vector containing a single *loxP* site in intron 1a and the floxed *neo-tk* cassette (see Materials and Methods) in intron 5, to conditionally remove exons 1b to 5 and thereby the translation-initiation codon of the β4 gene (Fig. 1A). After homologous recombination in ES cells and analysis of the correct recombination event by Southern blotting and PCR (Fig. 1B), the floxed *neo-tk* cassette was removed by transient expression of the Cre recombinase in two independent recombinant ES clones. One ES clone, lacking the *neo-tk* cassette but harboring the floxed exons 1b-5 ($\beta4^{flox}$ allele), was injected into blastocysts and germ line transmission of the $\beta4^{flox}$ allele occurred. Mice carrying the $\beta4^{flox}$ allele were then bred to homozygosity (Fig. 1C, Before K14-Cre).

Mice homozygous for the floxed β4 allele (hereafter referred to as $\beta4^{flox/flox}$ mice) appeared to be normal indicating that the genetic manipulation had not altered the function of the β4 subunit. To generate viable animals with a mosaic expression of β4 in the skin, we crossed the $\beta4^{flox/flox}$ mice with transgenic mice expressing the Cre-recombinase under the control of the Keratin 14 promoter (K14-Cre transgene). The removal of exons 1b-5 by Cre-mediated recombination and the presence of the K14-Cre transgene were confirmed by PCR analysis on DNA of the ear from adult progeny (Fig. 1C, After K14-Cre) and resulted in $\beta4^{flox/flox}$; K14-Cre mice.

Inactivation of the β4 gene in parts of the skin causes frequent ear skin abnormalities in adult mice

Mice with the genotype $\beta4^{flox/flox}$; K14-Cre were born without obvious skin defects. They were fertile and produced offspring with the same genotype as the parents when intercrossed. During adulthood, the $\beta4^{flox/flox}$; K14-Cre mice frequently (around 16%) developed an inflammation of the ear that might have been caused by repeated detachment of the epidermis (Fig. 2A). We assume that the skin of the ear is more liable to wounding because it is less hairy and thus more susceptible to mechanical stress, e.g. during cleansing by the mice with their forepaws.

The specificity and efficiency of the K14-Cre-mediated recombination in skin epithelium has been assayed previously in embryos and newborns of crosses between K14-Cre mice and mice bearing the ROSA26-*LacZ* reporter (Jonkers et al., 2001). Cre recombinase activity, as judged by 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal) staining, is observed from day E13.5 onwards. First in a patchy, mosaic pattern and, after birth, extending over most of the skin. However, in the case of the $\beta4^{flox}$ allele, the efficiency of K14-Cre-mediated recombination was such that expression of the β4 gene remained largely mosaic, even after birth (Fig. 2B).

Heamatoxylin and eosin staining of skin sections from newborn $\beta4^{flox/flox}$; K14-Cre animals showed normal organization of the skin. However, there were small blisters in some areas of the epithelium, probably because of mechanical stress in regions where β4 has been deleted (Fig. 2C). To investigate possible subtle defects that cannot be recognized by light microscopic analysis and to confirm the mosaic expression of β4, we performed an ultrastructural analysis of skin sections from $\beta4^{flox/flox}$; K14-Cre mice. Electron-microscopic analysis revealed large areas of skin with the same morphology as the control $\beta4^{flox/flox}$ mice (not shown), which were occasionally interrupted by areas in which basal keratinocytes, lacking discernible HDs, had detached from the dermis (Fig. 2D). In basal cells that were attached to the dermis, numerous HDs were detected that stained positively for the β4 protein (as shown by immunogold labeling in Fig. 2E). This finding further confirms that the α6β4 integrin is involved in the initial steps of HD assembly (Borradori and Sonnenberg, 1999; Koster et al., 2004).

Characterization of the cell adhesion defects

To further determine the extent to which the β4 gene is inactivated in the skin of $\beta4^{flox/flox}$; K14-Cre newborn (P2) mice, skin sections of mice were analyzed by immunofluorescence. As shown in Fig. 3, the areas in which β4 protein is synthesized vary in size in different animals, and range from a few cells to fairly large patches. Even in the large patches of skin lacking the β4 subunit, there were only a few micro-blisters, suggesting a compensatory adhesion mechanism(s).

Because the α6 subunit does not only associate with β4 but also with the β1 subunit, we first determined whether α6β1 was formed at all in the absence of β4. In areas that lacked β4 protein, α6 was not detectable indicating the absence of α6β1 (Fig. 3A,B), probably because free β1 subunits were unavailable and because of the rapid degradation of α6 when not associated with a β subunit. Thus, the loss of α6β4-mediated adhesion is not compensated by α6β1.

Another possibility was that other integrins, particularly α3β1, which – like α6β4 – binds to Ln-5, can compensate for the loss of α6β4-mediated adhesion. However, irrespective of whether α6β4 was present or not, the α3β1 integrin remained

