

## DEVELOPMENT AND DISEASE

# A crucial role of $\beta 1$ integrins for keratinocyte migration in vitro and during cutaneous wound repair

Richard Grose<sup>1,\*†</sup>, Caroline Hutter<sup>2,†</sup>, Wilhelm Bloch<sup>3</sup>, Irmgard Thorey<sup>1</sup>, Fiona M. Watt<sup>2</sup>, Reinhard Fässler<sup>4,5</sup>, Cord Brakebusch<sup>4</sup> and Sabine Werner<sup>1,‡</sup>

<sup>1</sup>Institute of Cell Biology, Department of Biology, ETH-Zürich, 8093 Zürich, Switzerland

<sup>2</sup>Keratinocyte Laboratory, Cancer Research UK London Research Institute, London WC2A 3PX, UK

<sup>3</sup>Institute of Anatomy, University of Cologne, D-50931 Köln, Germany

<sup>4</sup>Department of Experimental Pathology, Lund University, S-221 85 Lund, Sweden

<sup>5</sup>Max-Planck-Institute of Biochemistry, D-82152 Martinsried, Germany

\*Present address: Viral Carcinogenesis Laboratory, Cancer Research UK, London Research Institute, London WC2A 3PX, UK

†The first two authors have equally contributed to this work

‡Author for correspondence (e-mail: sabine.werner@cell.biol.ethz.ch)

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## SUMMARY

**Integrins are ubiquitous transmembrane receptors that play crucial roles in cell-cell and cell-matrix interactions. In this study, we have determined the effects of the loss of  $\beta 1$  integrins in keratinocytes in vitro and during cutaneous wound repair. Flow cytometry of cultured  $\beta 1$ -deficient keratinocytes confirmed the absence of  $\beta 1$  integrins and showed downregulation of  $\alpha 6\beta 4$  but not of  $\alpha v$  integrins.  $\beta 1$ -null keratinocytes were characterised by poor adhesion to various substrates, by a reduced proliferation rate and by a strongly impaired migratory capacity. In vivo, the loss of  $\beta 1$  integrins in keratinocytes caused a severe defect in wound healing.  $\beta 1$ -null keratinocytes showed impaired migration and were more densely packed in the hyperproliferative epithelium. Surprisingly, their**

**proliferation rate was not reduced in early wounds and even increased in late wounds. The failure in re-epithelialisation resulted in a prolonged inflammatory response, leading to dramatic alterations in the expression of important wound-regulated genes. Ultimately,  $\beta 1$ -deficient epidermis did cover the wound bed, but the epithelial architecture was abnormal. These findings demonstrate a crucial role of  $\beta 1$  integrins in keratinocyte migration and wound re-epithelialisation.**

Movies available on-line

Key words: Integrin, Migration, Epidermis, Mouse, Wound

## INTRODUCTION

Following cutaneous injury, a well-defined cascade of events is kick started, beginning with the plugging of the defect by a fibrin clot and culminating in the restitution of the epithelial barrier and the generation of fibrous scar tissue (reviewed by Martin, 1997). Re-epithelialisation, the process by which keratinocytes at the wound margin seal the epidermal fault, is a key event underpinning skin repair. It is achieved via a combination of proliferation and sustained migration of wound edge keratinocytes across the dermis and provisional wound matrix.

Several genetically modified mouse models have been used to investigate the molecular basis of the re-epithelialisation process. These studies revealed important roles for growth factor signalling, proteases and cell-surface molecules in epithelial repair (reviewed by Grose and Werner, 2002). However, despite a plethora of data on the involvement of integrins in skin

development and cell migration in vitro, there is little information on their in vivo functions during wound repair.

Integrins are heterodimeric transmembrane proteins consisting of an  $\alpha$  and a  $\beta$  subunit that play crucial roles in cell-cell and cell-matrix interactions. They underpin cell adhesion and migration, as well as being involved in cell proliferation, programmed cell death and differentiation (Brakebusch et al., 1997). The major integrins in the intact epidermis are  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$  and  $\alpha 9\beta 1$ , which bind various extracellular matrix proteins, including laminins, collagen I, tenascin C and fibronectin, and  $\alpha 6\beta 4$ , an integral component of hemidesmosomes that binds laminin 5 (Palmer et al., 1993; Yokosaki et al., 1994; Yamada et al., 1996). After wounding, the expression profile of integrins on wound margin keratinocytes changes, characterised by suprabasal integrin expression (Hertle et al., 1992) and induction of specific integrins that recognise proteins of the dermal and provisional matrix. These include the fibronectin receptor  $\alpha 5\beta 1$ ; the fibronectin and vitronectin

receptor  $\alpha v\beta 5$ ; and  $\alpha v\beta 6$ , which binds to fibronectin, vitronectin, tenascin C and transforming growth factor  $\beta$  (TGF $\beta$ ) (Larjava et al., 1993; Zambruno et al., 1995; Clark, 1990; Yamada et al., 1996; Plow et al., 2000).

A series of studies has revealed an essential role of  $\beta 1$  integrins in the regulation of keratinocyte proliferation and differentiation. Most interestingly, the  $\beta 1$  integrin subunit has been found to be a marker for stem cells in vitro and in vivo (Jones and Watt, 1993; Jones et al., 1995). Inhibition of  $\beta 1$  integrin function by introduction of a dominant-negative  $\beta 1$  mutant into keratinocytes reduced the activation of mitogen-activated protein kinase and stimulated exit from the stem cell compartment (Zhu et al., 1999). Furthermore, suprabasal expression of  $\beta 1$  is a feature of the hyperproliferative epidermis of psoriatic skin, and ectopic expression of the  $\beta 1$  integrin subunit in the suprabasal epidermal layers of transgenic mice caused keratinocyte hyperproliferation and a psoriasis-like phenotype (Carroll et al., 1995).

To determine the role of  $\beta 1$  integrins in skin morphogenesis and homeostasis, we and others generated mice that lack the  $\beta 1$  integrin subunit specifically in keratinocytes. The characterisation of the  $\beta 1$ -deficient mice revealed crucial roles for the  $\beta 1$  integrin subfamily for hair follicle development and skin integrity (Brakebusch et al., 2000; Raghavan et al., 2000). In this study, we used our mouse model to investigate cutaneous wound repair, a process that is reliant on both keratinocyte migration and proliferation, in the absence of epidermal  $\beta 1$  integrins. We demonstrate an important role for  $\beta 1$  integrins during wound re-epithelialisation, where  $\beta 1$ -null keratinocytes display a severely compromised epithelial migration.

## MATERIALS AND METHODS

### K5 $\beta 1$ -null mice

Mice that lack the  $\beta 1$  integrin subunit in the epidermis (K5 $\beta 1$ -null mice) have been described elsewhere (Brakebusch et al., 2000). Briefly, mice carrying two floxed  $\beta 1$  alleles were crossed with mice carrying one floxed  $\beta 1$  allele plus one copy of the *cre* recombinase gene under the control of the keratin 5 (K5) promoter (Ramirez et al., 1994). Littermate controls were maintained alongside the knockouts under identical husbandry conditions.

### Isolation and culture of mouse keratinocytes

Murine epidermal keratinocytes were isolated as described (Roper et al., 2001). Keratinocyte cell lines were generated by repeated subculturing at high density (Romero et al., 1999).

### Retroviral infection of keratinocytes

AM12 supernatants or concentrated VSV G-pseudotyped virus was added to subconfluent mouse keratinocyte cultures and incubated for 6–8 hours in the presence of 8  $\mu$ g/ml polybrene. The following retroviral vectors were used: pBabe chicken  $\beta 1$  integrin subunit (Levy et al., 1998), pBabe EGFP $\beta 1$  and LZRS $\beta 1$ BMN EGFP $\beta 1$  (Cre fusion protein and LZRS $\beta 1$ BMN, kind gifts from J. Muller, ICRF).

### Time-lapse recording

Frames were taken every 5 minutes for 22 hours using a Zeiss Axiovert 135 TV microscope fitted with a Hamamatsu CCD Orca ER camera (Hamamatsu, Japan). Motility was measured using a cell tracking extension (ICRF) written for IPLab (Signal Analytics, USA) and speed was calculated using a program written in Mathematica by Daniel Zicha (ICRF).

