Conditional Ablation of β1 Integrin in Skin: Severe Defects in Epidermal Proliferation, Basement Membrane Formation, and Hair Follicle Invagination

Srikala Raghavan, Christoph Bauer, Gina Mundschau, Qingqin Li, and Elaine Fuchs

Howard Hughes Medical Institute, Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

Abstract. The major epidermal integrins are α3β1 and hemidesmosome-specific α6β4; both share laminin 5 as ligand. Keratinocyte culture studies implicate both integrins in adhesion, proliferation, and stem cell maintenance and suggest unique roles for αβ1 integrins in migration and terminal differentiation. In mice, however, whereas ablation of α6 or β4 results in loss of hemidesmosomes, epidermal polarity, and basement membrane (BM) attachment, ablation of α3 only generates microblistering due to localized internal shearing of BM. Using conditional knockout technology to ablate β1 in skin epithelium, we have uncovered biological roles for αβ1 integrins not predicted from either the α3 knockout or from in vitro studies. In contrast to α3 null mice, β1 mutant mice exhibit severe skin blistering and hair defects, accompanied by massive failure of BM assembly/organization, hemidesmosome instability, and a failure of hair follicle keratinocytes to remodel BM and invaginate into the dermis. Although epidermal proliferation is impaired, a spatial and temporal program of terminal differentiation is executed. These results indicate that β1's minor partners in skin are important, and together, αβ1 integrins are required not only for extracellular matrix assembly but also for BM formation. This, in turn, is required for hemidesmosome stability, epidermal proliferation, and hair follicle morphogenesis. However, β1 downregulation does not provide the trigger to terminally differentiate.

Key words: integrins • epidermis • conditional knockout • proliferation • skin

Introduction

Integrins are heterodimeric transmembrane receptors composed of an α regulatory subunit and a β signal transducing subunit (for review see Howe et al., 1998; Miyamoto et al., 1998; Giancotti and Ruoslahti, 1999; Schoenwaelder and Burridge, 1999). Externally, activated integrins mediate cell–substratum attachment, while internally they transduce signals that regulate growth, differentiation, and migration. Most integrins use specific components of extracellular matrix (ECM)[1] as ligands to receive cues from their environment. Upon activation, integrins associate intracellularly with cytoskeleton to cluster, transmit, and translate these cues.

Integrin heterodimers containing the β1 subunit are broadly expressed in many cell types, and β1 is promiscuous, associating with many α partners. The α subunit imparts ligand specificity, enabling the heterodimer to bind to specific ECM or basement membrane (BM) components. Cultured fibroblast studies with the fibronectin receptor, α5β1, suggest that upon ligand engagement, β1's short (50 amino acid) cytoplasmic domain binds to proteins that in turn associate with and reorganize actin filaments to form focal adhesions. Upon further activation of Rho GTPases, changes in the actin cytoskeleton lead to integrin clustering, which facilitates the polymerization and assembly of ECM on the cell surface and enables stable substratum attachment (Wennerberg et al., 1996; for review see Schoenwaelder and Burridge, 1999). In contrast, α6β4 associates with transmembrane collagen XVII (BPAG2) and with two intermediate filament linker proteins, plectin and BPAG1 (for review see Jones et al., 1998). This enables the integrin to cluster into robust macrostructures referred to as hemidesmosomes, especially

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Address correspondence to Elaine Fuchs, Howard Hughes Medical Institute, Dept. of Molecular Genetics and Cell Biology, The University of Chicago, 5841 S. Maryland Ave., Rm. N314, Chicago, IL 60637. Tel.: (773) 702-1347. Fax: (773) 702-0141. E-mail: lain@midway.uchicago.edu

[1]*Abbreviations used in this paper:* BL, basal layer; BM, basement membrane; bs, backskin; DEJ, dermal–epidermal junction; ECM, extracellular matrix; ES, embryonic stem; GR, granular; LD, lamina densa; SC, stratum corneum; SP, spinous; WT, wild-type.

prominent in stratified squamous epithelia such as epidermis.

In the epidermis and its appendages, basal keratinocytes utilize integrins to adhere to their underlying BM, rich in ECM (for review see Burgeson and Christiano, 1997). The predominant epidermal integrins are $\alpha3\beta1$ and $\alpha6\beta4$, both of which bind laminin 5, the major ECM component of the BM (Carter et al., 1991; Rousselle et al., 1991; for review see Burgeson and Christiano, 1997). Minor epidermal integrins include $\alpha2\beta1$ (collagen/laminin), $\alpha5\beta1$ (fibronectin), and the wound healing–induced integrin $\alpha v\beta5$. Whereas $\alpha6\beta4$ and hemidesmosomes are restricted to the basal surface of epidermis, $\alpha\beta1$ heterodimers are not polarized (Kim and Yamada, 1997). As epidermal cells commit to terminally differentiate, they downregulate integrin expression, detach from the BM, and move outward towards the skin surface (Watt et al., 1993; for review see Fuchs, 1999).

The functions of $\alpha6\beta4$ in mice have been explored through gene targeting (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). When $\alpha6$ or $\beta4$ is missing, the partner is unstable, leading to a loss of the heterodimer. Hemidesmosomes are absent, and epidermal adhesion to the underlying BM is seriously impaired. Upon mild mechanical stress, the epidermis peels from its underlying substratum, a condition in humans known as junctional epidermolysis bullosa, also caused by mutations in laminin 5 (for review see Pulkkinnen and Uitto, 1999). Interestingly, ultrastructural signs of apoptosis are seen in $\beta4$ null basal cells, but pearls of relatively undifferentiated, mitotically active cells are found in the spinous (SP) layers of haired and nonhaired skin (Dowling et al., 1996).

In contrast, mice deficient in $\alpha3$ integrin exhibit a mild skin phenotype, with an epidermis that is normal in morphology, thickness, proliferation, and terminal differentiation, and that displays no overt signs of hair follicle defects (Kreidberg et al., 1996; DiPersio et al., 1997; Hodivala-Dilke et al., 1998). By immunofluorescence, laminin 5 and other ECM components still localize to the dermal–epidermal junction (DEJ), and ultrastructurally, hemidesmosomes are intact and seem unaffected, and most regions of the BM appear intact. However, microblisters occur in the limb skin regions due to rupturing within the BM, which has led to the postulate that without $\alpha3\beta1$, laminin 5 assembly may be perturbed.

The loss of $\alpha3$ still leaves $\beta1$ with several partners thought to be minor in normal skin, but in fact, fibronectin receptor ($\alpha5\beta1$) and collagen IV receptor ($\alpha2\beta1$) activities are increased in cultured $\alpha3$-deficient keratinocytes (Hodivala-Dilke et al., 1998). This leaves open the possibility, presently unaddressed, that the physiological importance of $\alpha\beta1$ integrins in skin extends beyond merely a role for $\alpha3\beta1$ in laminin 5 assembly and/or BM integrity. Furthermore, there are considerable differences between the relatively mild skin defects in the $\alpha3$ knockout mouse and the many different putative functions ascribed to $\beta1$ from cell culture studies. This begs the question of what might happen when $\beta1$ is missing, leaving $\alpha2$, $\alpha3$, and $\alpha5$ without partners. The $\beta1$ knockout is lethal in the early embryo, making it impossible to assess its role in skin (Fassler and Meyer, 1995; Stephens et al., 1995). Further complications arise in assessing function, because $\beta1$ null embryonic stem (ES) cells only survive in the absence of fibroblast feeder cells, whereas their wild-type (WT) counterparts only survive in the presence of feeders. Thus, although $\beta1$ null ES cells fail to generate keratinocytes when induced to differentiate (Bagutti et al., 1996), this could be either due to the lack of a feeder layer or to the absence of $\beta1$ integrin. In WT/$\beta1$ null chimeric mice, $\beta1$ null keratinocytes survive, and it has been reported that the skin is normal (Bagutti et al., 1996), raising additional questions regarding possible functional redundancies and the physiological role(s) of $\beta1$ in skin.