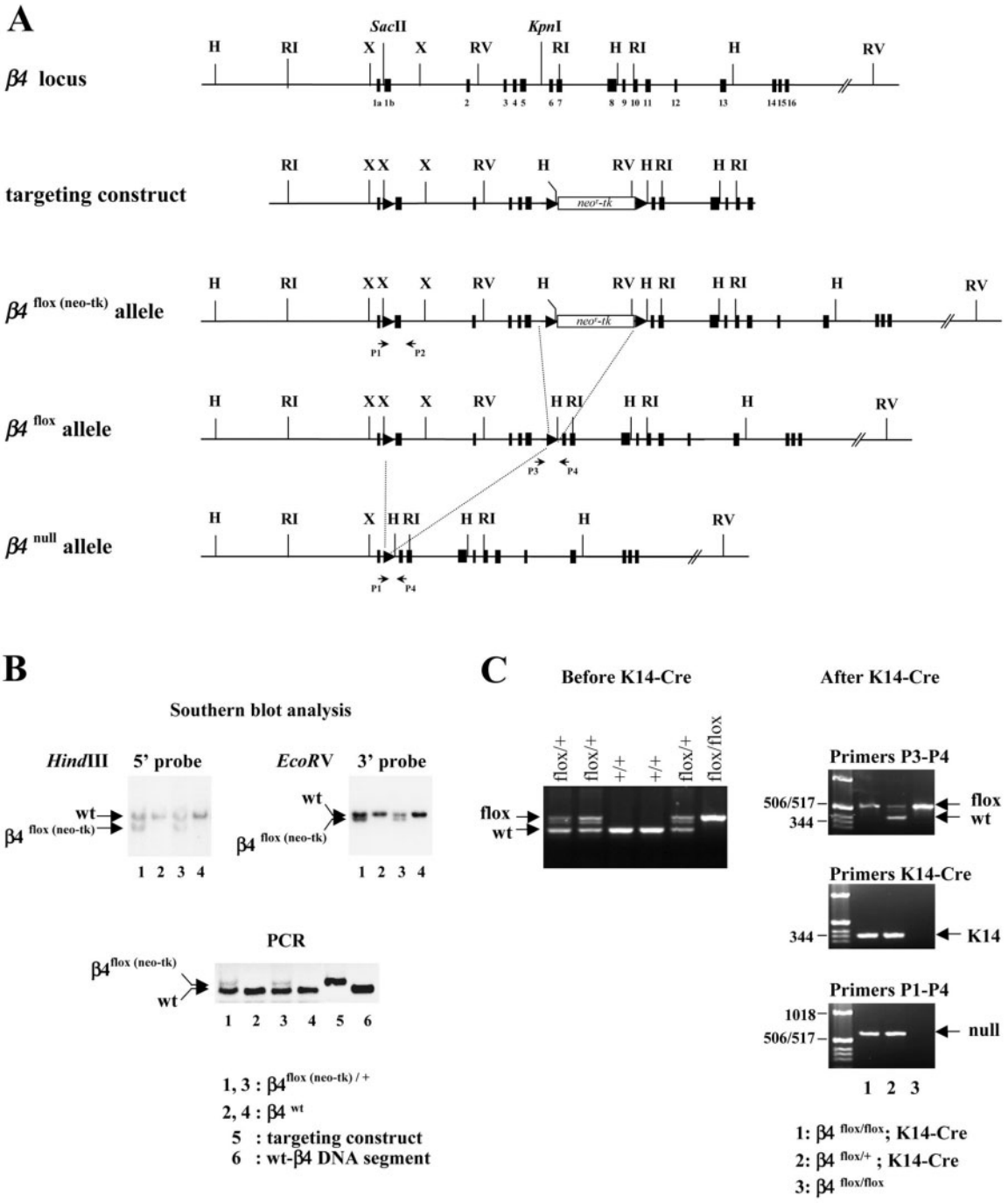


Fig. 1. Targeting strategy and molecular analysis of recombinant ES cells and conditional β4 knockout mice. (A) Restriction map of the 5′ end of the β4 locus, the targeting construct and different β4 mutant alleles. Exons are indicated as numbered black boxes and *loxP* sites as triangles. Position of the unique *Sac*II and *Kpn*I sites used for insertion of the single *loxP* and the floxed *neo-tk* cassette are indicated, as are the primers used for PCR analyses to detect the different mutant alleles of the β4 gene. Restriction sites are: H, *Hin*dIII; RI, *Eco*RI; X, *Xho*I; RV, *Eco*RV. (B) Southern blot and PCR analyses of recombinant ES clones. DNA from two independently targeted ES clones (lanes 1, 3) and two wild-type ES clones (lanes 2, 4) were digested with *Hin*dIII (left panel) or *Eco*RV (right panel), subjected to agarose gel electrophoresis, and transferred to nitrocellulose. Wild-type and mutant alleles were detected by hybridization of the filters with radiolabeled mouse β4 genomic probes corresponding to exons 2-7 (5′ probe) or exons 7-9 (3′ probe) (left and right panels, respectively). The presence of the first *loxP* site in intron 1a of the targeted ES clones was confirmed by PCR using primers P1 and P2 (bottom panel). (C) PCR analysis on genomic DNA of conditional β4 knockout mice before and after their crossing with K14-Cre mice. The conditional allele of the β4 gene was detected by PCR analysis on tail DNA using primers P3 and P4 (Before and After K14-Cre, left and top right panel), and the K14-Cre transgene using the primers K14-cre3 and K14-cre5 (After K14-Cre, middle panel). The removal of exons 1b-5 by Cre-mediated recombination, thereby generating a β4 null allele, was detected by PCR analysis using primers P1 and P4 (After K14-Cre, bottom panel). PCR fragments were resolved by agarose gel electrophoresis and visualized by ethidium bromide. Bands corresponding to the wt, floxed and null alleles of the β4 gene as well as to the K14-Cre transgene are indicated (wt, flox, null, K14). Sizes of molecular weight markers are indicated in bp.

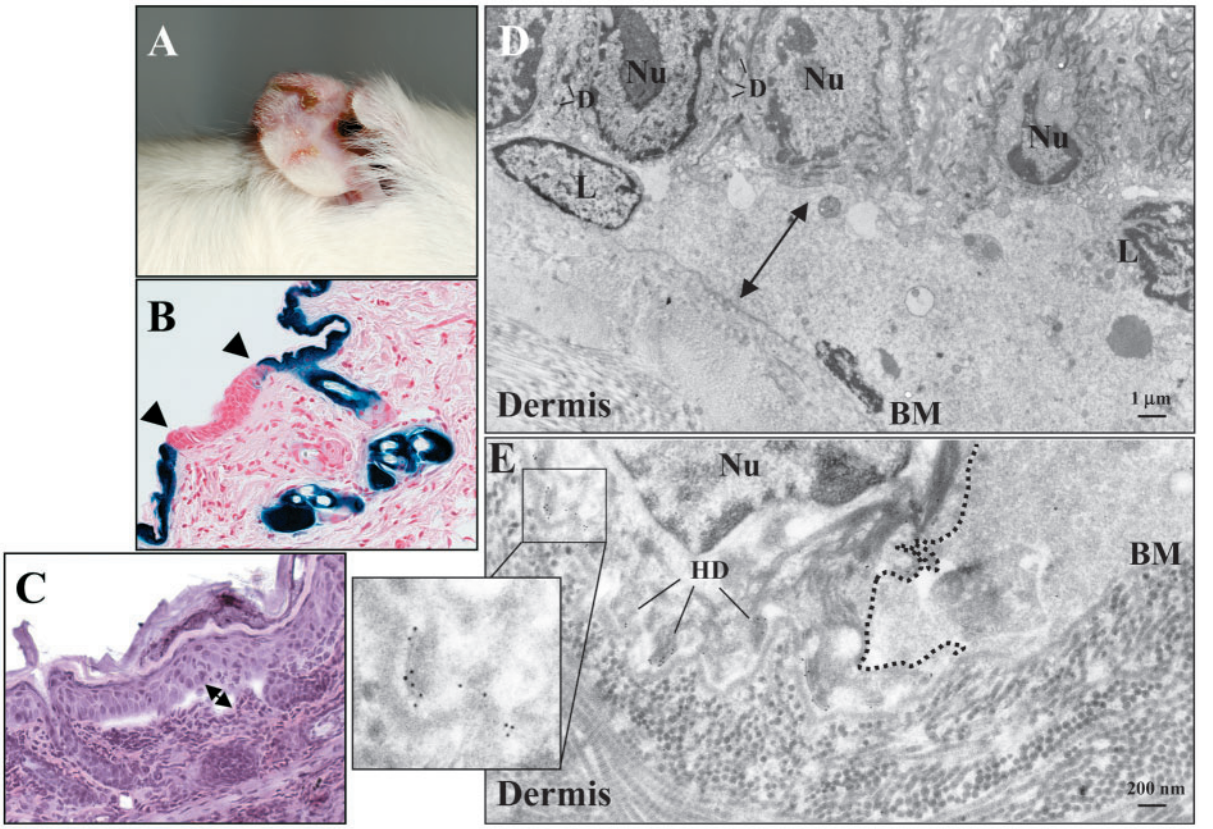


Fig. 2. Histological and ultrastructural abnormalities in β4 null skin. (A) Inflamed ear of a 4-month-old mouse of the genotype $\beta4^{flox/flox}$; K14-Cre. (B) Expression pattern of the Cre-recombinase in the skin of a 15.5-day-old embryo of the genotype ROSA26-pGK-*neo*-pA-*LacZ*-pA; K14-Cre, as assessed by X-gal staining. The non-stochastic expression of the Cre-recombinase at that stage has been described previously (Jonkers et al., 2001). The efficiency of K14-Cre-mediated recombination towards the $\beta4^{flox}$ alleles is such that expression of the β4 gene remained largely mosaic even after birth. (C) Cryosection of skin from a newborn $\beta4^{flox/flox}$; K14-Cre mouse stained with hematoxylin and eosin showing the presence of a small blister. The double-headed arrow denotes separation of the epidermis from the dermis. (D) Electron microscopy of the skin of a $\beta4^{flox/flox}$; K14-Cre mouse shows a blistered area (double-headed arrow) infiltrated by leukocytes (L). The keratinocytes that had detached from the dermis lacked discernible HDs. (E) Immuno-electron microscopy of $\beta4^{flox/flox}$; K14-Cre skin with primary antibodies against β4 demonstrates gold particles associated with HDs at the base of a non-recombined keratinocyte. The neighboring cell to the right has probably degenerated as a result of an inflammatory reaction. The dotted line demarcates the boundary of the cell. BM, basement membrane; Nu, nucleus; HD, hemidesmosome; D, desmosome; L, leukocyte.