### FACS analysis

Single cell suspensions of freshly isolated keratinocytes were incubated with a rat antibody against the mouse  $\beta 1$  integrin subunit (MB1.2; 1:2 diluted; kind gift from B. Chan), a rat antibody against the  $\beta 4$  integrin subunit (CD104; 1:100 diluted, BD Pharmingen, Franklin Lakes, NJ) or a hamster antibody against the  $\alpha v$  integrin subunit (1:100 diluted; RDI, Flanders, NJ), and subsequently with AlexaFluor488-conjugated secondary antibody directed against rat IgG or biotinylated antibody directed against hamster IgG (Jackson ImmunoResearch, West Grove, PA), followed by incubation with RPE-Cy5 conjugated streptavidin (Dako, Hamburg, Germany). Immediately before analysis on a Becton-Dickinson FACScan, TO-PRO-3 (Molecular Probes, Leiden, NL) was added to the sample for viability gating.

### Adhesion assays

Keratinocytes isolated from 2-day-old mice were seeded into 96-well plates ( $5 \times 10^4$  cells/well), pre-coated with fibronectin, laminin 1, poly-D-lysine (PDL), collagen type I or collagen type IV (BD Biocoat Cellware, Franklin Lakes, NJ). After overnight incubation, cells were washed with phosphate-buffered saline (PBS) and adhesion was quantified using a CytoTox 96 colorimetric kit (Promega, Madison, WI).

### Wounding and preparation of wound tissue

Mice were anaesthetised by intraperitoneal (i.p.) injection of 100  $\mu$ l ketamine (10 g/l)/xylazine (8 g/l) solution. Two full-thickness excisional wounds, 3 mm in diameter, were made on either side of the dorsal midline by excising skin and panniculus carnosus. Wounds were left uncovered and harvested 1, 2, 3, 5, 10 or 15 days after injury ( $n \geq 4$  mice for each timepoint). For expression analyses, the complete wounds including 2 mm of the epithelial margins were excised and immediately frozen in liquid nitrogen. Non-wounded back skin from 10 days post partum (d.p.p.) and 20 d.p.p. mice served as controls. Alternatively, two full-thickness incisional wounds were made on either side of the dorsal midline, left uncovered and harvested at 3 or 6 days after injury ( $n = 3$  mice for each time point). For histological analysis, the complete excisional or incisional wounds were isolated, bisected, fixed overnight in 4% paraformaldehyde in PBS and embedded in paraffin wax. Sections (7  $\mu$ m) from the middle of the wound were stained with Haematoxylin/Eosin (H/E) or Masson trichrome. All experiments with animals were carried out with permission from the local veterinary authorities.

### Labelling with 5'BrdU

BrdU labelling was performed as described (Werner et al., 1994). Sections (7  $\mu$ m) from the middle of the wound were incubated with a peroxidase-conjugated monoclonal antibody directed against BrdU (Roche Diagnostics, Rotkreuz, Switzerland) and stained with a diaminobenzidine-peroxidase substrate kit (Vector Laboratories, Burlingame, CA).

### Immunofluorescence

To stain keratinocyte cultures for paxillin, cells were fixed with 4% paraformaldehyde in PBS containing 0.1% Triton X-100, washed in PBS, blocked in 2% FCS in PBS and incubated with anti-paxillin antibody (1:100 diluted; BD Transduction Laboratories), followed by AlexaFluor488-conjugated anti-rabbit IgG. To stain for filamentous actin, cultures were incubated in a 1:1000 dilution of rhodamine-conjugated phalloidin (Sigma-Aldrich, St Louis, MO). Wax and cryosections (7  $\mu$ m) from the middle of the wound were incubated with antibodies directed against the  $\beta 1$  integrin subunit (a kind gift from Dr Staffan Johansson, Uppsala, Sweden), the  $\beta 4$  integrin subunit,  $\beta$ -catenin and E-cadherin (kind gifts from Dr Rolf Kemler, Freiburg, Germany), caspase 3 (Roche Diagnostics Ltd, Rotkreuz, Switzerland), keratin 6 (Babco, Richmond, CA), laminin 5 ( $\alpha 2$ LE4-6, kind gift from Dr Rupert Timpl, Munich, Germany), and PECAM1 (BD Biosciences, Heidelberg, Germany), followed by Cy2- or Cy3- conjugated secondary

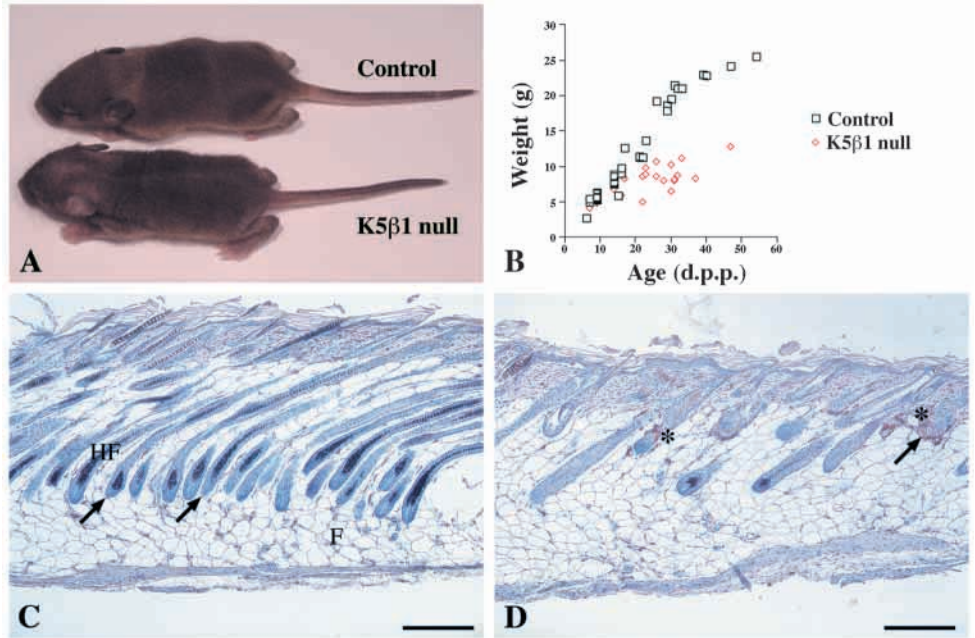
antibodies (Jackson Immunoresearch). Specimens were mounted with Mowiol (Hoechst, Frankfurt, Germany) prior to photographing on a Zeiss Axioplan fluorescence microscope or confocal imaging on a Leica confocal laser scanning microscope.

### Immunohistochemistry

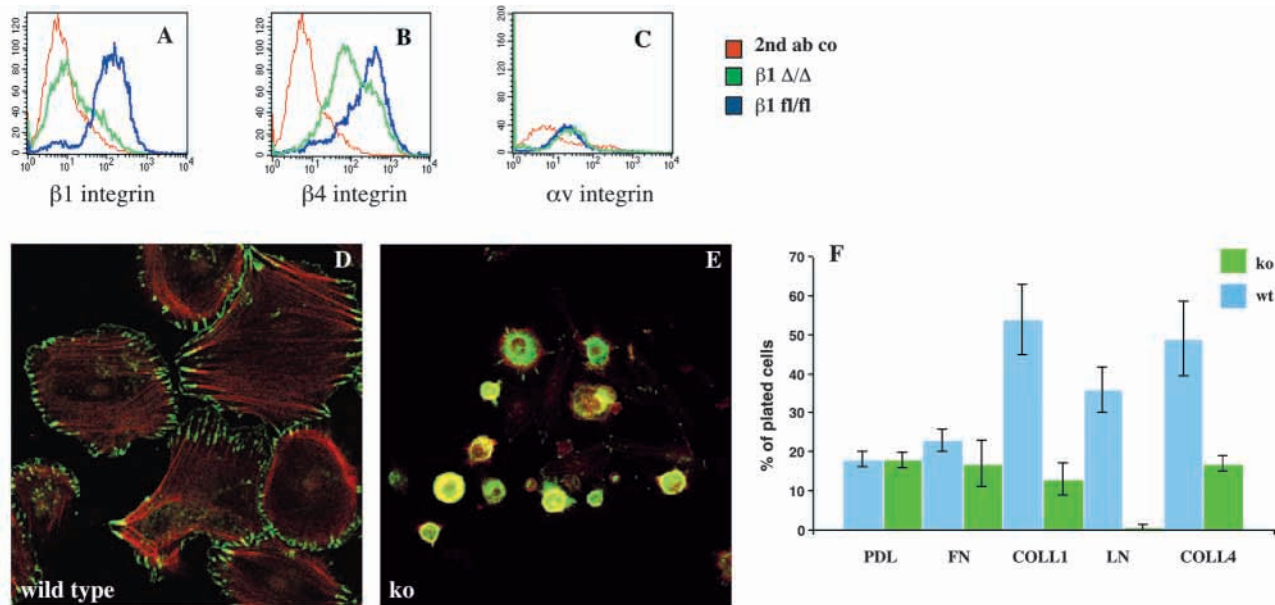
Wax sections (7  $\mu\text{m}$ ) from the middle of the wound were incubated with the appropriate primary antibody: neutrophils – 1:125 dilution of a biotinylated rat antibody targeted to Ly-6G (BD Biosciences); and macrophages – 1:125 dilution of a rat antibody targeted to F4/80 (Serotec, Oxford, UK). Anti-F4/80 was incubated with biotinylated donkey anti-rat IgG (Dianova GMBH, Hamburg, Germany). The VectaStain avidin-biotin-peroxidase complex kit was then used according to the manufacturer's instructions, prior to peroxidase detection with a diaminobenzidine-peroxidase substrate kit (both from Vector Laboratories). Sections were counterstained with Mayer's Hemalum.