Using conditional knockout technology, we now explore the in vivo function of $\beta1$ integrin in epidermis and its appendages. The phenotype of $\beta1$ null epidermis is markedly distinct from that seen in other knockouts of epidermally expressed integrins and could not have been predicted based upon prior in vivo or in vitro studies conducted on the role of $\alpha\beta1$ integrins in skin. Most notable are a near complete loss of BM, a reduction in hemidesmosomes, a severely impaired proliferative compartment in the epidermis, and failure of developing hair follicles to invaginate into the underlying dermis. Surprisingly, however, the spatial and temporal program of terminal differentiation is preserved. Taken together, our results implicate $\alpha\beta1$ integrins in controlling proliferative potential in the epidermis by virtue of their ability to organize and assemble a BM at the DEJ. In this regard, they also are necessary for maintaining hemidesmosomes, but they are not required for maintaining the gene expression program that defines a keratinocyte, nor is their downregulation a trigger to induce terminal differentiation. Finally, our data suggest that $\alpha\beta1$ integrins play a major role in the BM remodeling and keratinocyte migration that is essential for hair follicle morphogenesis.

Materials and Methods

Engineering $\beta1$ Conditional Knockout Mice

The $\beta1$ integrin gene was isolated from mouse RW4 genomic DNA, and a 5-kb BamHI restriction endonuclease fragment was subcloned and used for preparation of the targeting vector. Electroporations of DNAs into RW4 Agouti ES cells were carried out at 270 V, 500 mF in a GenePulser (Bio-Rad Laboratories). ES cells harboring the desired recombinations were injected into mouse C57BL blastocysts, which were then transferred to CD1 mothers. After breeding, heterozygous and homozygous mice were identified by PCR analysis of toe skin DNAs.

Histology and Immunofluorescence

For routine histology, tissues were fixed in Bouin's fixative, processed, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin and examined and photographed using an Axiophot microscope (Carl Zeiss, Inc.). For immunofluorescence, frozen sections of tissues or cells on the glass coverslips were fixed in 4% paraformaldehyde in PBS for 10 min and were subjected to indirect immunostaining (DasGupta and Fuchs, 1999) and analyzed using a confocal microscope LSM 410 (Carl Zeiss, Inc.).

Unless otherwise indicated, primary antibodies were polyclonal and raised in rabbits. Antibodies and dilutions used were: rat monoclonal $\beta1$ (1:100), $\alpha3$ (1:100), rat monoclonal $\alpha4$ (1:50), rat monoclonal $\alpha6$ (1:100) (Chemicon); K1 (1:200), loricrin (1:250), filaggrin (1:2000) (BabCo); laminin (1:200), mouse monoclonal Ki67 (1:100) (Sigma-Aldrich); K17 (1:1000; gift of P. Coulombe, Johns Hopkins University School of Medicine, Baltimore, MD); guinea pig polyclonal K5 (1:300); and Lef1 (1:250). Fluorescence-conjugated secondary antibodies were from Jackson Immuno-Research Laboratories. DAPI was used to stain nuclei.

Ultrastructural Analyses

Skin tissues were processed for conventional electron microscopy by fixing in 2% glutaraldehyde in 0.05 M cacodylate buffer, 2 mM CaCl$_2$, pH 7.4, followed by a second fixation with 1% OsO$_4$ in water for 60 min on ice. Ultrathin sections on copper grids were treated with uranyl acetate and lead citrate and were examined with a Phillips CM120 electron microscope.

Results and Discussion

Generation of Mutant Mice Conditionally Targeted for β1 Gene Inactivation in Skin Epithelium

To conditionally inactivate the β1 gene in skin, we engi-neered the targeting vector to contain loxP sequences flanking the third exon (referred to as floxed), which once removed would produce an early frame shift, translation termination, and quantitative loss of β1 protein. A restriction map of the WT allele, the targeting vector, and the mutated allele is shown in Fig. 1 A. This vector was used to generate a single homologous recombination event in three independently derived RW4 ES clones, and this was confirmed by Southern analysis (Fig. 1 B, 5′ probe).

To delete the floxed neo gene, two ES clones were transiently transfected with a Cre recombinase gene under the control of the cytomegalovirus (CMV) promoter. By Southern analysis, the desired clones lacked the PGK-neo gene but still harbored the floxed exon 3 (Fig. 1 B, exon3
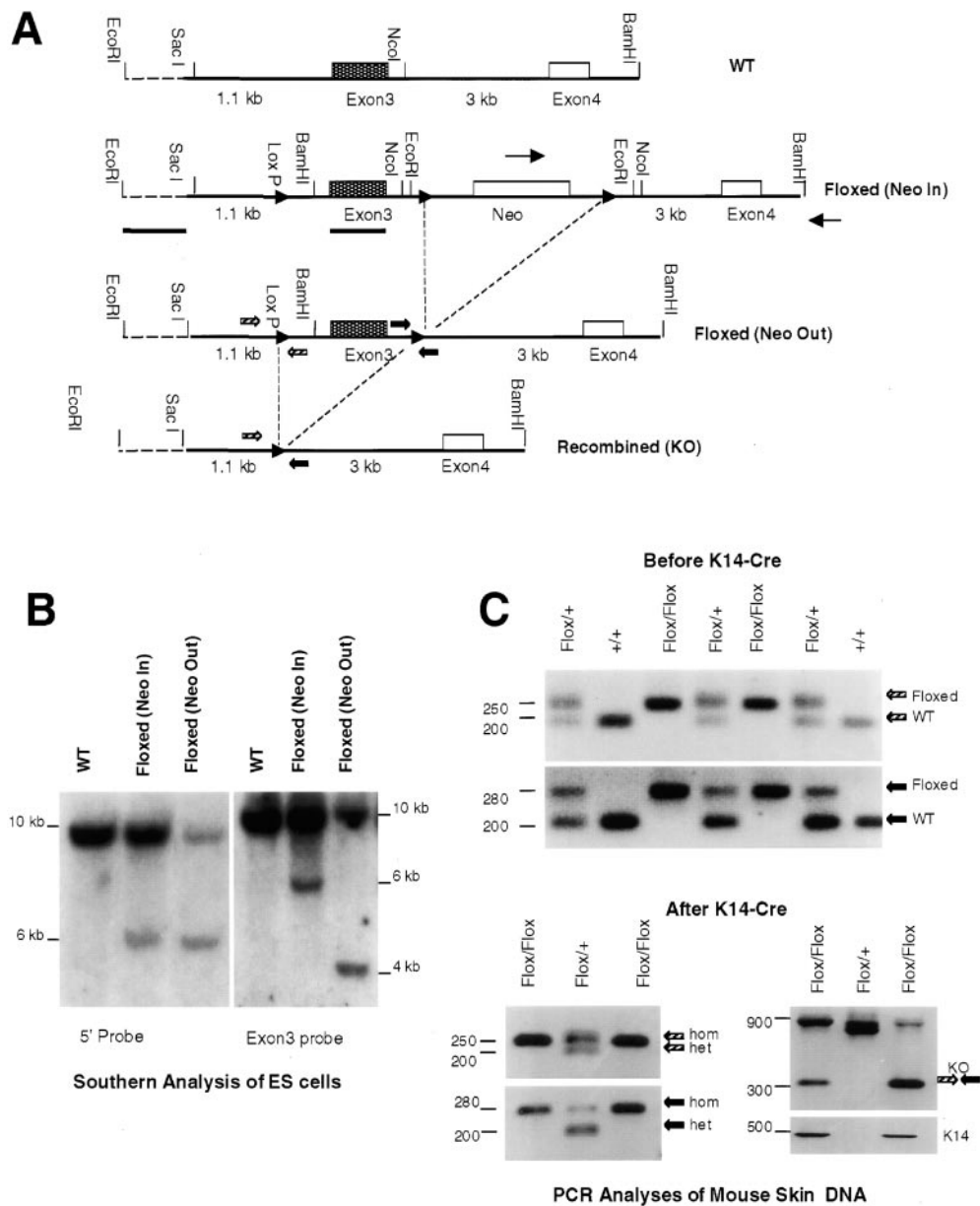