localized at the periphery of basal cells, with a slight tendency to concentrate at the basal surface (Fig. 3C). Therefore, although the absence of α6β4 results in relatively more Ln-5 available for an interaction with α3β1, we did not detect a change in the distribution levels of this integrin. Moreover, the distribution patterns and levels of αvβ5, a receptor for vitronectin, and dystroglycan, another laminin receptor, appeared to be unaltered (Fig. 3D,E). Taken together, these data do not reveal a compensatory mechanism for adhesion in the skin of mice with the $\beta4^{flox/flox}$; K14-Cre genotype. Therefore, the normal architecture of the skin may be due to the localized expression of β4.

A prominent feature of β4 null mice is the loss of HDs and the resulting reduction in the expression of the hemidesmosomal components plectin, BP180 and BP230 at the dermal-epidermal junction (Dowling et al., 1996; van der Neut et al., 1996). A marked reduction in the concentration of these components was also observed in areas of the epidermis where the β4 gene had been deleted (not shown). On the whole, the distribution and the amount of plectin that directly interacts with β4 (Geerts et al., 1999; Niessen et al., 1997; Rezniczek et al., 1998) were most strongly affected. However, unexpectedly, there were also regions where the distribution and the levels of all three hemidesmosomal components did not seem to be significantly changed, suggesting that they can cluster in the absence of α6β4 (Fig. 3G-I) (Niessen et al., 1996). However, as confirmed by the ultrastructural analysis, the formation of stable HDs requires α6β4 (Nievers et al., 1999; Schaapveld et al., 1998).

The expression and distribution of ECM components were not disturbed in the β4-null epidermis (van der Neut et al., 1996). In the regions of skin lacking β4, the distribution of Ln-5, Ln-10 and type IV collagen (Fig. 3A and not shown) was normal, whereas fibronectin was restricted to the dermis (Fig. 3F). Furthermore, in the few regions where microblisters had formed, the different BM proteins remained present in the blister floor. These data support the view that α6β4 has no role in the assembly and maintenance of the BM (DiPersio et al., 1997; Dowling et al., 1996; van der Neut et al., 1996).

However, in the skin of the ear, which is less hairy and

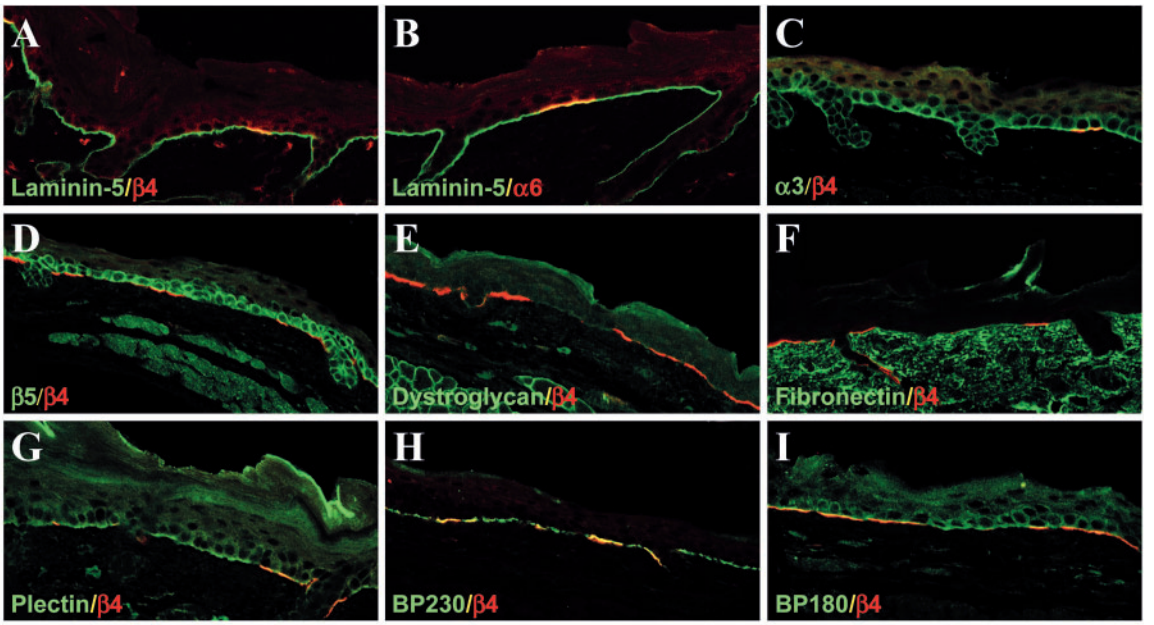


Fig. 3. Expression and distribution pattern of different cell-adhesion receptors, BM components and hemidesmosomal proteins in regions of skin lacking β4. (A-I) Cryosections of skin from a 2-day-old $\beta4^{flox/flox}$; K14-Cre mouse were processed for indirect immunofluorescence and visualized by confocal microscopy. Primary antibodies are against proteins indicated in the left lower corner of each image, and colors are coded according to FITC or Texas Red secondary antibodies. In all panels, the epidermis is at the top and the dermis at the bottom. Notice that only the distribution of the hemidesmosomal proteins plectin, BP180 and BP230 is affected by the absence of α6β4.

therefore more susceptible to mechanical stress, the loss of α6β4 frequently led to chronic inflammation in the $\beta4^{flox/flox}$; K14-Cre mice (Fig. 2A). Indeed, detachment of skin lacking α6β4 induces permanent activation of the keratinocytes as shown by the expression of keratin 6 (Fig. 4A), which is associated with a disorganization of the BM (Fig. 4B-F). Probably, as a result of continuous wound healing, the deposition of a provisional BM is induced as shown by the presence of fibronectin in the BM (Fig. 4B,E) and the lamination of the BM components collagen IV and Ln-5 (Fig. 4C,F).