### Electron microscopy

Wounds were fixed overnight in 2%



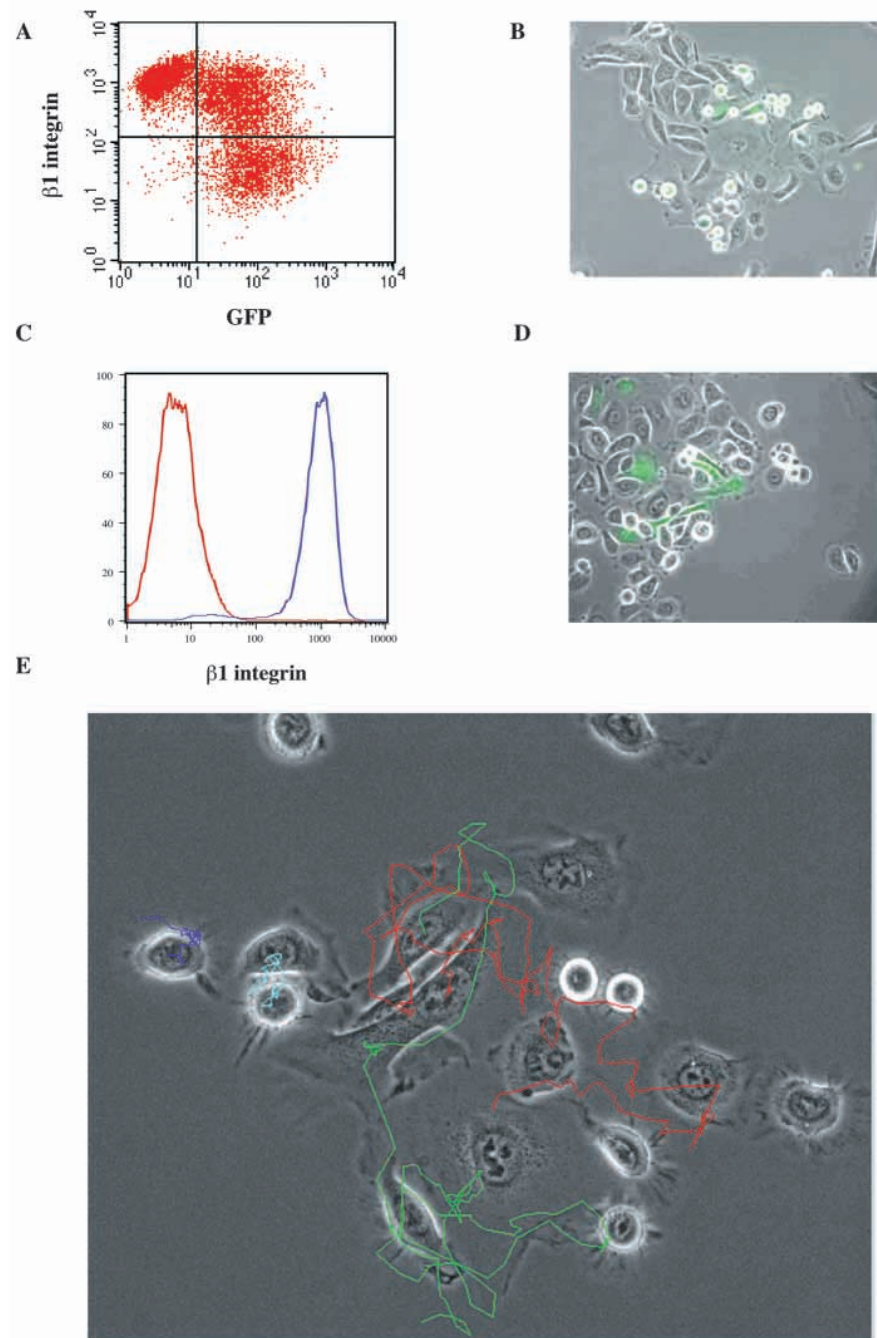
**Fig. 1.** Postnatal development of K5 $\beta$ 1-null mice. Ten d.p.p. K5 $\beta$ 1-null mice have a thinner coat (A), but there is no significant difference in their size or weight (A,B). Transverse sections through the back skin of control (C) and K5 $\beta$ 1-null mice (D) at day 10 p.p. reveal a decreased number of hair follicles in K5 $\beta$ 1-null mice, with concomitant recruitment of macrophages (stained in brown using anti-F4/80 antibody) to the dying follicles (asterisks in D). F, fatty tissue; HF, hair follicle. Scale bars: 200  $\mu\text{m}$ .



**Fig. 2.** Effect of  $\beta 1$  integrin deletion on adhesion of primary keratinocytes. (A-C) Surface expression of integrins in freshly isolated keratinocytes from 2-day-old K5 $\beta$ 1-null mice ( $\beta 1 \Delta/\Delta$ ) and control littermates that were homozygous for the floxed  $\beta 1$  integrin allele but did not express Cre recombinase ( $\beta 1 \text{ fl/fl}$ ), as determined by FACS analysis. Orange lines represent the second antibody control. Keratinocytes were stained with antibodies against  $\beta 1$ ,  $\beta 4$  and  $\alpha v$  integrins. (D,E) Loss of focal adhesions and actin stress fibres in  $\beta 1$ -null keratinocytes. Wild-type (D) and  $\beta 1$ -deficient (E) keratinocytes were cultured for 2 days and examined for focal adhesions (green) and F-actin (red) by immunofluorescence staining with an antibody against paxillin or by staining with phalloidin, respectively. Note that the  $\beta 1$ -null cells are completely rounded and that the green fluorescence is cytoplasmic paxillin. (F) Adhesion of wild-type and  $\beta 1$ -deficient keratinocytes. Keratinocytes were plated onto 96-well plates ( $5 \times 10^4$  cells/well), pre-coated with fibronectin (FN), laminin (LN), poly-D-lysine (PDL), collagen type I (COLL 1) and collagen type IV (COLL 4). Adhesion was quantified using a CytoTox 96 colorimetric kit. Error bars represent standard deviation of the mean of triplicate samples within one experiment.

paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, rinsed in 0.1 M cacodylate buffer and stained in 1% uranyl acetate. They were then incubated in 70% ethanol for 8 hours, dehydrated through an ethanol series and embedded in araldite resin.

Semi-thin sections (1  $\mu\text{m}$ ) were cut with a glass knife using an ultramicrotome (Reichert, Bensheim, Germany) and stained with Methylene Blue. Ultra-thin sections (30–60 nm) for electron microscopic observation were processed on the same microtome with a diamond knife and placed on copper grids. The transmission electron microscopy was performed with a Zeiss 902A electron microscope (Zeiss, Oberkochen, Germany).



**Fig. 3.** Effect of  $\beta 1$  integrin deletion on keratinocyte migration in vitro. (A)  $\beta 1$  fl/fl keratinocytes were infected with an EGFP-Cre-expressing retrovirus and examined 5 days later. Flow cytometry demonstrates that the  $\beta 1$  integrin subunit is missing in 50% of EGFP-positive keratinocytes. (B) The GFP positive keratinocytes fail to spread. (C)  $\beta 1$  fl/fl keratinocytes transduced with chick  $\beta 1$  integrin. Red, second antibody control; blue, chick  $\beta 1$  integrin-positive cells. (D) Chick transduced keratinocytes remain spread after infection with the EGFP-Cre retrovirus. (E) Phase and photograph of  $\beta 1$  fl/fl keratinocytes infected with EGFP-Cre at  $t=0$  hours of the movie. In colour are the tracks of two EGFP-Cre infected (dark and light blue tracks) and uninfected (red and green tracks) keratinocytes. For detailed migration movies, see <http://dev.biologists.org/supplemental/>.