Figure 1. Targeting vector, Southern, and PCR analyses of ES cells and mice conditionally targeted to lack β1 integrin gene expression in skin epithelium. A, Four genomic states of the β1 integrin allele. WT, WT allele; Floxed, allele containing loxP sequences flanking β1's exon 3, either before (Neo In) or after (Neo Out) removal of floxed PGK-Neo by transient expression of ES cells with CMV-Cre. Recombined (KO), the desired recombined allele, lacking exon 3. Triangles, loxP sequences; black bars, sequences used to make the 5′ and exon 3 probes. Matched arrow sets denote oligonucleotides used for various PCR screens. B, Southern analysis. Shown are data for one of three independently derived ES clones that gave identical restriction patterns. Genomic DNAs were digested with BamHI restriction endonuclease, and fragments were separated by agarose gel electrophoresis. After transfer, DNA blots were hybridized with radiolabeled mβ1 genomic probes to sequences either 5′ from those in the targeting vector or within exon 3. C, PCR analyses. Either before or after breeding with K14-Cre mice, skin DNAs from toes of homozygous β1-floxed animals were subjected to PCR using oligonucleotide primers indicated by the short arrows (A). PCR fragments were re-solved by agarose gel electrophoresis and visualized by ethidium bromide staining. Matched primer sets are denoted by single arrow at right, with the genomic identity of the band (WT or Floxed, Neo Out); mixed primer sets are denoted by double arrow. K14, primer set specific for the K14-Cre recombinase transgene; hom, homozygous; het, heterozygous. Sizes of PCR fragments are indicated in base-pairs.

probe). ES technology was then employed to produce germ-line homozygous mice, and PCR analyses were used to verify that the targeting of both β1 alleles had been successful (Fig. 1 C, before K14-Cre).

The homozygous floxed animals appeared normal, indicating that the genomic manipulations had not interfered with β1 function. We then bred the mice to generate newborn animals that were both transgenic for K14-Cre and homozygous for the floxed β1 exon. PCR confirmed the successful conditional removal of exon 3 in skin and the presence of the K14-Cre transgene (Fig. 1 C, after K14-Cre). Previously, we documented the near absolute efficiency and specificity of K14-Cre–mediated recombination in skin epithelial stem cells and their progeny (Vasioukhin et al., 1999). The activity of the K14 promoter is strongly upregulated at embryonic day 14.5 (E14.5), and by birth, nearly all skin epidermal and hair follicle cells score positive for the targeted recombination event when mated to

our highest expressing animals. We have previously verified this with conditional knockouts that either activate a foreign gene (β-galactosidase) or inactivate a gene (α-E-catenin) expressed in all transcriptionally active epithelial cells of the skin (Vasioukhin et al., 1999).

Conditional β1 Null Mice Display Extremely Severe Skin Blistering

The phenotype of newborn conditional β1 null (KO) mice was unmistakable. These animals displayed thin and fragile skin, leading to separation at the DEJ upon mechanical trauma (Fig. 2 A). However, in contrast to α6 or β4 null mice, which exhibited extensive epidermal denuding (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996), β1 mutant newborns only rarely displayed denuding, and this was upon rigorous physical exertion (example shown in Fig. 2 A). Thus, adhesiveness

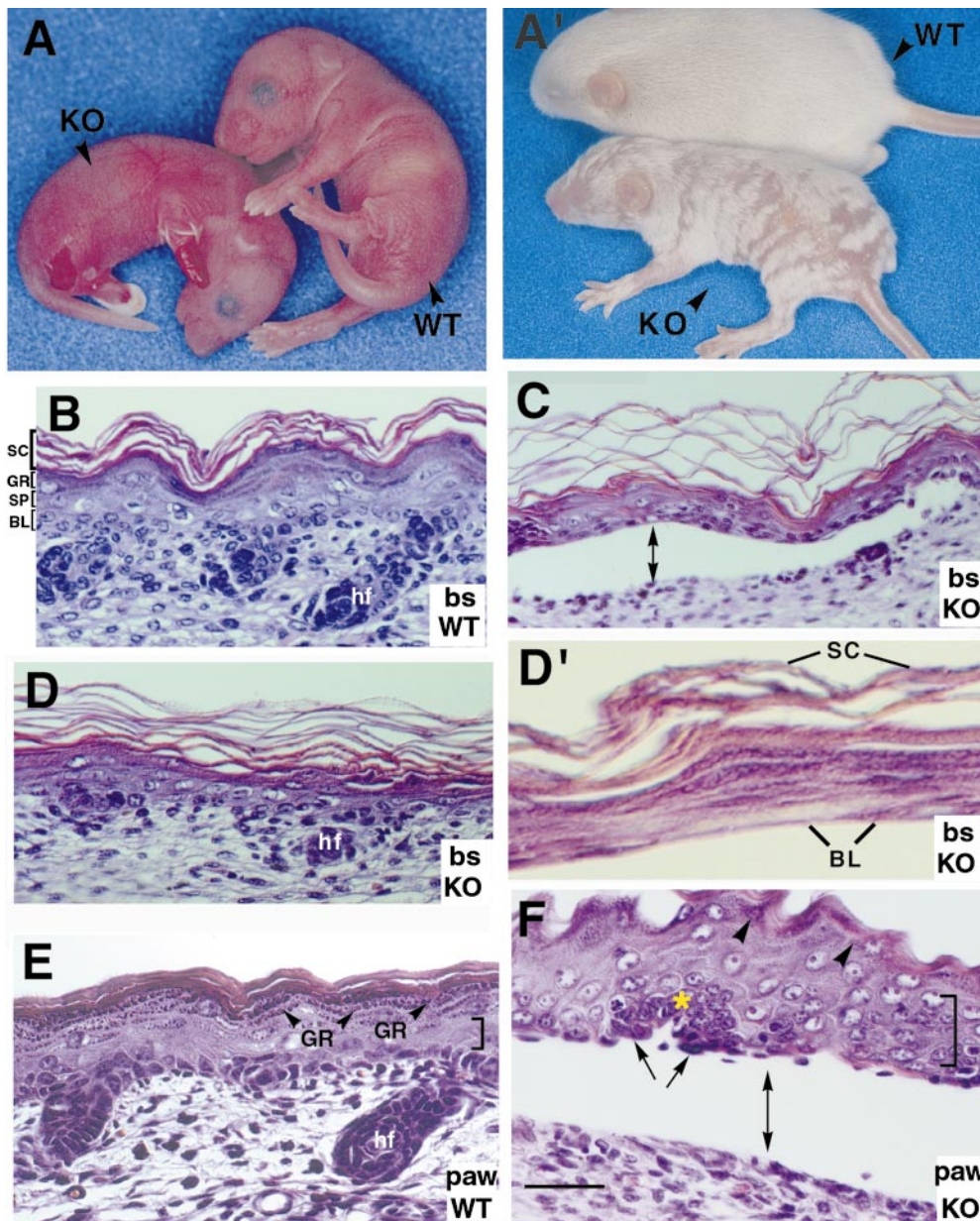*Figure 2.* Visible and histological abnormalities in β1 null skin. A, Complete β1 null (KO) and control (WT) mouse from newborn (nb) litter. A′, Juvenile littermates from a mass producing KO animals expressing lower Cre levels, leading to mosaic β1 mutant skin in the first 2 wk after birth. Note bald areas of skin, reflecting the β1 null state. B–F, Skin sections stained with hematoxylin and eosin. bs, Back skin; hf, hair follicle; paw, paw skin. Double arrows denote separation between epidermis and dermis. D′ shows a severely affected region of bs with highly flattened basal cells. Asterisk in F denotes a pocket of cells likely to be a hair follicle that failed to invaginate; arrowheads in E and F denote keratohyalin granules typical of GR cells. Compare SP layers (brackets in E and F), where an increase in number of cells exists in KO skin. Bar: (B, C, and D) 120 μm; (E and F) 75 μm; (D′) 48 μm.

was not as perturbed by the loss of β1 as it was by β4 ablation.