Establishment of a NMK-1 cell line homozygous for a conditional mutation in the β4 gene

To confirm the in vivo data and define any possible adhesive-independent functions of α6β4, we isolated keratinocytes from newborn $\beta4^{flox/flox}$ mice and cultured them in vitro. We obtained one, spontaneously immortalized, clonal cell line (NMK-1) (Fig. 5A). Analysis by immunobloting revealed that this cell line did not express p53 at detectable levels (Fig. 5B), which is probably the molecular explanation for its immortalization (Sedman et al., 1992). Furthermore, this cell line did not show signs of cell transformation in vitro and did not form tumors when injected subcutaneously into nude mice (10^7 cells, n=6). Adenoviral-mediated delivery of Cre-recombinase in the β4-positive NMK-1 cells [NMK-1(+)], resulted in the removal of β4. Subsequently, a pure β4-negative population [NMK-1(–)] was obtained by FACS sorting (Fig. 5C). The absence of the β4 subunit did not lead to an obvious change in cell morphology (Fig. 5A). Both NMK-1(+) and (–) cells express the hemidesmosomal components BP180 and BP230 (Fig. 5B,C), as well as keratin 5, 6 and 14 (Fig. 5B). They also expressed E-cadherins, α- and β-catenin, whereas they did not contain the mesenchymal marker vimentin (Fig. 5B). FACS and immunoprecipitation analyses of NMK-1(+) and (–) cells revealed that neither expressed the α4 subunit, whereas they did express integrin α2β1, α3β1 and αvβ5 at similar levels (Fig. 5C,D). The levels of α5β1 were very low on both NMK-1(+) and (–) cells, and αvβ6 was not detectable in either of them. The lack of β4 in NMK-1(–) had no effect on the protein levels of α6, whereas it caused a slight increase in the levels of β1, probably because α6β1 was formed at the expense of α6β4. Thus, in the absence of β4, α6 can associate with β1 in vitro. Adhesion assays indicated that the NMK-1(+) and (–) cells adhered equally well to Ln-5, indicating that under static conditions, both α3β1 and α6β1 fully compensated for the loss of the adhesion function of α6β4 (Fig. 5E). As expected, the binding to fibronectin was not affected by the loss of α6β4. However, when adhesion was measured in detachment assays, there appeared to be a clear role for α6β4 regarding the strength by which cells adhere to Ln-5 (Fig. 5F). Ten minutes after trypsinisation, most NMK-1(–) cells had become detached from the substratum, whereas almost all NMK-1(+) cells were still attached to the plate. This data indicates that neither α3β1 nor α6β1 can compensate for the strength of the adhesion conferred by α6β4. Moreover, detachment-analysis by centrifugal-force assay showed that at the lowest force (500 ***g***) detachment of both cell populations was similar (around 25% (Fig. 5G) but that an increase to 750 ***g*** resulted in the detachment of about 30% and 50% of NMK-1(+)- and NMK-1(–)-cell populations, respectively. This indicates that the presence of β4 strengthens the adhesion to Ln-5.

Confocal-microscopy analysis showed colocalization of β4 with Ln-5 and the hemidesmosomal components plectin and BP230, indicating that type I HDs were formed in NMK-1(+) cells (Fig. 5H). This was further confirmed by ultra-structural analysis (Fig. 5I). In contrast to the NMK-1(+) cells, no HDs were detected in NMK-1(–) cells and plectin and BP230 were diffusely distributed over the cytoplasm (Fig. 5H). Ln-5 was

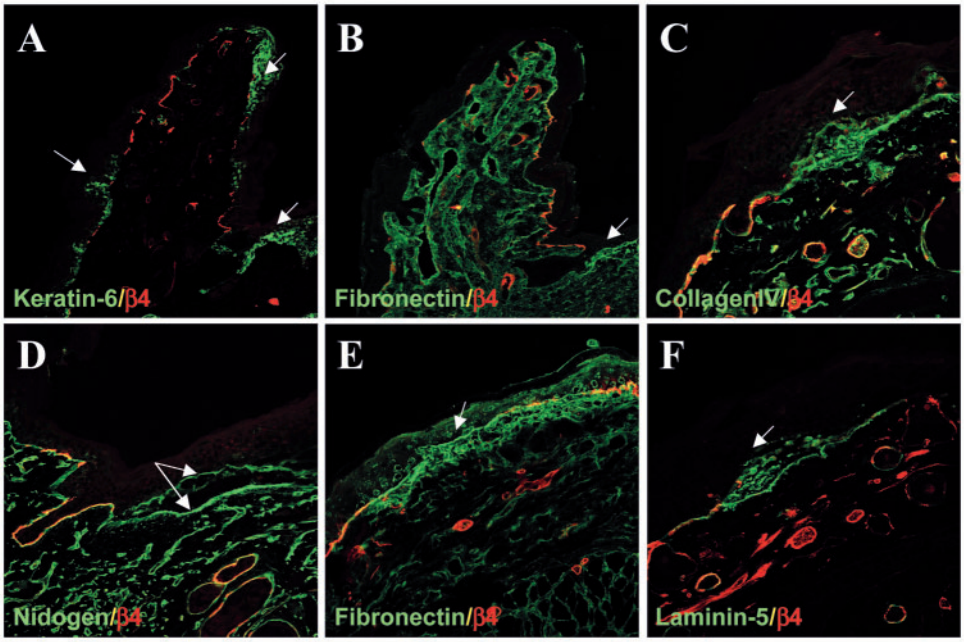


Fig. 4. The BM is disorganized in the inflamed ear of a 4-month-old $\beta4^{flox/flox}$; K14-Cre mouse. (A-F) Cryosections of inflamed ear processed for indirect immunofluorescence and visualized by confocal microscopy. Primary antibodies are against proteins that are indicated in the left lower corner of each image and colors are coded according to FITC or Texas Red secondary antibodies. (A,B) Images show that the removal of β4 is associated with keratinocyte activation as visualized by keratin-6 staining (A) and the deposition of fibronectin into the BM (B). (C-F) Higher magnifications illustrating the presence of blisters when α6β4 is absent (D, arrows), the alteration of the BM components collagen IV and Ln-5 in inflamed regions of the skin showing lamination (C,F, arrows) and the deposition of fibronectin into the BM (E, arrow).

normally deposited in clusters in NMK-1(–) cells, confirming that α6β4 does not play a role in the assembly of the epidermal BM (Dowling et al., 1996; van der Neut et al., 1996).

During wound healing, basal keratinocytes at the wound edge disassemble HDs to be able to migrate on a newly deposited Ln-5 matrix (Martin, 1997; Decline and Rousselle, 2001; Frank and Carter, 2004). We have previously shown that the motility of human keratinocytes is inhibited when ligated α6β4 is bound to plectin (Geuijen and Sonnenberg, 2002). Similarly, we found that the absence of HD-like structures in the NMK-1(–) cells leads to an increased rate of migration after wounding. Compared to NMK-1(+), the NMK-1(–) cells migrated twice as fast (815 μm/hour versus 475 μm/hour) (Fig. 5J). Furthermore, when the α6-blocking mAb GoH3 was added to the cultures to block the interaction of α6β4 with Ln-5, the migration of NMK-1(+) cells was enhanced and became as fast as that of the NMK-1(–) cells, indicating that the binding of α6β4 to its substrate was a crucial step in the stabilization of adhesion (not shown). We conclude that the function of α6β4 during wound healing appeared to be conserved from mice to men.

Analysis of the biological defects

It has been proposed that α6β4 contributes to signals that regulate cell proliferation (Dans et al., 2001; Mainiero et al., 1997; Murgia et al., 1998). Through its adhesion function, this integrin might ensure that cells with a strong proliferative potential remain present in the basal compartment of the skin. We therefore wondered whether the loss of α6β4 results in a premature differentiation and a reduced proliferation of basal keratinocytes. No abnormalities in epidermal differentiation were revealed by routine histology of areas of skin from newborn mice that lacked β4. Even in regions where small blisters were visible, the differentiation of the skin appeared to be normal (not shown). Furthermore, the distribution patterns of keratin 14 and a variety of differentiation markers were similar in areas of skin lacking or containing α6β4 (Fig. 6A-C). Keratin 14 was expressed in the basal cells, whereas keratin 1 was confined to the suprabasal cell layers. The late-stage differentiation marker involucrin was faithfully expressed in the superficial cell layers. Other proteins such as CD9 and CD44 (Fig. 6D,E) as well as F-actin (not shown), were also expressed in patterns indistinguishable from those in epidermis that expressed α6β4. Finally, the formation of hair follicles and their epidermal breaching, a process that is associated with the induction of expression of mucin which is recognized by the mAb 33A10, appeared to be normal (Fig. 6F). To confirm these observations in vitro, NMK-1(+)- and NMK-1(–)-cell populations were grown to confluency in the culture conditions routinely used to propagate them. RT-PCR analysis was then performed to detect potential differences between NMK-1(+) and (–) cells in the levels of differentiation markers, such as envoplakin and desmoglein-1 (Dsg-1). Whereas NMK-1(–) cells did not express β4, specific markers for basal cells such as the α6 integrin, Dsg-2 and Dsg-3, the γ2 chain of Ln-5, and the differentiation markers Dsg-1 and envoplakin were expressed at similar levels (Fig. 6G). This observation suggested that, under normal culture conditions with relatively high Ca^{2+} levels (0.09 mM), the signals for cells to differentiate are identical in the NMK-1(+)- and NMK-1(–)-cell populations.