### RNA isolation and RNase protection assay

RNA isolation and RNase protection assays were performed as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) or by Werner et al. (Werner et al., 1994), respectively. As a loading control, 1  $\mu\text{g}$  of each RNA sample was resolved through a 1% agarose gel and stained with Ethidium Bromide. Alternatively, the RNAs were hybridised with an antisense RNA probe to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The templates we used have been published previously: IL1 $\beta$ , IL1 $\alpha$  and TNF $\alpha$  (Hübner et al., 1996); VEGF (Frank et al., 1995); MMP-10 and MMP-13 (Madlener et al., 1998); fibronectin (Munz et al., 1999); and collagen  $\alpha 1$  (I) (Bloch et al., 2000).

## RESULTS

### Normal skin thickness and lack of fibrosis in 10 day old K5 $\beta 1$ -null mice

Because K5 $\beta 1$ -null mice lose their hair and develop a severe skin fibrosis in the first 5 weeks post partum, we decided to use mice at 10 d.p.p. for our experiments. At this stage, K5 $\beta 1$ -null animals are distinguishable from control littermates by virtue of their thinner coats (Fig. 1A). However, there is no significant difference in their size or weight (Fig. 1A,B).

Skin of 10 d.p.p. K5 $\beta 1$ -null mice was characterised by a decreased number of hair follicles, a recruitment of macrophages to the dying follicles (Fig. 1C,D), and a significant difference in the depth that individual hair follicles had invaded the subcutaneous fat. By contrast, the thickness of the skin was similar in the knockout mice and their control littermates, and fibrosis was not yet observed at this age.

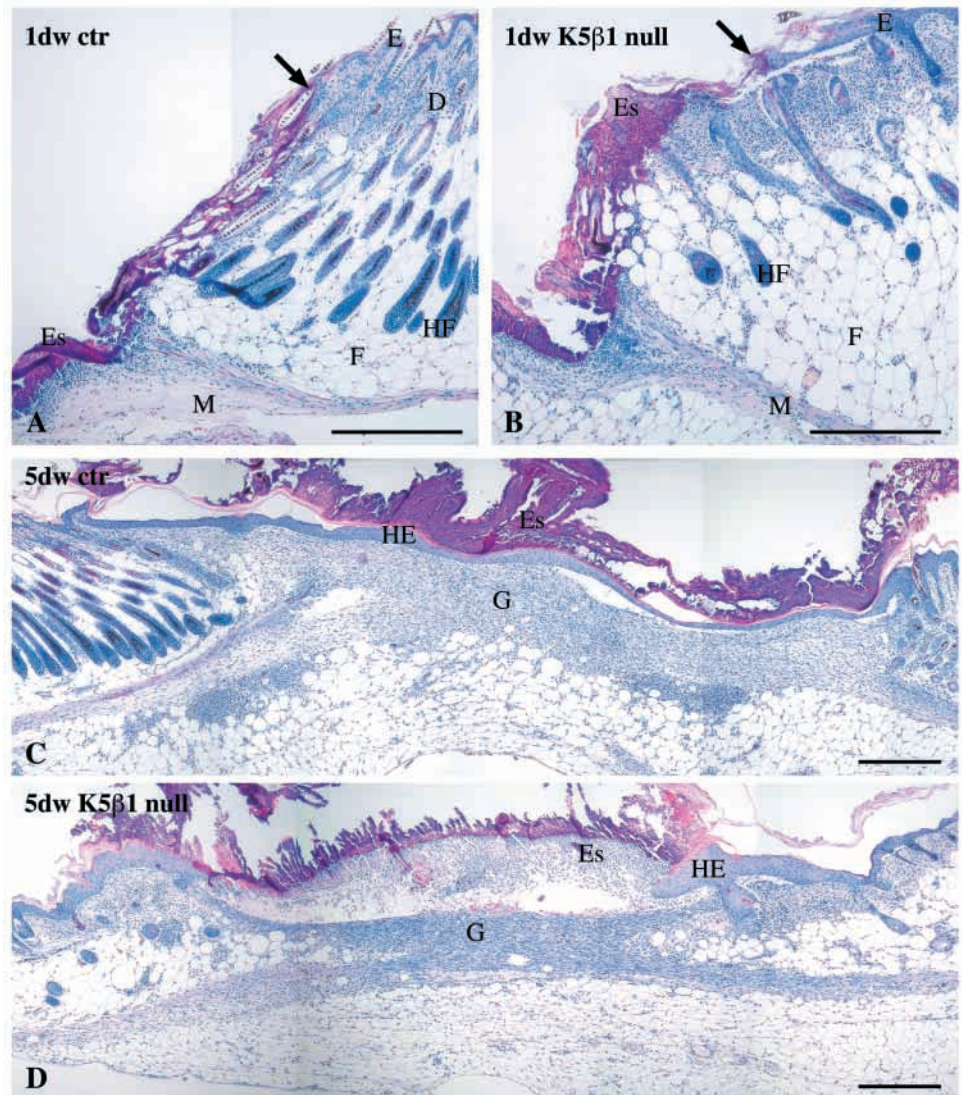
### Impaired adhesion, spreading, proliferation and migration of $\beta 1$ -null keratinocytes in culture

To determine the role of  $\beta 1$ -integrins in keratinocyte adhesion, proliferation and migration in vitro, we isolated keratinocytes from 2-day-old mice. As shown by FACS analysis (Fig. 2A),  $\beta 1$  integrins were almost completely absent on keratinocytes of K5 $\beta 1$ -null mice at this

early time point, demonstrating efficient Cre-mediated deletion of the gene. Deletion of only one copy of the  $\beta$ 1 integrin gene did not affect the levels of  $\beta$ 1 integrins on the cell surface.  $\beta$ 4 integrin levels were reduced to about 50% of wild-type control levels in K5 $\beta$ 1-null keratinocytes (Fig. 2B), whereas  $\alpha$ v integrins were expressed at equally low levels in K5 $\beta$ 1-null and wild-type cells (Fig. 2C).

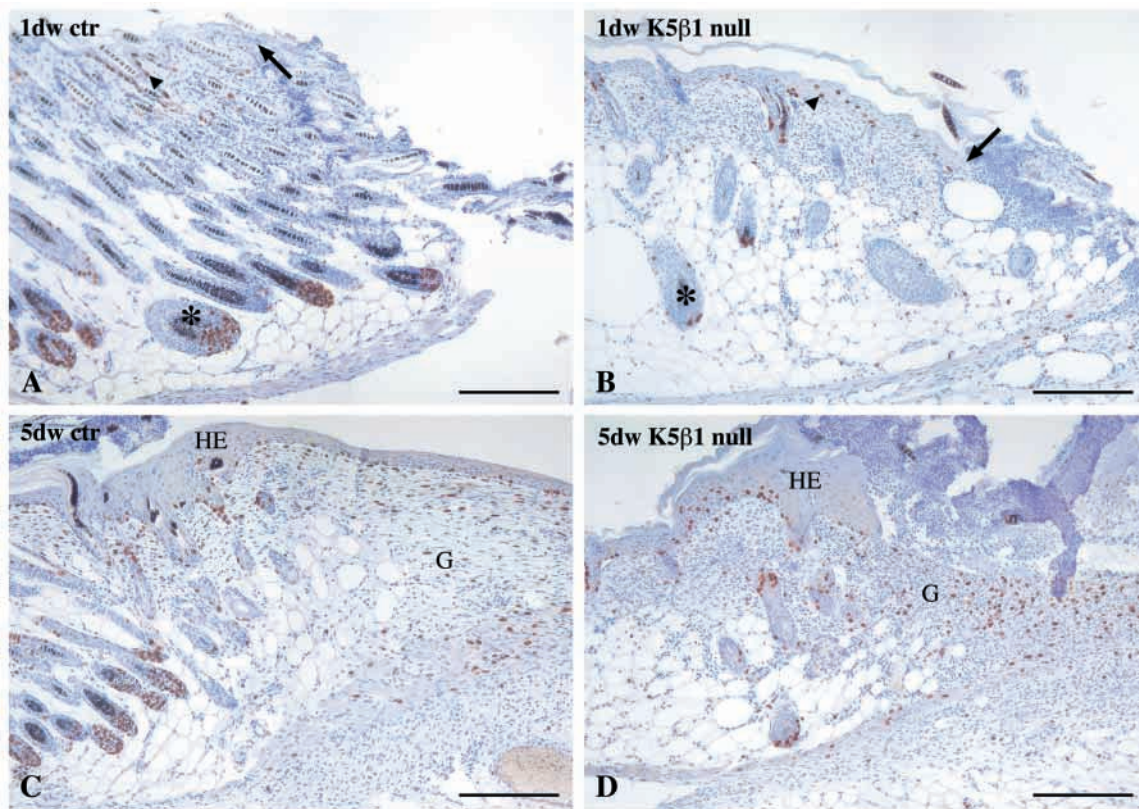
Keratinocytes from K5 $\beta$ 1-null mice and control littermates were then plated onto type I collagen, and co-stained with an antibody against paxillin (to test if cells form focal adhesions) and with TRITC-conjugated phalloidin (to visualise actin microfilaments).  $\beta$ 1-null cells were not able to organise their actin cytoskeleton or to form focal adhesions, with paxillin being diffusely distributed in the cytoplasm (Fig. 2E). Most of the cells remained rounded and even those that were able to spread to a small extent failed to display the stress fibres or focal adhesions that are characteristic of wild-type keratinocytes (Fig. 2D). To quantitate the extent of adhesion of  $\beta$ 1-null keratinocytes, they were plated onto various substrates. Because freshly isolated keratinocytes need several hours to adhere, we determined the adherence after overnight incubation. Adhesion on poly-D-lysine and fibronectin was not impaired by the lack of  $\beta$ 1 integrins, but adhesion to collagen type I, laminin 1 and collagen type IV was severely reduced (Fig. 2F). In addition to the adhesion defect, we found a 40% reduction in the proliferation rate of these cells as determined by BrdU labelling of first passage keratinocytes. Thus 9% of wild-type, but only 5.5% of  $\beta$ 1-null keratinocytes were labelled. Furthermore, a strong stimulation of terminal differentiation, as evaluated by expression of involucrin and cornifin, was observed (5% of wild-type cells were involucrin positive versus 25% of  $\beta$ 1-null cells on all substrates, 3 days after plating), but apoptosis was not affected (data not shown).