β1 null animals usually died within a few hours after birth, most likely due to a loss of the epidermal barrier required to prevent dehydration and death (Segre et al., 1999). A few animals survived longer, but these came from breedings involving mice with lower Cre levels, delaying the quantitative loss of β1 and leading to intermediate mosaic stages in postnatal animals. The appearance of these mice revealed a marked defect in body hairs (Fig. 2 A′). Below, we provide additional evidence to demonstrate that skin mosaic for β1 null cells is clearly not normal, in contrast to a previously held notion (Bagutti et al., 1996).

Conditional β1 Null Skin Displays Separations at the DEJ, Changes in Epidermal Morphology, and Impairment of Hair Follicle Downgrowth

Sections of newborn backskin (bs) of conditional β1 null animals revealed gross abnormalities in the epithelium. In WT bs, epidermis consists of four morphologically distinct stages of differentiation, with a mitotically active basal layer (BL) of columnar keratinocytes, three to four spinous (SP) layers, and one to two granular (GR) layers of transcriptionally active but terminally differentiating cells, and a stratum corneum (SC) of dead, enucleated squames (Fig. 2 B). In contrast, β1 null epidermis was thinner, consisting of a flattened BL and only one or two layers of suprabasal layers before the SC (Fig. 2, C, D, and D′). Due in part to the altered morphology of the BL, it was often difficult to discern the boundary between β1 null epidermis and dermis.

Many areas of β1 null skin showed extensive separations at the DEJ (Fig. 2 C, double arrow). In severely affected areas, sections of thin, flat epidermis detached entirely from dermis (Fig. 2 D′). In contrast, no morphological abnormalities were reported in the epidermis of α3 null skin (DiPersio et al., 1997; Hodivala-Dilke et al., 1998).

In animals where β1 null bs was very thin (e.g., Fig. 2 D′), thickness differences were not as pronounced between WT and β1 null paw skin (Fig. 2, E and F; dorsal surface shown). However, morphological perturbations were still prevalent and included an increase in the density of cells within the SP layers (brackets). As the surface area of β1 null pawskin appeared to be normal and because mitotic indexes were not detected suprabasally, we surmise that the suprabasal β1 null cells may not be as metabolically active as their WT counterparts, leading to a reduction in cell size and increase in cell density.

On the dorsal (haired) side of β1 null paw, follicles did not extend into the dermis. Instead, epithelial masses were seen within the epidermis at comparable spacing to hair follicles and with morphology similar to the early stages of this process (Fig. 2 F, single arrows and asterisk). Later, we provide further evidence to support this notion. Mutant bs also exhibited a paucity of developing hair follicles relative to WT littermate skin (Fig. 2, compare frame B with C, D, and D′). A few mature hair follicles were detected, and these most likely represented guard hairs, which develop first during embryonic development (not shown). Although these severely affected mice did not survive to develop a hair coat, a near quantitative absence of hair coat would have been predicted based upon this morphology. This could be seen in juvenile animals expressing lower Cre, where their mosaic β1 null skin resulted in alternating stripes of bald skin (Fig. 2 A′).

Loss of BM at the DEJ and Abnormal Distribution of Integrins in Conditional β1 Null Epidermis

The gross morphological abnormalities at the DEJ of β1 null epidermis were also reflected at the immunofluorescence level (Fig. 3). As expected from the activity of our high-expressing K14-Cre recombinase mice (see above), antibodies against β1 showed no staining in the newborn epidermis of mutant skin but exhibited normal staining in the underlying dermis (Fig. 3 A and A′, WT; and Fig. 3 B, B′, and B″, KO).

In normal skin, the BM at the DEJ stains as a continuous line with a variety of antibodies, including laminins, collagen IV, and fibronectin (Fig. 3; examples shown are laminin 5 in A and a panel laminin in A′). In β1 null skin, however, all of these antibodies showed highly discontinuous staining patterns, often with long stretches of little or no laminin 5 staining at the DEJ (Fig. 3, B and B′). Remarkably, as judged by immunofluorescence, the majority of BM proteins resided in the upper dermis rather than the BM (Fig. 3 B). In the few regions where anti-ECM labeling was detected near or at the DEJ, blistering and splits were often seen, with staining on both sides of the split (Fig. 3 B″, double arrow).

Consistent with the well established staining patterns for epidermal integrin antibodies, β1 and its partners localized at the perimeter of WT basal cells (Fig. 3, A, A′, and C; shown are β1 and α3). As expected, whereas α3 was restricted to the epidermal BL, β1 was present in dermal fibroblasts (Fig. 3, A and A′). In addition, antibodies against β4 and α6 stained only the base of WT basal epidermal cells, i.e., at the DEJ (Fig. 3, C and E, respectively).

Remarkably, the patterns of all integrins were markedly perturbed in β1 mutant skin. It was already known from in vitro studies (for example see Bagutti et al., 1996) that when β1 is absent, its α partners are unstable, and hence we both anticipated and observed an absence of staining with antibodies against α3 (Fig. 3 D). However, given that α6β4 is localized to hemidesmosomes whereas α1β1 integrins are not, we were very surprised to see that in many regions of β1 null epidermis, anti-β4 and anti-α6 staining was weak and/or discontinuous (Fig. 3, D and F, arrowheads). The loss of α6β4 was seen irrespective of whether separations were visible at the DEJ, and in most severely affected β1 null newborn mice analyzed, only a few skin regions could be found where antibody staining appeared normal (see left sides of Fig. 3, D and F). Thus far, we have not found evidence for induction of potential compensatory integrins when β1 is ablated in the skin.

Perturbations in laminin 5, α6, and β4 antibody stainings were not seen in α3 KO skin (DiPersio et al., 1997), leading us to wonder how these perturbations might arise upon β1 ablation and what might be the underlying explanation for the few areas of seemingly normal patterns in epidermis that were absent for anti-β1 staining. To gain further insights into this issue, we examined the skin from a 4-d-old,