To investigate the effects that the deletion of α6β4 has on proliferation in vivo, newborn mice were injected intraperitoneally with BrdU and sacrificed 1 hour later. By triple-labeling of corresponding skin sections with anti-BrdU, anti-β4 antibodies and antibodies against nidogen (a component of the BM), fibronectin (a dermal marker) or with TOPRO-3 (to stain the nuclei), it can be investigated whether proliferation is spatially and/or quantitatively disturbed in the absence of β4. In both β4-positive and β4-negative areas of the epidermis, proliferating cells were exclusively present in the basal cell compartment (Fig. 7A-C) and the number of BrdU labeled cells in the interfollicular areas of the skin was similar (26.1% in β4-positive and 25.7% in β4-negative areas of the

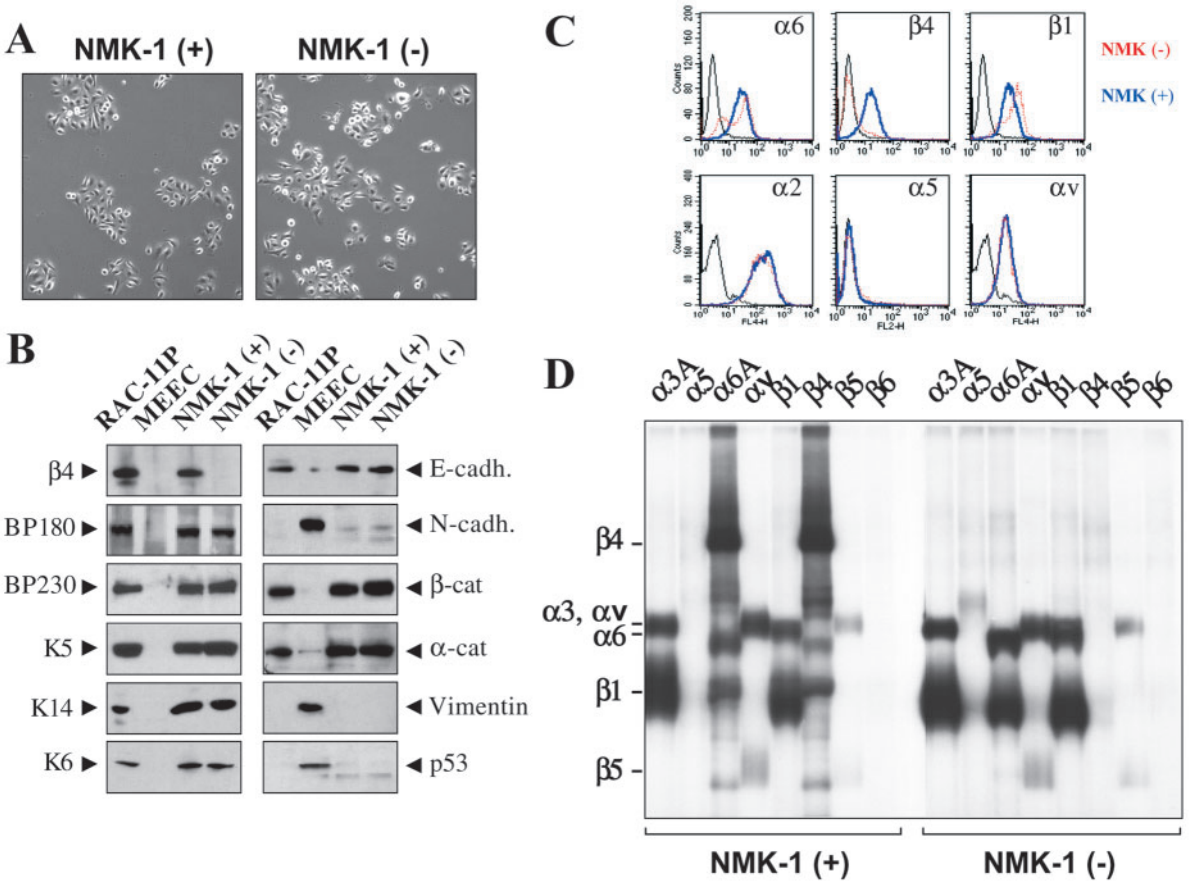
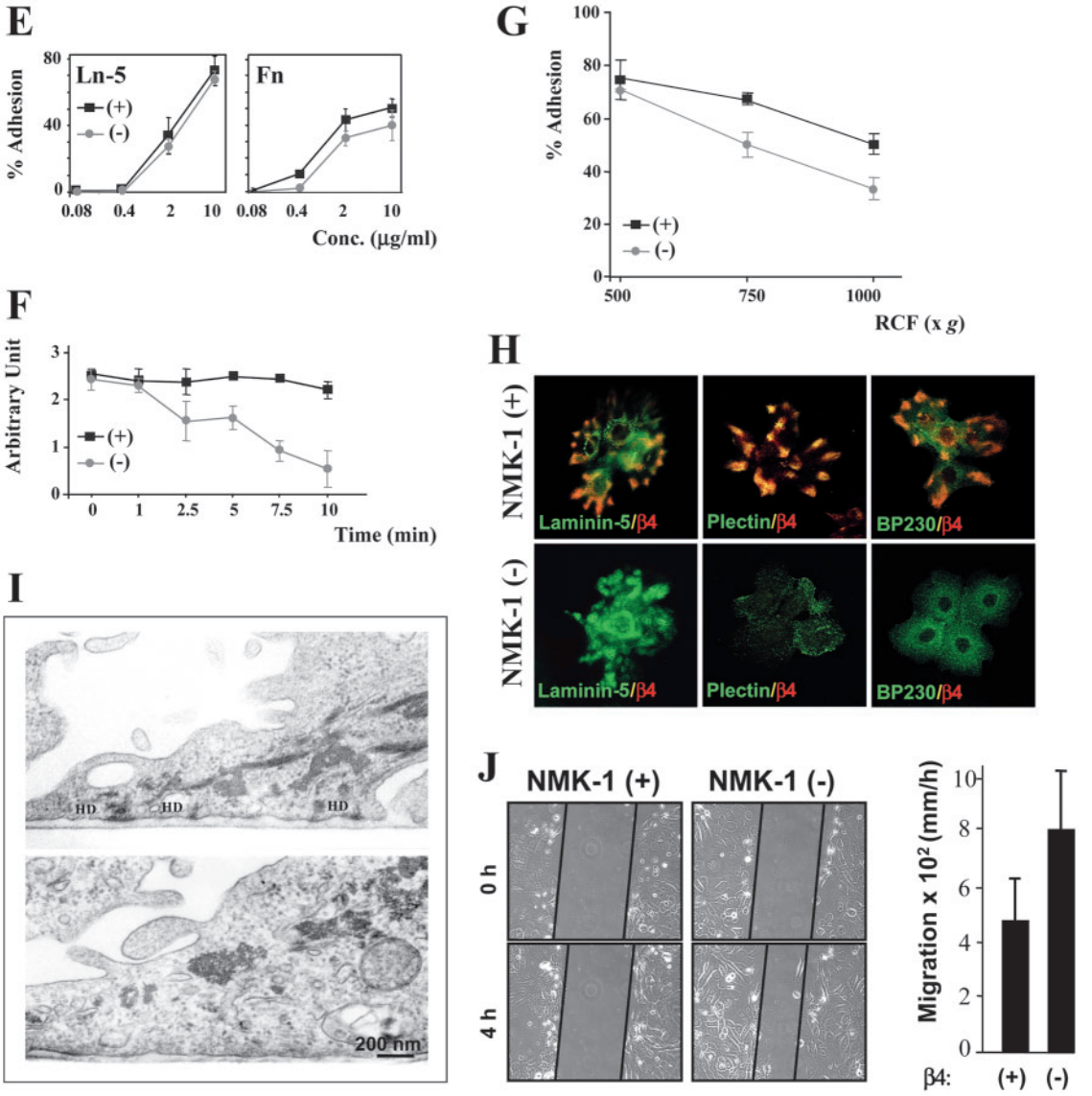