To determine whether the lack of  $\beta$ 1 integrins affects migration of keratinocytes, we generated keratinocyte cell lines from mice homozygous for the floxed  $\beta$ 1-integrin allele and deleted this gene in culture using a retrovirus that expressed a fusion protein of enhanced green fluorescent protein (EGFP) and Cre recombinase. Five days after infection, 50% of EGFP-positive cells had lost the integrin  $\beta$ 1 subunit (Fig. 3A).



**Fig. 4.** Impaired re-epithelialisation in K5 $\beta$ 1-null mice. Haematoxylin and Eosin staining of sections from 1-day wounds reveals similar histology between wounds of control (A) and K5 $\beta$ 1-null (B) mice (arrows indicate the epithelial margin). By 5 days post-wounding, 60% of wounds to control mice had re-epithelialised, with the hyperproliferative epithelium clearly covering the wound bed (C), whereas those of their K5 $\beta$ 1-null littermates (D) had never closed, with the hyperproliferative epithelium remaining almost static at the wound margins ( $n=10$  for each). The tissue covering the wound bed in D is eschar, which will eventually be shed when the epithelial fronts meet several days later. D, dermis; E, epidermis; Es, eschar; F, fatty tissue; G, granulation tissue; HE, hyperproliferative epithelium; HF, hair follicle; M, muscle. Scale bars: 200  $\mu$ m.

Consistent with the results obtained with keratinocytes isolated from K5 $\beta$ 1-null mice, the  $\beta$ 1-deficient keratinocytes generated by retroviral deletion were not able to spread (Fig. 3B). This defect was rescued by infection of the cells with a retrovirus that expressed the chick  $\beta$ 1 integrin subunit (Fig. 3C,D). The cells were filmed over a period of 24 hours on days 4 and 5 after infection. Interestingly, the migration capacity of  $\beta$ 1-deficient keratinocytes was severely impaired (Fig. 3E and Movies). Thus, the average migration speed of wild-type cells was 21.2 ( $\pm$ 0.506)  $\mu$ m/hour, whereas  $\beta$ 1-deficient cells only migrated at a speed of 2.56 ( $\pm$ 0.858)  $\mu$ m/hour. These results demonstrate the importance of  $\beta$ 1 integrins for keratinocyte migration in vitro. Although the  $\beta$ 1-null keratinocytes had a



**Fig. 5.** Cell proliferation in 5-day wounds. Mice were injected with BrdU 2 hours before sacrifice. Sections of 1-day (A,B) and 5-day (C,D) wounds from control mice (A,C) and K5 $\beta$ 1-null mice (B,D) were stained with an anti-BrdU antibody (stained nuclei are indicated by arrowheads and the epithelial wound edge by arrows in A,B). There was an obvious impairment of keratinocyte proliferation in the hair follicles of K5 $\beta$ 1-null mice (A-D; compare asterisks in A and B). G, granulation tissue; HE, hyperproliferative epithelium. Scale bars: 200  $\mu$ m.

defect in spreading and migration, they were still able to undergo cell division (see Movies).

#### Delayed re-epithelialisation in K5 $\beta$ 1-null mice

To determine the importance of  $\beta$ 1 integrins for re-epithelialisation of skin wounds, we analysed full-thickness excisional wounds from knockout animals and control littermates at days 1, 2, 3, 5, 10 and 15 after injury ( $n \geq 4$  for each time point and genotype). No obvious abnormalities in wound contraction or appearance were observed during the first 5 days after injury, but at later time points, the loss of the scab was delayed in K5 $\beta$ 1-null mice (not shown). At the histological level, one-day wounds from K5 $\beta$ 1-null mice appeared grossly similar to those from control littermates (Fig. 4A,B). By 5 days post-wounding, 60% of control wounds were fully covered with a thin neoepidermis (Fig. 4C). By contrast, in K5 $\beta$ 1-null mice, the epidermis at the wound margin remained almost static, and the number of wound keratinocytes was reduced in the mutant mice. Only a small area of the granulation tissue was covered by the new epidermis, whereas the remaining part was still covered by eschar. Surprisingly, the keratinocytes at the wound edge had clearly increased in number, resulting in an overtly hyperthickened epidermis at the wound edge (Fig. 4D; Fig. 5D). Most noticeably, no clear migrating epithelial tongue was distinguishable. By contrast, granulation tissue formation appeared unaffected.

#### Cell proliferation is not reduced in early wounds of control and K5 $\beta$ 1-null mice

To investigate if the delayed re-epithelialisation in K5 $\beta$ 1-null mice was due to a difference in cell proliferation, we performed BrdU labelling studies. No difference in the percentage of BrdU-positive cells was observed in the epidermis of 1-day-old wounds (Fig. 5A,B). At day 5 after injury, the percentage of proliferating cells was still similar in the hyperthickened wound epidermis of control and K5 $\beta$ 1-null mice, with 10–15% of keratinocytes being labelled in each case ( $n=5$  wounds for each). However, the distribution of the labelled cells was altered, with more BrdU-positive suprabasal cells being found in the wound epidermis of K5 $\beta$ 1-null mice (Fig. 5C,D). At day 15 after injury, the epidermal keratinocytes had returned to their normal proliferation rate in control mice, but the epidermis was still hyperproliferative in K5 $\beta$ 1-null mice (data not shown). No difference in cell proliferation was observed in the granulation tissue at any stage of repair (Fig. 5; data not shown).

#### Cell-cell and cell-matrix interactions during wound repair

To confirm the lack of  $\beta$ 1 integrins in wound keratinocytes, we stained 10-day-old wound sections with an antibody against this integrin subunit. It was expressed in basal cells of the hyperproliferative wound epithelium in control mice (Fig. 6A), but absent in keratinocytes of K5 $\beta$ 1-null animals (Fig. 6B).

The reduction in the number of hemidesmosomes seen in

non-wounded skin of K5 $\beta$ 1-null mice (Brakebusch et al., 2000) was also observed in the hyperproliferative epithelium of their 5-day-old wounds, as reflected by the punctate  $\beta$ 4 integrin staining of the basal keratinocytes (Fig. 6D, red) compared with the continuous line of staining seen in the epithelium of control mice (Fig. 6C). The reduced number of hemidesmosomes was also confirmed by electron microscopy (data not shown). The expression of keratin 6, a classical marker for the hyperproliferative epithelium used for counterstaining, was unchanged (Fig. 6C,D, green).