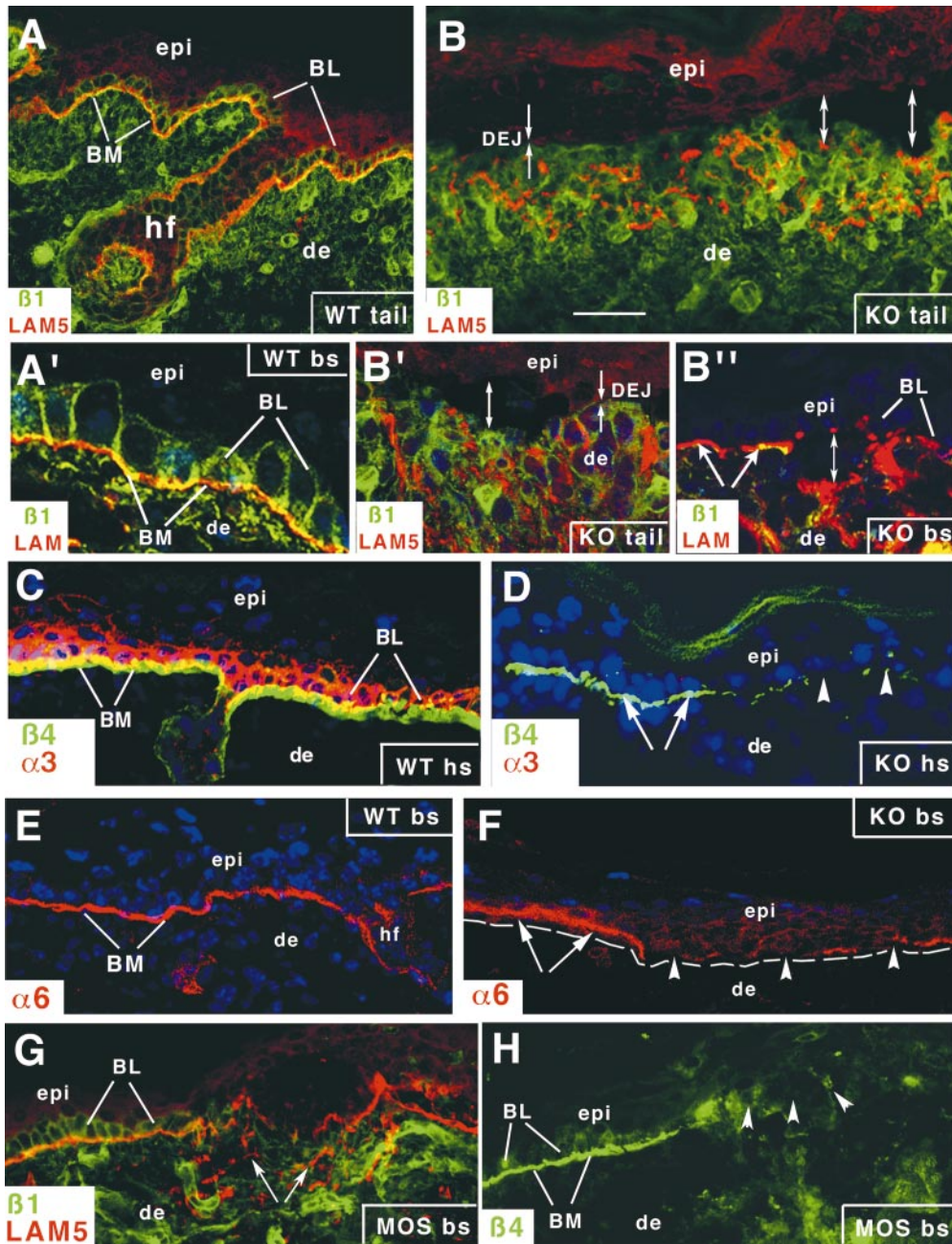*Figure 3.* Loss of BM and other integrins in β1 integrin null epidermis. A–F, Frozen sections of WT and KO skins (hs, headskin; tail, tailskin) from neonatal animals were processed for indirect immunofluorescence and visualized by confocal microscopy. Primary antibodies were against proteins indicated at lower left of frames, color coded according to FITC or Texas Red secondary antibodies; some sections show DAPI-stained nuclei in blue. A, B, and B′ are all stained with anti-β1 and antilaminin 5 (LAM5); A′ and B″ are stained with panel antilaminin antibody (LAM), which also stains blood vessels in dermis. Skin in B′ is shown at higher magnification to illustrate that the LAM5 labeling is in the dermis rather than the DEJ of β1 null skin. Collagen IV and fibronectin antibodies also gave staining analogous to LAM5 (not shown). Note complete absence of anti-β1 staining in BL of β1 null epidermis (epi) but not in fibroblasts in the dermis (de). B″ shows a rare region of LAM staining still at the DEJ (arrows), with a microblister where LAM has partitioned to both sides (double arrow). Similar regions of intact DEJ can be seen in D and H at left (arrows), with discontinuous labeling denoted by arrowheads; these skin sections were stained with antiintegrin antibodies as indicated. Hatched white lines are just beneath DEJ. G and H, Frozen sections of 4-d-old, low Cre–expressing, floxed β1 mutant mouse skin depicting mosaic epidermis that is positive for β1 on left and null for β1 on right. Note correlation between loss of anti-β1 staining and perturbations in BM and β4. Bar: (A–H) 100 μm; (A′, B′, and B″) 30 μm.

β1-floxed mouse expressing lower levels of Cre, and therefore still mosaic, exhibiting some regions still positive for β1 interspersed with areas that were β1 null. As shown in the serial sections of Fig. 3, G and H, the areas of epidermis that still expressed β1 (left sides of Fig. 3, G and H) showed normal laminin 5, β1, and β4 staining, and in many areas where β1 expression was no longer detected, the laminin 5 and β4 staining patterns were perturbed (centers of Fig. 3, G and H). However, in these mice, it was easier to find stretches where ECM and/or α6β4 staining were still localized to the DEJ, even though β1 was absent (for example, see area at right of Fig. 3 G). Taken together,

these findings suggest that at early times after the β1 integrin gene is mutated, the BM and hemidesmosomes remain intact, but soon afterwards, BM assembly is compromised, leading to a loss of ECM and hemidesmosomes at the DEJ.

Previous in vitro studies have indicated a role for α5β1 integrins in the assembly of fibronectin (Wennerberg et al., 1996), and a role for α3β1 in laminin 5 assembly has been postulated based upon the occasional areas of BM perturbations and microblistering seen in the α3 knockout mouse (DiPersio et al., 1997; Hodivala-Dilke et al., 1998). Our data provide the strongest functional evidence to date
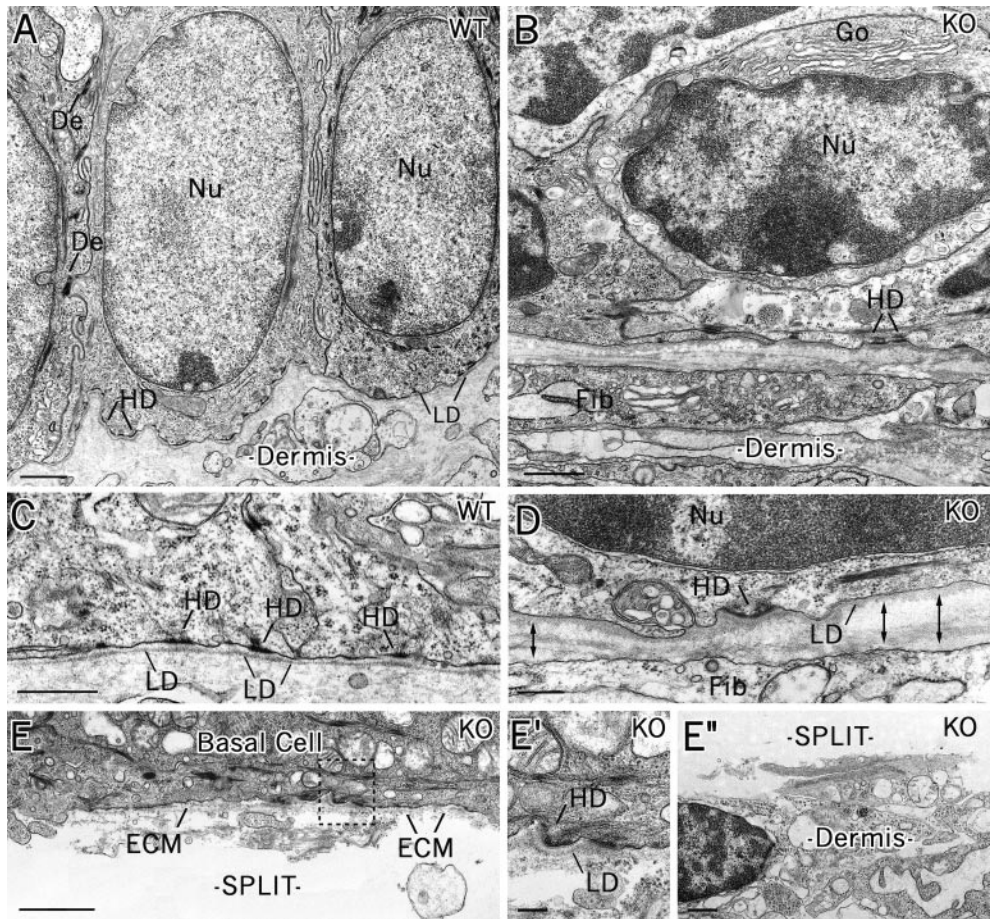
Figure 4. Ultrastructural analysis reveals gross disorganization of the BM and reductions in hemidesmosomes. bs samples, processed for electron microscopy, were from WT or conditionally β1 null (KO) 4-d-old mice as indicated. Regions are from epidermal–dermal border. Nu, nuclei of basal epidermal cells; BM, BM composed of lamina lucida, contiguous with the base of the epidermis, and LD an electron dense line of highly organized ECM; HD, hemidesmosome; De, desmosome; Go, golgi; Fib, dermal fibroblast; double arrows, separation within the LD. E, Blistered area, with epidermis in E and underlying dermis in E''. Hatched box in E is enlarged and shown as E'. All bars represent 1 μm, except E' which is 200 nm.