Fig. 5. Establishment of a mouse keratinocyte cell line, carrying a conditional allele of the β4 gene. (A) Phase-contrast image of subconfluent NMK-1(+) and (–) keratinocytes showing their morphology. (B) Immunoblot analysis of the expression levels of p53 and different epithelial and mesenchymal markers in the NMK-1(+) and (–) cell lines. RAC-11P cells (Sonnenberg et al., 1993) and mouse embryonic endothelial cells (MEEC) (Larsson et al., 2001) are used as control cell lines. (C) FACS analysis of the levels of α6, β4, β1, α2, α5 and αv integrin subunits expressed by NMK-1(+) and (–) cells. (D) Immunoprecipitation analysis of surface-labeled components from NMK-1(+) and (–) cells with antibodies specific for a range of integrin subunits. (E) NMK-1(+) and (–) keratinocytes adhered equally well to Ln-5 and fibronectin. (F,G) Detachment assays based on resistance to trypsin treatment (F) and centrifugal force (G) demonstrate the importance of α6β4 in strengthening the adhesion. (H) Indirect immunofluorescence analysis of hemidesmosomal protein localization in NMK-1 cells visualized by confocal microscopy. Primary antibodies are against proteins specified in the left lower corner of each image and colors are coded according to FITC or Texas Red secondary antibodies. The colocalization of the different hemidesmosomal components indicates that these cells form type I HDs in culture. (I) Ultrastructural analysis of NMK-1(+) and (–) cells (upper and lower panels, respectively) further confirms the presence of HDs in NMK-1(+) cells whereas they are lost in NMK-1(–) population. (J) Wound healing assay shows that the motility of NMK-1(–) cells is increased compared with those of NMK-1(+). In the bar graph, results are expressed as the unadjusted means ± s.d. of four separate experiments with six replicates each ($P<0.001$).

skin, Fig. 7G). Similarly, in vitro, we found that the ability of the NMK-1(+) and (–) cells to grow under various conditions does not differ. Neither population formed colonies in semi-solid medium (Fig. 7H, left panels) and they did not grow in the absence of exogenous growth factors (Fig. 7I), indicating that they are not transformed. Growth curves and clonogenic assays showed that under normal conditions of culture, when the concentration of exogenous growth factors was reduced (1/10 or 1/20 of the normal concentration of EGF and pituitary gland extract) (data not shown) and at low density, the growth capacities of the NMK-1(+) and (–) cells were similar (Fig. 7H, right panels, I). Furthermore, cell-cycle analysis of NMK-1(+) and (–) cells by flow cytometry and BrdU incorporation revealed no difference in cell proliferation (Fig. 7J,K). Finally, activation of Erk1 and Erk 2 was similar in the two cell lines in response to growth factor stimulation (Fig. 7L). Taken together, these results indicate that α6β4 does not play a role in the control of cell growth in vitro.

We also examined the effects of the absence of α6β4 on apoptosis by TUNEL labeling of the skin in newborn mice (Fig. 7D-F). We observed that in interfollicular regions of the skin, apoptosis took place only in the suprabasal layers and that the loss of β4 did not lead to an accelerated death of the basal keratinocytes. Increased apoptosis was observed in regions of detached epidermis, indicating that epidermal adhesion to the BM was critical for keratinocyte survival. Taken together, these data clearly show that in regions where the adhesion of the epidermis to the dermis is not compromised by the loss of α6β4, keratinocytes of adult mice display normal survival, proliferation and differentiation. Obviously, when basal



keratinocytes in the blisters did not receive extracellular cues over prolonged periods of time, this resulted in abnormalities in differentiation and proliferation potential.

Discussion

The generation of mice that lack specific integrins has greatly contributed to our understanding of their role in the development of tissues and organs, and have provided conclusive evidence that integrins not only mediate adhesive events, but also play an instructive role in the formation of these structures (Danen and Sonnenberg, 2003; Bouvard et al., 2001). The integrin α6β4 is expressed in many tissues, but most strongly in the epidermis, where it is concentrated in HDs (Stepp et al., 1990; Sonnenberg et al., 1991). In these structures, α6β4 links the intermediate filament system to the plasma membrane. Mice carrying a null mutation of the α6 or β4 subunit do not form HDs and die shortly after birth because of severe blistering of their skin (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). Similarly, humans who have an autosomal recessive mutation in the genes for either of these integrin subunits exhibit epidermal blistering, the degree of severity depending on the nature of the mutation (Ashton et al., 2001). Because of the perinatal lethality of the α6 and β4 knockout mice, post-developmental studies on the effects of the loss of α6β4 were impossible. In the present study, we used a Cre-*loxP* approach to introduce a conditional mutation in the β4 gene. We crossed the conditional β4 knockout mice with transgenic mice expressing the Cre enzyme at low levels in basal keratinocytes (Jonkers et al., 2001). This generated viable mice, in which only small parts of skin were entirely devoid of α6β4. Most probably, blister formation is rare in these mice because the cells that do express α6β4 are sufficient to prevent epidermal detachment. This may be particularly important during the first one or two days after birth when mice do not yet have hair, which provides an additional means of adherence of the epidermis to the dermis.