A distinct line of laminin-5, a ligand for  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 6 $\beta$ 4 integrins, was observed beneath the newly formed epidermis of 5-day wounds from control mice (Fig. 6E), whereas a diffuse distribution in the granulation tissue was seen beneath the hyperthickened epidermis at the margin of wounds of K5 $\beta$ 1-null mice (Fig. 6F). This finding confirms that  $\beta$ 1 integrins are required for correct localisation of laminin-5 in the basement membrane, as previously reported in studies of unwounded skin architecture in the same conditional knockout mouse (Brakebusch et al., 2000). By contrast, cadherin-dependent cell-cell contacts appeared normal as demonstrated by the normal expression of E-cadherin (Fig. 6G,H) and of  $\beta$ -catenin (data not shown) in K5 $\beta$ 1-null keratinocytes, and their appropriate association with cell membranes.

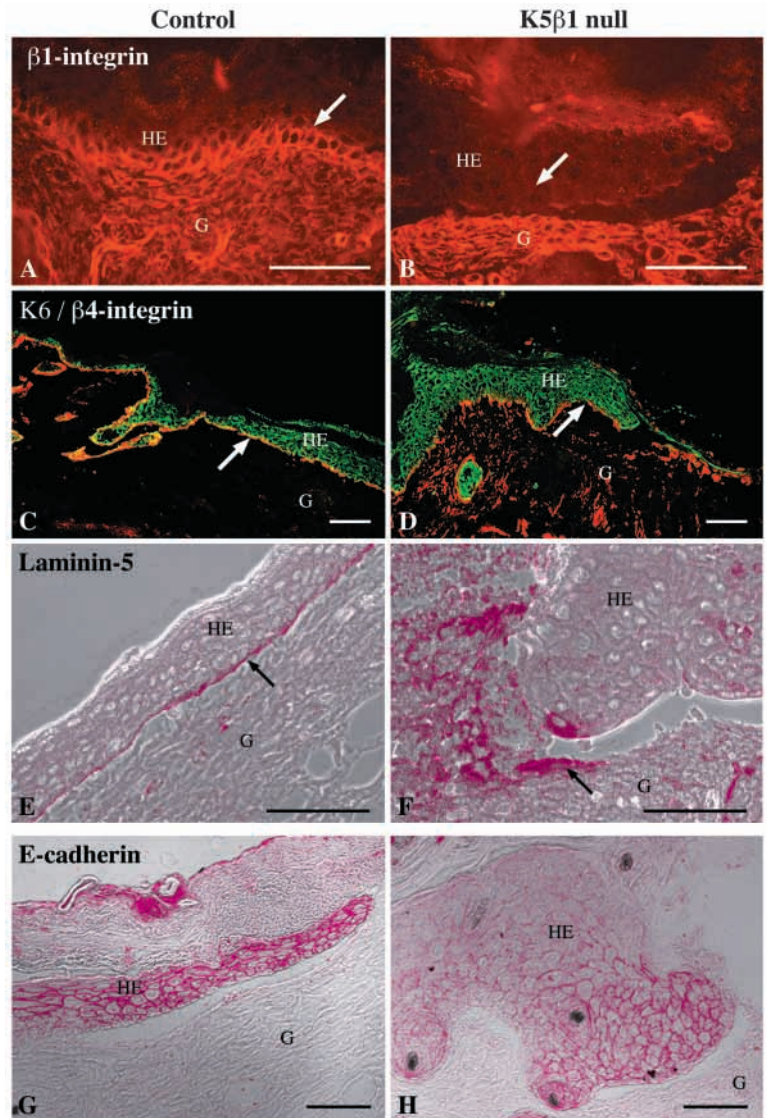
#### Lack of $\beta$ 1 integrins in the keratinocytes of the hyperproliferative epithelium causes narrowing of the intercellular spaces

In the hyperproliferative epithelium of 5-day-old wounds from control mice, semi-thin sectioning revealed clear intercellular spaces between the migrating cells (Fig. 7A,C). In comparison, the keratinocytes of K5 $\beta$ 1-null mice were much more closely packed (Fig. 7B,D).

Transmission electron microscopy confirmed these differences and revealed many cytoplasmic processes bridging the wide intercellular spaces between control keratinocytes (Fig. 7E). By contrast, K5 $\beta$ 1-null keratinocytes showed greatly reduced intercellular spaces (Fig. 7F). The number of desmosomes and their ultrastructure was not obviously altered in the keratinocytes of K5 $\beta$ 1-null mice (Fig. 7G,H, and data not shown). However, the number of tonofilaments, thin filaments that course through the basal cells and insert into the desmosomes, was significantly reduced (Fig. 7H).

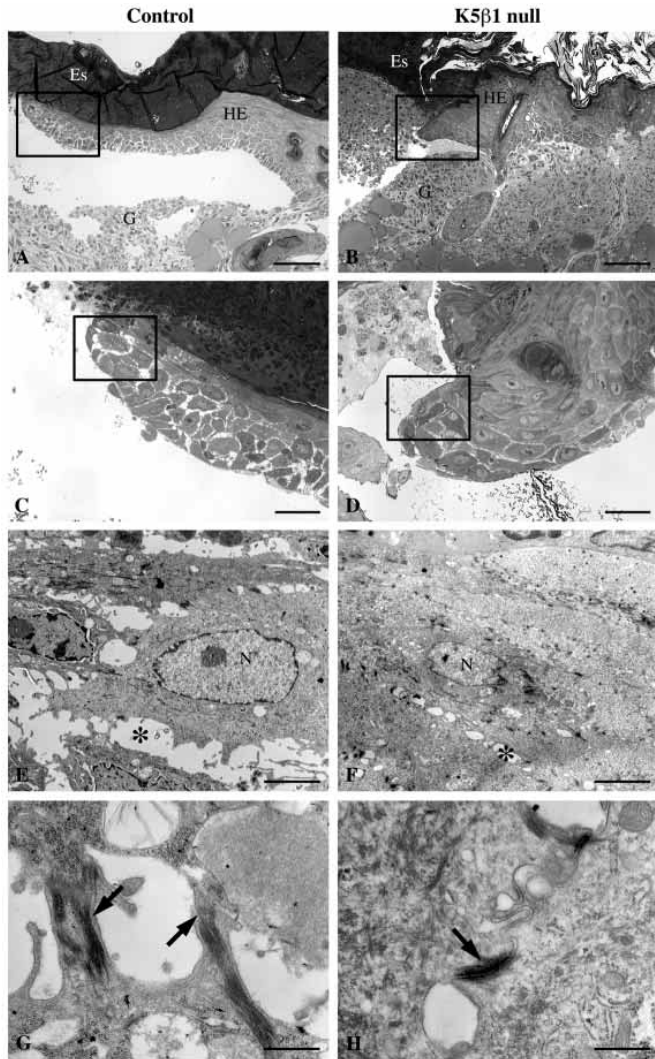
#### Defective epidermal architecture in late wounds of K5 $\beta$ 1-null mice

K5 $\beta$ 1-null mice never showed complete re-epithelialisation at day 5-10 after injury (Fig. 4D and data not shown). By contrast, 60% of wounds in control mice had re-epithelialised by 5 days and all were completely healed by 10 days ( $n \geq 5$  for each). By 15-days post-injury, wounds from control mice were completely covered with a thin, uniform epithelium (Fig. 8A) ( $n=5$ ), which was characterised by normal



**Fig. 6.** Cell-cell and cell-matrix interactions during wound repair. Immunostaining on sections from the middle of 10-day wounds, with an anti- $\beta$ 1 integrin antibody reveals expression in basal keratinocytes of the healed epidermis of control mice and confirms the absence of  $\beta$ 1 in the epidermis of K5 $\beta$ 1-null mice (arrows in A,B). Lack of  $\beta$ 1 integrins also results in a disturbance in hemidesmosome localisation, as detected by immunostaining on sections from 5-day wounds for  $\beta$ 4 integrin (red; arrows in C,D). The hyperproliferative epithelium is stained with an anti-keratin 6 antibody (green). G, granulation tissue; HE, hyperproliferative epithelium. This difference in hemidesmosome staining is recapitulated in the staining for the laminin 5 substrate (magenta) to which they bind (arrows in E,F). By contrast, expression of E-cadherin (magenta) is similar in the hyperproliferative epithelium of 5-day wounds of control and K5 $\beta$ 1-null mice (G,H). G, granulation tissue; HE, hyperproliferative epithelium. Scale bars: 50  $\mu$ m.