in support of these earlier predictions and further suggest essential roles for all of the epidermal αβ1 integrins, not only in ECM assembly/organization but also in assembly of the BM. Thus, in striking contrast to α3 null skin, where most of the laminin 5 still resided at an intact BM (DiPersio et al., 1997; Hodivala-Dilke et al., 1998), BM assembly was severely compromised in the β1 null epidermis, and laminin 5 diffused into the dermis. The differences in laminin 5 localization in the β1 conditional and the α3 knockouts lead us to wonder whether one of the minor αβ1 integrins also participates in laminin 5 assembly or whether properly assembled collagen and fibronectin provide a BM scaffold to support laminin 5 and retain it at the DEJ.

Marked Ultrastructural Differences Between β1 Integrin Null and WT Epidermis

To gain further insights into the perturbations we have described thus far, we performed ultrastructural analyses on skin of WT littermate and conditional β1 null mice (Fig. 4). WT basal cells exhibit a classical columnar morphology, with nuclei oriented perpendicularly to the BM (Fig. 4 A). At the mesenchymal–epithelial junction of WT skin is a BM, composed of a lamina lucida at the base of the epidermis, and a parallel lamina densa (LD) of ECM beneath it (Fig. 4, A and C). Contiguous with the lamina lucida are numerous electron dense hemidesmosomal plaques. Tiny anchoring filaments, in part composed of

collagen XVII, extend from the base of each hemidesmosome to the LD (Pas et al., 1999).

In β1 null epidermis of a mouse that survived to four days, most basal cells were flat, and the nucleus was oriented parallel to the BM (Fig. 4 B). In regions where β1 null epidermis was attached to its underlying BM, hemidesmosomes were present (Fig. 4, B and D). Although their morphology was indistinguishable from control skin, these structures were markedly reduced in number, and often long stretches along the base of each basal cell lacked discernible hemidesmosomes. Another marked difference between WT and KO skin was the discontinuity of the electron dense LD, which existed beneath the hemidesmosomes but not in most areas between hemidesmosomes. In many areas, neither hemidesmosomes nor LD were detected. In these regions, what appeared to be disorganized remnants of ECM dangled from the underlying surface of the basal epidermal layer (Fig. 4 D, double arrows). These long stretches of disorganized BM were typical of conditional β1 null animals that survived for several days (examples shown), but in more severely affected animals, even traces of ECM material at the DEJ were rare (not shown).

In β1 null animals that still showed some skin areas with traces of BM, ECM material, and hemidesmosomes at the DEJ, it was evident that where the DEJ separated, splits occurred within the disorganized BM (Fig. 4 E). This left fragments of ECM at both the base of the epidermis and

the upper surface of the dermis. In addition, the hemidesmosomes often contained an intact LD beneath them, even though elsewhere LD was not detected (Fig. 4 E′). We surmise that through the ability of hemidesmosomes to anchor to and maintain organized BM through their collagen XVII anchoring fibers, these areas of LD are the last to survive in the β1 null epidermis.

The dramatic reduction in hemidesmosomes, not seen in the α3 knockout but a prominent feature of the β1 null epidermis, suggests strongly that α6β4 relies upon αβ1 integrins for BM assembly, and without a BM, they are unable to assemble into stable hemidesmosomes. Although the progressive loss of hemidesmosomes and α6β4 antibody staining would seem to favor this hypothesis, we cannot exclude the possibility that hemidesmosome assembly and/or stability might be governed by an αβ1-regulated intracellular signaling pathway. In support of this view is the finding that in vitro hemidesmosomes can be formed upon adhesion of α6β4-expressing cells to fibronectin, the receptor for α5β1 (Nievers et al., 1999). Irrespective of mechanism, however, our results reveal a functional interdependency between αβ1 and αβ4 integrins.

Terminal Differentiation Is Spatially and Temporally Maintained in β1 Null Epidermis, but Proliferation Is Impaired

Epidermal cells downregulate integrin expression as they detach from the BM, terminally differentiate, and move outward towards the skin surface. Based upon a number of gene transfection and keratinocyte suspension studies, investigators have postulated that downregulation of β1 integrin expression may be a trigger for inducing terminal differentiation (Watt et al., 1993; Hotchin et al., 1995; Levy et al., 2000). On the basis of these in vitro studies, we anticipated that loss of β1 in the epidermis should induce premature differentiation in the BL. This did not happen.

In normal epidermis, an early hallmark of terminal differentiation is the switch from expression of keratins 5 and 14 to keratins 1 and 10 (Fig. 5 A; Fuchs, 1999). This mutually exclusive expression pattern was faithfully maintained in β1 null epidermis, irrespective of the severity of the morphological aberrations (Fig. 5 B). We did note atypical pockets of K5/K14-positive cells (asterisks) in β1 null skin regions where late follicle morphogenesis is characteristic of the WT counterpart (example shown). However, these pockets did not label with antibodies against the epidermal differentiation markers K1, K10, or involucrin, and they very likely represent developing hair follicles that failed to invaginate (see below). Moreover, even in the thinnest regions of epidermis found in β1 null skin, K5/K14 was expressed in the BL and K1/K10 was not (Fig. 5 B, inset).

Expression of late stage markers of epidermal differentiation, including loricrin and filaggrin, were also faithful in their gene expression patterns (Fig. 5, C–F), despite the fact that some differences in epidermal morphology had been noted in the upper skin layers (Fig. 2). Finally, cornified envelopes, an end-stage product of terminal differentiation, appeared morphologically normal when isolated from β1 null skin and examined under the light microscope (data not shown; for procedures see Segre et al.,

1999). Thus, despite the loss of β1 and its associated α partners, basal epidermal cells still maintained their basal-like properties and refrained from premature execution of the program of terminal differentiation. This result argues against the long-standing hypothesis that downregulation of β1 integrin is a molecular trigger of terminal differentiation (Levy et al., 2000). The result, however, is consistent with the findings of Stephens et al. (1993), who previously reported that in F9 teratocarcinoma cells lacking β1 integrin, cell type–specific programs of gene expression were not perturbed.

Another hypothesis regarding the possible function of αβ1 integrins is that they control proliferation in epidermal cells (for review see Watt et al., 1993). This notion is based upon the findings that (a) cultured keratinocytes with the highest proliferative potential are those that possess the highest levels of surface β1 integrins (Jones et al., 1995), (b) mitogen-activated kinase levels are suppressed when keratinocytes are transfected with dominant negative forms of β1 integrin (Zhu et al., 1999), and (c) transgenic mice expressing suprabasal β1 integrin exhibit signs of epidermal hyperproliferation (Carroll et al., 1995). Although this is intriguing, a critical role has also been proposed for α6β4 in regulating epidermal proliferation (Mainiero et al., 1996; Li et al., 1998; Murgia et al., 1998).