The ability of α6β4 to effect and consolidate epidermal cell adhesion appears to be a specific function of this integrin because, when absent, it is not compensated by the integrin α3β1, the other Ln-5 receptor present on keratinocytes. In

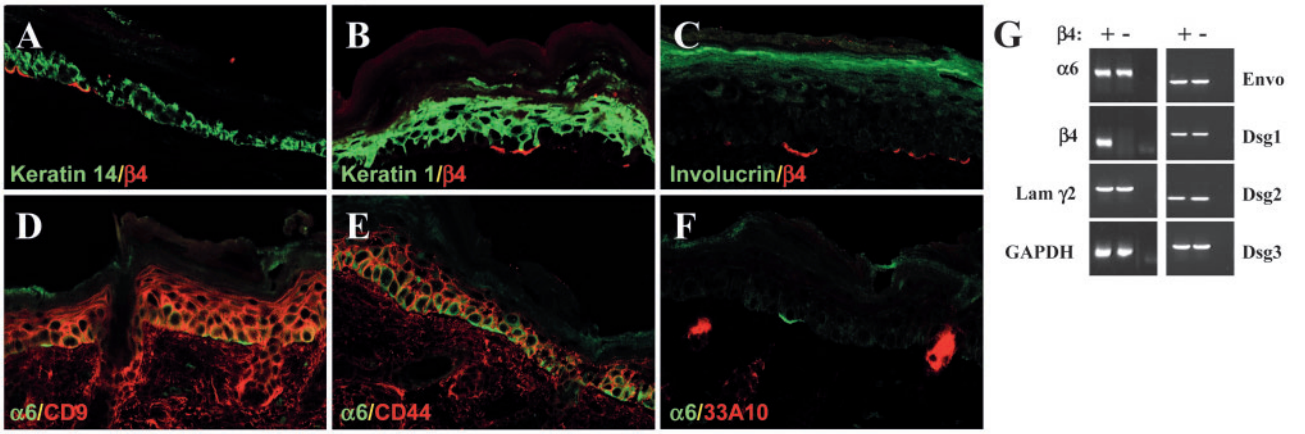


Fig. 6. Loss of the α6β4 integrin does not affect stratification and differentiation of keratinocytes. (A-F) Cryosections of skin from two-day-old $\beta4^{flox/flox}$; K14-Cre mice processed for indirect immunofluorescence and visualized by confocal microscopy. Primary antibodies are against proteins indicated in the left lower corner of each image, and colors are coded according to FITC or Texas Red secondary antibodies. In all panels, the epidermis is at the top and the dermis at the bottom. (G) Semi-quantitative RT-PCR showing that there were no significant changes in the level of transcripts for a range of basal (α6 integrin, Dsg-2 and -3 and the γ2 chain of Ln-5) and suprabasal (Dsg-1 and envoplakin) cell markers in NMK-1(+) and (–) cells.

addition, the severity of blistering is not exacerbated when α3β1 is also deleted (DiPersio et al., 2000). Consistent with this observation, we found, by using immunofluorescence analysis, that the levels of α3β1 and other receptors that can possibly compensate for the loss of α6β4, had not changed. As expected, the expression of α6β4 leads to a considerable increase in the strength of adhesion of cells to the substratum. In detachment assays, immortalized keratinocytes of conditional β4 knockout mice, detached considerably more slowly than the same cells without β4 (deleted by Cre-mediated recombination). This ability of α6β4 to strengthen adhesion is probably mediated by the binding of the β4 cytoplasmic domain to plectin, which links this integrin to the intermediate filament system (Geerts et al., 1999; Niessen et al., 1997; Rezniczek et al., 1998). This conclusion is supported by observations that, plectin-deficient mice have defects in the mechanical integrity of the epidermis (Andra et al., 1997) and deletion of the cytoplasmic domain of the integrin β4 subunit in mice produces the same phenotype as the removal of the entire β4 subunit (Murgia et al., 1998). In this context, it is noteworthy that the defects in the $\beta4^{flox/flox}$; K14-Cre mice generated by us, mainly occurred in their ears, probably because of the low density of hair follicles in this part of the skin compared to the rest of the body. This may render the skin more susceptible to the effects of stress, thus increasing the tendency to form blisters in the absence of α6β4. A chronic inflammation of the ear may result from this because of an impaired wound healing, ultimately leading to an aggravation of the pathological condition.

As adhesion receptors organizing the actin cytoskeleton, β1 integrin and β3 integrin play a key role in the control of many important cellular processes (DeMali et al., 2003; Giancotti and Ruoslahti, 1999; Schwartz and Ginsberg, 2002). However, integrin α6β4 is not connected to the actin cytoskeleton but to intermediate filaments, whose primary role is to impart mechanical strength to cells (Fuchs and Cleveland, 1998). Nevertheless, a function of this integrin in cell migration, proliferation and survival of cells has been clearly demonstrated (Dajee et al., 2003; Murgia et al., 1998; Santoro et al., 2003; Trusolino et al., 2001; Weaver et al., 2002); although how α6β4 exerts these diverse functions is not clear. An obvious possibility is that α6β4 regulates these processes by strengthening the adhesion of cells to the substrate – as we have shown to occur. For example, in cells that can only form unstable interactions with the substrate through β1 and/or β3 integrins, α6β4 might be required to reinforce the resulting associations. This would allow the cells to organize their actin cytoskeleton and to adopt a spread-morphology, conditions known to be essential for the above-mentioned processes. Such an adhesion function of α6β4 might be far more important for tumor cells than normal cells because of the strong effects that activated oncogenes can have on cell adhesion and the actin cytoskeleton (Hughes et al., 1997). Indeed, there is evidence that, in carcinoma cells α6β4 is translocated from HDs to actin-containing structures, such as lamellipodia, where it contributes to their stability (Rabinowitz et al., 1999) and protects cells from apoptosis (Dajee et al., 2003; Weaver et al., 2002). In apparent contrast to the reported positive effect of α6β4 on the migration of carcinoma cells, our results with immortalized keratinocytes, expressing or lacking α6β4, clearly revealed a negative effect of this integrin on cell migration. In these cells, the number of β1 and β3 (or β5) adhesion receptors is probably already optimal for migration, and under these circumstances, β4 will only stabilize the already existing adhesions to impede cell migration. Indeed, the anti-α6 mAb GoH3 that blocks the interaction between α6β4 and its ligand Ln-5 accelerates the migration of keratinocytes. Consistent with the notion that adhesion strengthening is facilitated by the interaction of α6β4 with plectin, we previously found that disruption of this association leads to an accelerated migration of human keratinocytes (Geuijen and Sonnenberg, 2002).

Several reports have suggested that α6β4 also participates directly in signaling processes that control cell proliferation, apoptosis and migration. For example, it has been shown that ligation of α6β4 promotes tyrosine phosphorylation of the

cytoplasmic domain of β4 by activation of Fyn kinase, followed by recruitment of the adaptor protein Shc and activation of the Erk pathway (Dans et al., 2001; Mainiero et al., 1997; Gagnoux-Palacios et al., 2003). Additionally, phosphorylation of β4 by growth-factor-receptor-stimulation has been associated with the disassembly of HDs and increased cell motility (Gambaletta et al., 2000; Santoro et al., 2003; Trusolino et al., 2001). Finally, there is evidence suggesting that α6β4 activates PI3-K through tyrosine phosphorylation of IRS-1. For this stimulation a specific tyrosine residue on the β4 subunit was shown to be crucial (Shaw, 2001). These data suggest that, α6β4 has a specialized function in the regulation