expression of the differentiation-specific keratin 10 in the first suprabasal layer (data not shown). The epidermis was well attached to the underlying granulation tissue. In K5 $\beta$ 1-null mice, the whole wound bed was covered by epidermis, but in two out of five cases the converging epithelial tongues had failed to fuse (Fig. 8B). Where fusion had occurred, the hyperproliferative epithelium was about twice as thick as that in control mice (Fig. 8C), keratin 10 was only expressed in

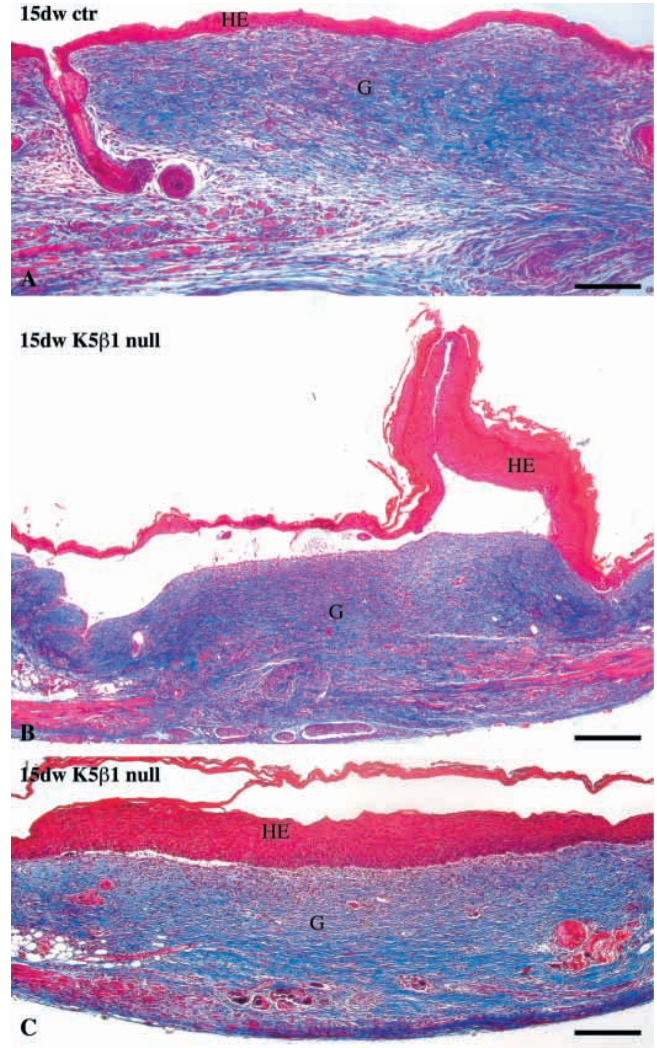


**Fig. 7.** Structural analysis of the epithelial tongue. Light microscopical (A-D) and ultrastructural (E-H) views of the epidermal tongue at the margin of 5-day wounds as it cuts a path between the granulation tissue and the overlying eschar. The migrating keratinocytes of the hyperproliferative epithelium in control mice are relatively loosely associated with each other, showing extensive intercellular spacing (compare asterisk in E with that in F). By contrast, wound margin keratinocytes of K5 $\beta$ 1-null mice are densely packed (compare A,C,E with B,D,F). The boxes represent the approximate areas shown in the higher magnification figures. The number of tonofilaments attached to the desmosomes was significantly reduced in K5 $\beta$ 1-null mice (H) compared with controls (G) (indicated by arrows). Es, eschar; G, granulation tissue; HE, hyperproliferative epithelium; N, nucleus. Scale bars: 50  $\mu$ m in A,B; 20  $\mu$ m in C,D; 1  $\mu$ m in E-H.

the most upper layer (not shown), and the keratinocytes were poorly attached to the underlying tissue.

#### Repair of incisional wounds is delayed in the absence of the $\beta$ 1 integrin subunit

In control mice, incisional wounds were fully re-epithelialised within 3 days ( $n=3$ ; Fig. 9A), but 3-day wounds in K5 $\beta$ 1-null mice ( $n=3$ ) showed no evidence of re-epithelialisation (Fig. 9B). Similar to excisional wounds, the epidermis at the wound



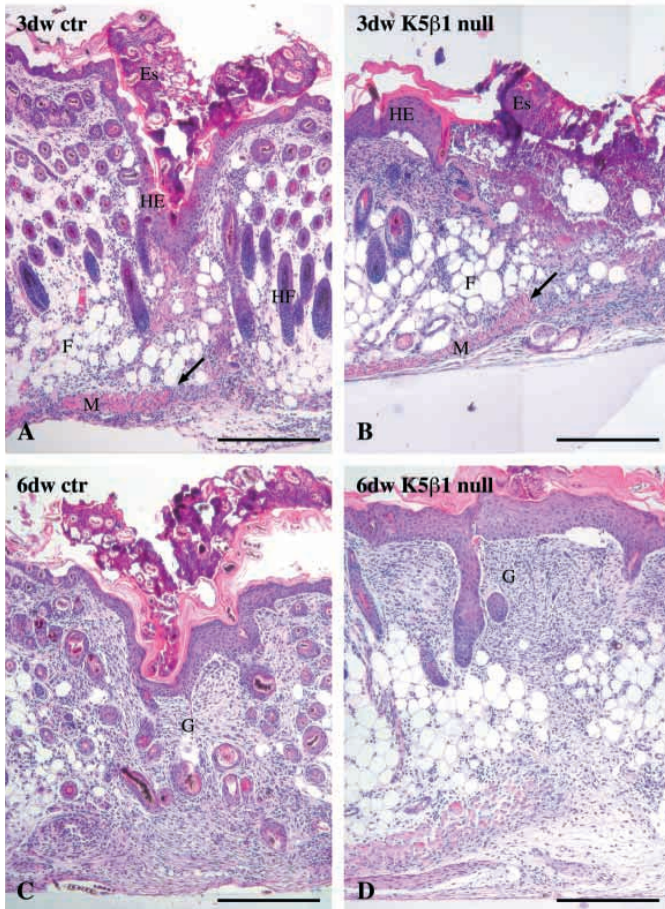
**Fig. 8.** Histology of 15-day wounds reveals marked differences in the outcome of the repair process. Masson trichrome staining of 15-day wounds of control mice reveals complete re-epithelialisation (A). However, in several wounds of K5 $\beta$ 1-null mice, the epithelial tongues fail to fuse and detach from the underlying granulation tissue (B) and, where fusion does occur, the epidermis is far thicker than that seen in control mice (compare A with C). G, granulation tissue; HE, hyperproliferative epithelium. Scale bars: 200  $\mu$ m.

edge was markedly thickened, but keratinocyte proliferation was not altered (data not shown). By 6 days post-wounding, K5 $\beta$ 1-null mice had also healed their wounds ( $n=3$ ), though interestingly the regenerated epidermis showed a different morphology. In control wounds, the keratinocytes had migrated down the margins of the wound, resulting in a V-shaped morphology (Fig. 9A,C), whereas the hyperproliferative epithelium of K5 $\beta$ 1-null mice healed over the surface of the wound bed, resulting in a flat morphology (Fig. 9D).

#### Enhanced inflammation but normal angiogenesis in the wounds of K5 $\beta$ 1-null mice

We next determined if the delayed re-epithelialisation altered the inflammatory response. F4/80 staining of 2-, 5-, 10- and 15-day wounds from K5 $\beta$ 1-null mice revealed no difference in the infiltration of monocytes/macrophages (Fig. 10A,B; data not





**Fig. 9.** Repair of incisional wounds is delayed in K5 $\beta$ 1-null mice. Haematoxylin and Eosin staining reveals that full-thickness incisional wounds on the backs of 10 d.p.p. control mice had healed by day 3 post-injury (A; arrows indicate where the panniculus carnosus has been cut), but wounds of K5 $\beta$ 1-null mice are wide open at the same stage (B). By 6 days after wounding, wounds of both control and K5 $\beta$ 1-null mice have healed (C,D). Es, eschar; F, fatty tissue; G, granulation tissue; HE, hyperproliferative epithelium; HF, hair follicle; M, muscle. Scale bars: 200  $\mu$ m in A-D.

shown). However, in their still exposed 5-day wounds, neutrophils were still present in large numbers throughout the granulation tissue (Fig. 10D), although no signs of infection were observed. There was no obvious difference in angiogenesis as determined by immunofluorescence analysis with an antibody against the endothelial-specific protein PECAM (Fig. 10E,F).