In the most severely affected conditional β1 null mice, few mitoses were detected in the basal epidermal layer. In contrast, ∼5% of basal cells in littermate skin displayed mitotic figures. Antibodies against Ki67, a proliferating nuclear antigen present throughout the cell cycle, permitted more rigorous examination of proliferation within the newborn epidermis. The majority of WT basal cells were Ki67 positive, and labeled cells were especially abundant in hair follicles (Fig. 5 G). In contrast, very few Ki67-positive cells were found in β1 null newborn epidermis. Fig. 5 H illustrates a region where at least some basal cells were labeled, but most stretches of epidermis were entirely negative. Taken together, these findings demonstrate that although terminal differentiation is spatially and temporally defined in β1 null epidermis, the proliferative potential is markedly reduced.

Although we cannot rule out a participatory role for α6β4 in this process, the marked inhibition of proliferation seen in β1 null epidermis was not seen in β4 null skin (Dowling et al., 1996; van der Neut et al., 1996). Conversely, whereas we detected clear ultrastructural signs of apoptosis in the BL of β4 null skin (Dowling et al., 1996), we did not see this in the sections examined from β1 null epidermis. Thus, our data support the view that distinctions may exist between the signal transducing capacities of the two β subunits.

As a final evaluation of the early consequences of β1 ablation, we examined skin from a 4-d-surviving animal that expressed lower Cre levels and thus was still undergoing homologous recombination in some basal epidermal cells (same skin as that analyzed in Fig. 3, G and H). In this skin, many areas could be found where β1 expression had been ablated, but laminin 5 and hemidesmosomal markers still localized to the DEJ. Since rupturing at the BM was the earliest sign that β1 had been ablated, we focused on one of these areas (Fig. 5; serial sections shown in I–K). Ki67-positive cells were fewer in number but still found
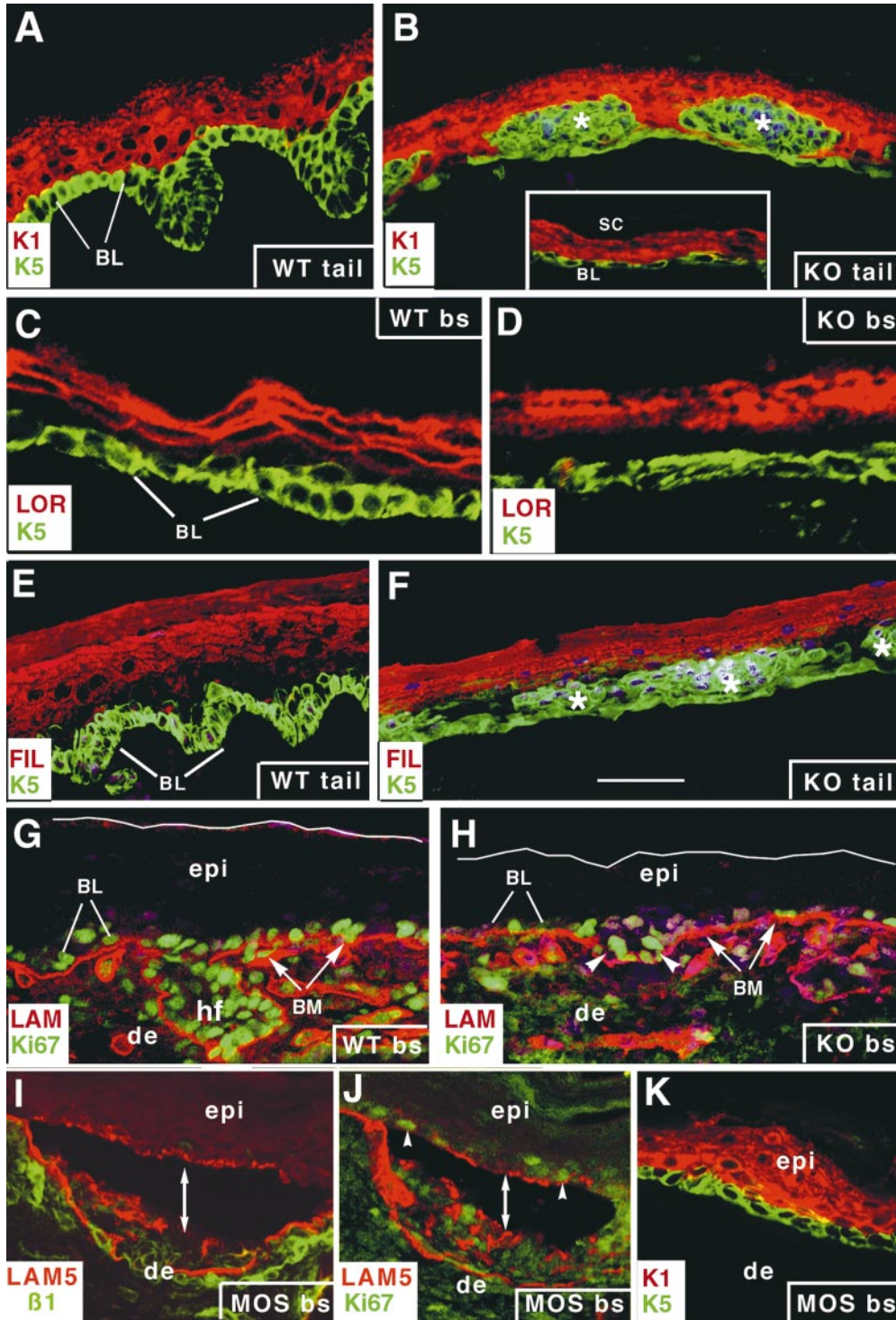
Figure 5. Spatially and temporally regulated terminal differentiation but reduced proliferation in β1 integrin null epidermis. A–H, Frozen sections of WT and KO newborn skins (tail or back) were processed for indirect immunofluorescence and visualized by confocal microscopy. Note: in tail, the last wave of hair follicle morphogenesis occurs relatively late and exists in newborn skin; asterisks denote atypical masses of epithelial cells, likely to be hair follicles that failed to invaginate. Inset to B denotes severely affected bs, with very thin epidermis, and yet the flat basal cells are still K5 positive and K1 negative. See Fig. 2 D for hematoxylin and eosin stain. White line denotes skin surface. I–K, Serial sections of 4-d-old, β1-floxed mouse in which the process of Cre-mediated homologous recombination was still ongoing. Note that even in regions where β1 was recently ablated and Ki67-positive cells were still seen, early markers of terminal differentiation had not been induced in the β1 null basal cells. Note: the serial section in K shows epidermis immediately over the DEJ split, as in I and J, but in this case, the split is not evident, because the K1/K5 antibodies are restricted to the epidermis. Primary antibodies were against proteins indicated in lower left. Fil, filaggrin; LOR, loricrin; LAM5, laminin 5; epi, epidermis; de, dermis. Bar: (A, B, and E–K) 80 μm; (C and D) 50 μm.

within these areas (Fig. 5 J). However, no basal cell was found that was positive for K1 or any other terminal differentiation marker that we examined, and no basal cell was found that was negative for K5 or K14 (Fig. 5 K). Thus, despite the clear loss of β1, in a skin area where this was a very recent event, some cell proliferation still existed and terminal differentiation was not induced in the BL. Therefore, whether we examined skin at early or late times after β1 ablation, our results did not support the notion that downregulation of β1 is the trigger for terminal

differentiation in vivo. Rather, the results suggest that keratinocyte culture may not always be reliable as a model system for studying the roles of integrins in controlling the balance between epidermal proliferation and terminal differentiation.