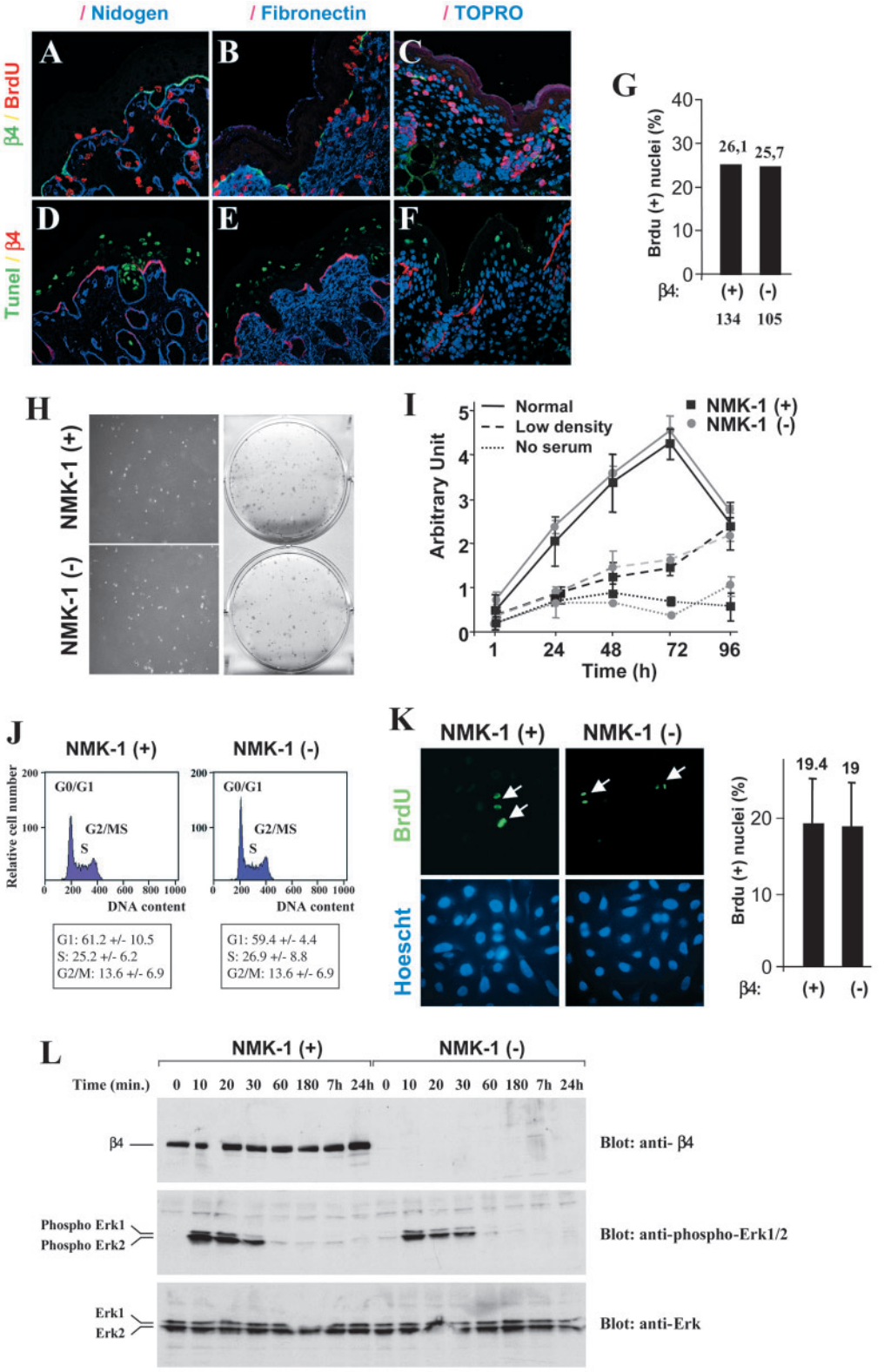


Fig. 7. Loss of α6β4 integrin does not affect proliferation/survival of basal keratinocytes when cells are adhered to the BM. (A-F) Cryosections of skin from newborn $\beta4^{flox/flox}$; K14-Cre mice, injected intraperitoneally with BrdU for 1 hour (A-C) or assayed by TUNEL for the presence of apoptotic cells (D,F) that were subsequently processed for indirect immunofluorescence and visualized by confocal microscopy. Primary antibodies are against proteins indicated, and colors are coded according to FITC or Texas Red secondary antibodies. In all panels, epidermis is at the top and dermis at the bottom. (G) Quantitative analysis of cell proliferation in the interfollicular regions of epidermis that lack or contain the β4 integrin. (H) The NMK-1(+) and (–) cell populations do not form colonies in semi-solid medium (left panels) and show the same growth capacities at low density (right panel). (I) Growth curves indicate that the NMK-1(+) and (–) cell populations have the same growth characteristics under the various conditions tested. (J) Representative DNA histograms of exponentially growing NMK-1(+) and (–) cells. (K) Immunofluorescent detection and quantification of BrdU incorporation in NMK-1(+) and (–) cells. (L) Erk1/2 phosphorylation in growth-factor-stimulated NMK-1(+) and (–) cells. Cells were cultured for 16 hours in keratinocyte serum-free medium after which EGF and pituitary gland extract were added for the time periods indicated. Lysates were blotted and probed with antibodies against β4, phospho-Erk and total Erk as control.

of important cellular processes, independent of its function as an adhesion receptor.

The coexistence of β4-positive and β4-negative stretches of skin in the same animal permitted an accurate analysis of the role of α6β4 in cell adhesion and signaling. Our findings do not provide evidence for a specific role of α6β4 in differentiation and proliferation that is independent of its function as an adhesion receptor. The same conclusion was reached in previous studies in which the role of α6β4 was studied during embryonic development of the skin (DiPersio et al., 2000; Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). In these studies, it was shown that integrin-mediated adhesion to Ln-5 was not essential for epidermal development and differentiation, and that mechanisms of epidermal adhesion other than those mediated by α6β4 and α3β1, were sufficient to ensure normal embryogenesis. Furthermore, the absence of α6β4 did not cause a difference in the survival of cells. The present study extends these findings by showing that, after birth, differentiation and proliferation is normal in the absence of α6β4. Apoptotic cells were detected in the superficial epidermal layers, but only in certain areas and in regions where the skin had been detached over prolonged periods of time. The finding that the absence of α6β4 does not lead to an increase in the number of apoptotic basal keratinocytes – where the skin remains attached to the dermis – excludes an adhesion-independent role of β4 in this process and rather suggests that adhesion per se protects cells from apoptosis.

Our findings are in excellent agreement with those of Dipersio et al., who showed that epidermal proliferation in α6 or β4 null E16.5 embryos is not different from that in wild-type embryos (Dipersio et al., 2000) but they are in apparent contrast with those of Murgia et al., who reported a two-fold reduction in epidermal proliferation in mutant E18.5 embryos, whose β4 subunit lacked the cytoplasmic domain (Murgia et al., 1998). This discrepancy might be explained in different ways. First, whereas in our study the β4 subunit is completely absent in some parts of the skin, the study presented by Murgia et al. (Murgia et al., 1998) involved a truncated β4 protein lacking the cytoplasmic domain. It is possible that this truncated β4 integrin exerts a dominant-negative effect on signaling pathways controlling cell proliferation. In this respect, it is worth mentioning that associations have been observed between α6β4 and various growth factor receptors (Gambaletta et al., 2000; Santoro et al., 2003; Trusolino et al., 2001) as well as CD151 (Sterk et al., 2000). Second, because we have studied the effect of β4 on epidermal proliferation by comparing β4-negative and β4-positive parts of the skin in the same animal, biological variations between animals can be ruled out. By contrast, Murgia et al. compared different embryos (wild-type embryos versus embryos with a truncated β4) and thus the selection of their embryos may explain their results (Murgia et al., 1998). Moreover, the effect of β4 on cell proliferation might depend on the stage of embryogenesis.

In summary, the present study shows that the adhesion of α6β4 to Ln-5, which ensures normal function of keratinocytes, might be far more important than its possible signaling function in controlling epidermal cell proliferation and differentiation. Furthermore, our results show that at least in immortalized keratinocytes, α6β4 impedes cell migration, consistent with its role in adhesion strengthening and the promotion of HD formation.

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