Hair Follicle Invagination and Differentiation Is Impaired in Conditional β1 Null Skin
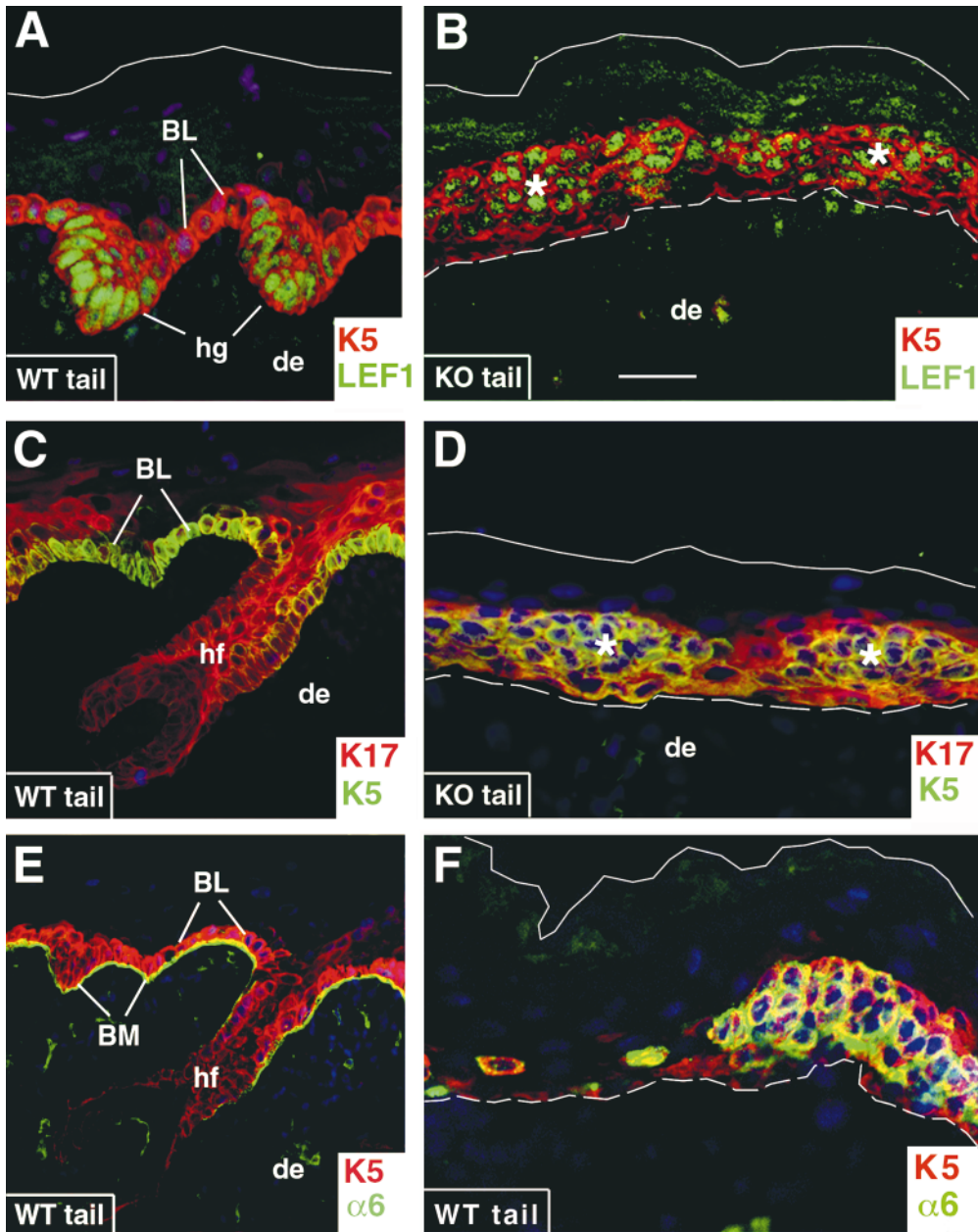
The striking perturbations of the hair coat of β1-floxed an-

Figure 6. The K5-positive epithelial masses in β1 null epidermis are positive for hair germ markers. Frozen sections of littermate (WT) and knockout (KO) skins were processed for indirect immunofluorescence and visualized by confocal microscopy. Primary antibodies were against proteins indicated at lower right. Asterisks in KO samples denote K5-positive epithelial masses not seen in WT controls; hf and hg denote hair follicles (hf) or hair germs (hg) in WT samples, not seen in KO skin. de, dermis. White line, upper epidermal border; white hatched lines are just beneath DEJ. Bar: (A, B, D, and F) 60 μm; (C and E) 100 μm.

imals expressing low Cre recombinase levels and the paucity of hair follicles in the dermis of conditional β1 null skin led us to suspect that the epithelial masses represented hair germs that failed to invaginate into the underlying dermis. We therefore examined the β1 null skin for markers of hair follicle differentiation.

In WT skin, the leading front of developing hair follicles express nuclear Lef1, a DNA binding protein that collaborates with stabilized β-catenin to activate downstream target genes (Fig. 6 A; see also DasGupta and Fuchs, 1999). In β1 null skin, nuclear Lef1 was significantly more prevalent and appeared throughout the epithelial masses that were K5 positive (Fig. 6 B). Another prominent marker of developing hair follicles is K17, also expressed in embryonic epidermis (McGowan and Coulombe, 1998). By birth, K17 is largely restricted to hair follicles or to a few patches of differentiating epidermal layers (Fig. 6 C), and thereaf-

ter, only follicle staining is seen (McGowan and Coulombe, 1998). In β1 null skin, the K5- and Lef1-positive masses stained with anti-K17 (Fig. 6 D). In contrast, these masses did not stain with antibodies against terminally differentiated hair-specific markers, which had begun to appear in the developing hair shafts of WT follicles (not shown). Finally and unexpectedly, the epithelial masses in β1 null epidermis stained with antibodies against β4 and α6, even though regions flanking these masses showed little or no labeling (Fig. 6 F; compare with WT in Fig. 6 E). Currently, we have no molecular understanding for why these cells express α6β4; however, it may explain why these regions display more Ki67-positive cells and a more organized BM than other areas of β1 null epithelium.

Little is known about the molecular mechanisms underlying the ability of developing hair follicles to locally remodel their ECM and invaginate into underlying mes-

enchyme. Our findings suggest a critical role for $\alpha\beta1$ integrins in this process, a feature hitherto unrecognized. This is in agreement with Stephens et al. (1995), who reported that $\beta1$ null embryos appear unable to invade properly into the uterine epithelium and decidua (Stephens et al., 1995; Fassler and Meyer, 1995). How the hair follicle utilizes $\alpha\beta1$ integrins to remodel its BM and grow downward remains an intriguing issue that awaits further exploration.

In summary, the conditional ablation of the $\beta1$ integrin gene in mouse epidermis has provided major new insights into the functions of $\alpha\beta1$ integrins and into the differential roles of $\beta1$ and $\beta4$ integrins in the skin. Our findings argue against an essential role for $\alpha\beta1$ integrins in regulating the spatial and temporal program of epidermal terminal differentiation, a function predicted from keratinocyte culture studies conducted with mutant $\beta1$ transgenes (Watt et al., 1993; Hotchin et al., 1995; Levy et al., 2000). In contrast, our results underscore a critical role for $\beta1$ in maintaining proliferative potential in developing skin epithelium and provide compelling support for previous in vitro studies correlating proliferative potential with surface levels of $\beta1$ integrins in keratinocytes (Jones et al., 1995). Perhaps most interesting in this regard is that our findings reveal that the underlying reason for this long-standing observation is likely the unique ability of $\alpha\beta1$ integrins to assemble BM. This insight was not obtained from the $\alpha3$ knockout, where BM was largely intact and proliferation unaffected, although some clues to potential roles for $\alpha\beta1$ integrins in BM assembly have emerged from studies on other tissues (Sasaki et al., 1998). The dramatic defect in the BM, not seen yet for any other epidermal integrin knockout, enabled us to uncover an unanticipated new role for $\alpha\beta1$ integrins in hemidesmosome assembly/stabilization and in hair follicle invagination into the underlying dermis. The challenge that faces us now will be to dissect the molecular pathways used by $\alpha\beta1$ integrins in orchestrating these events.

Note Added in Proof. A related paper was recently published by Fässler and colleagues (Brakebusch, C., R. Grose, F. Quondamatteo, A. Ramirez, J.L. Jorcano, A. Pirro, M. Svensson, R. Herken, T. Sasaki, R. Timpl, S. Werner, and R. Fässler. 2000. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:3990–4003).

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