#### Changes in gene expression in the wounds of K5 $\beta$ 1-null mice

To gain insight into the molecular mechanisms underlying the observed phenotype, we performed RNase protection assays for genes that encode important regulators of repair. For this purpose, we used two independent pools of total RNA, each isolated from two wounds (1, 5 and 10 day) from each of at least five control and five K5 $\beta$ 1-null mice (Fig. 11). Owing to the development of fibrosis in the K5 $\beta$ 1-null mice, we used unwounded skin from 10 and 20 d.p.p. wild-type and K5 $\beta$ 1-null mice as controls, thus enabling us to distinguish between developmental and wound-specific changes in gene expression.

Expression of the pro-inflammatory cytokines interleukin 1 $\beta$  (IL1 $\beta$ ), IL1 $\alpha$  and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) was increased in 10 and 20 d.p.p. unwounded back skin of K5 $\beta$ 1-null mice (Fig. 11; data not shown). Pro-inflammatory cytokine expression was further increased at days 5 and 10 post-wounding compared with expression in wild-type animals. Similar expression profiles were observed for IL1 $\alpha$  and TNF $\alpha$  (data not shown). The levels of activin  $\beta$ A and keratinocyte growth factor (KGF) mRNAs were both increased during repair in K5 $\beta$ 1-null mice compared with controls. By contrast, expression of vascular endothelial growth factor (VEGF), TGF $\beta$ 1 and TGF $\beta$ 3, as well as of connective tissue growth factor (CTGF) remained unchanged in the absence of epidermal  $\beta$ 1 integrins (Fig. 11; data not shown).

We also observed a slight increase in mRNA levels of fibronectin, collagen  $\alpha$ 1 (I) and tenascin-C both in non-wounded skin and during wound repair of K5 $\beta$ 1-null mice (Fig. 11 and data not shown). Concomitant with elevated levels of mRNAs encoding matrix metalloproteinase 10 (MMP10; stromelysin 2) and MMP13 (collagenase 3), also both in skin and wounds of K5 $\beta$ 1-null mice (Fig. 11). These MMPs are expressed mainly at the migrating edge of the epidermis in wounds of wild-type mice (Madlener et al., 1998). Taken together, these findings demonstrate that several major players of the wound repair process are aberrantly expressed in K5 $\beta$ 1-null mice.

## DISCUSSION

A series of expression studies have suggested an important role of  $\beta$ 1 integrins in keratinocyte migration during wound healing, but this has not yet been experimentally proven. The availability of genetically engineered mice lacking this integrin subunit in the epidermis offered the unique possibility to address this question.

#### Proliferation of $\beta$ 1-null wound keratinocytes is reduced in vitro but not in vivo

Within a few hours of cutaneous injury, keratinocytes start to migrate over the injured dermis. Subsequently, the cells behind the migrating epithelial sheet increase their proliferation rate and constitute a pool of extra cells that replace those lost during injury (reviewed by Martin, 1997). Thus, delayed re-epithelialisation can result from either impaired migration or proliferation. Interestingly, the results of the present study revealed no difference in the percentage of proliferating cells in the hyperthickened epidermis at the wound edge in early wounds of K5 $\beta$ 1-null mice and even prolonged keratinocyte hyperproliferation. This was unexpected, as high expression of the  $\beta$ 1 integrin subunit has been shown to be a marker for proliferation competent stem cells (Jones and Watt, 1993; Jones et al., 1995). Furthermore, keratinocyte proliferation is reduced in non-wounded skin of K5 $\beta$ 1-null mice (Brakebusch et al., 2000) and of K14 $\beta$ 1-null mice, which revealed a similar, but more severe, phenotype (Raghavan et al., 2000). Finally, the proliferation rate of our cultured  $\beta$ 1-deficient keratinocytes was almost halved and terminal differentiation was stimulated in these cells. These differences between the in vitro and in vivo situation could reflect the lack of dermal inflammatory cytokines and growth factors in culture, the presence of a

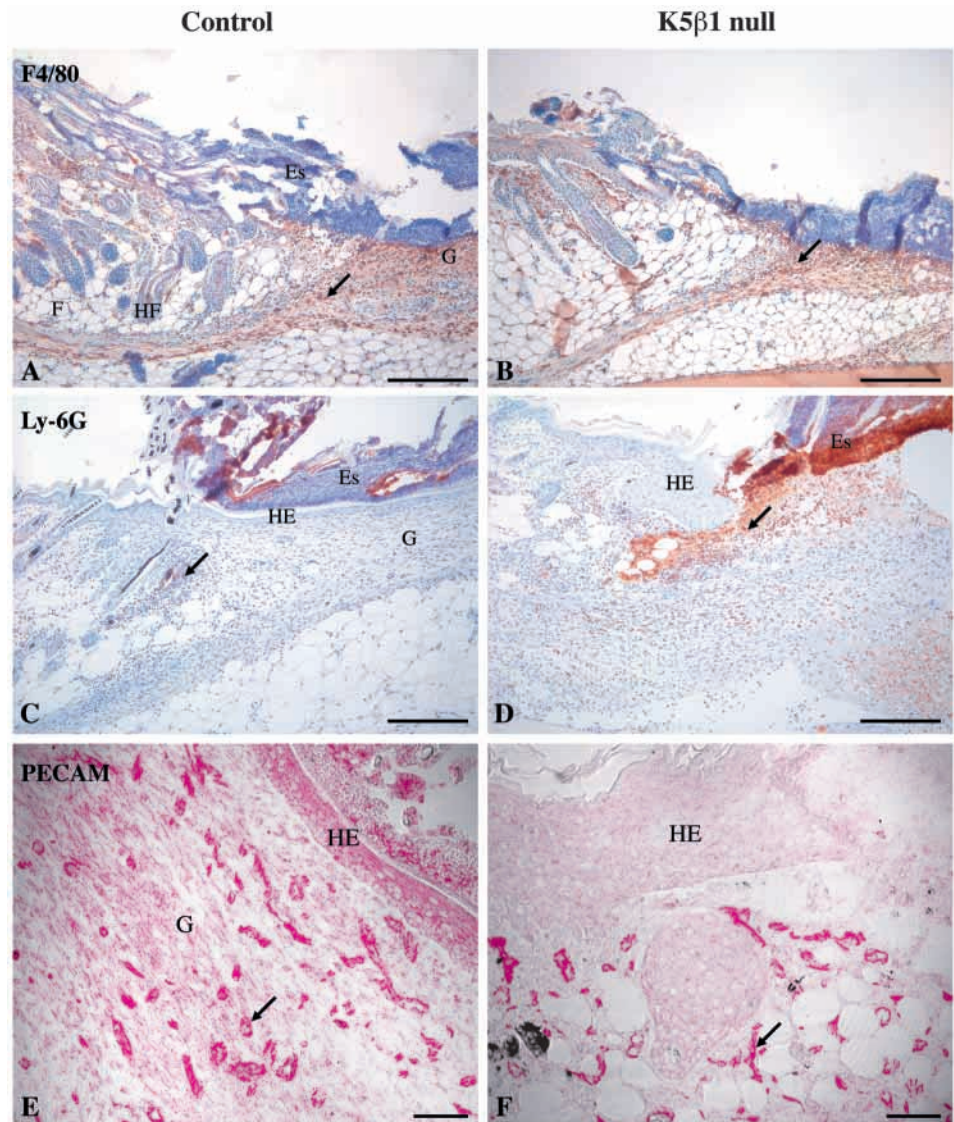
different matrix and the three-dimensional organisation *in vivo*.

### Keratinocyte migration is impaired in the absence of $\beta 1$ integrins *in vitro* and *in vivo*

Because the total number of wound keratinocytes was reduced in the mutant mice, although the percentage of proliferating cells was unchanged, reduced migration of  $\beta 1$ -null keratinocytes is likely to be the reason for the decrease in the total number of keratinocytes in the wound. This observation is strengthened by the observation that the  $\beta 1$ -null keratinocytes are piling up at the wound edges instead of forming a thin epithelial tongue across the wound. Most importantly, this hypothesis is strongly supported by the impaired migratory capacity of  $\beta 1$ -deficient cultured keratinocytes.

Under normal circumstances, keratinocytes first migrate down over the injured dermis where they contact fibrillar type I collagen (Pilcher et al., 1997). Based on *in vivo* expression studies and functional *in vitro* data, it has been suggested that the  $\alpha 2\beta 1$  integrin is required for this migration over the wounded dermis (Pilcher et al., 1997; Hodiola-Dilke et al., 1998; Goldfinger et al., 1999). The results presented in the present study thus provide the first *in vivo* evidence for this hypothesis.

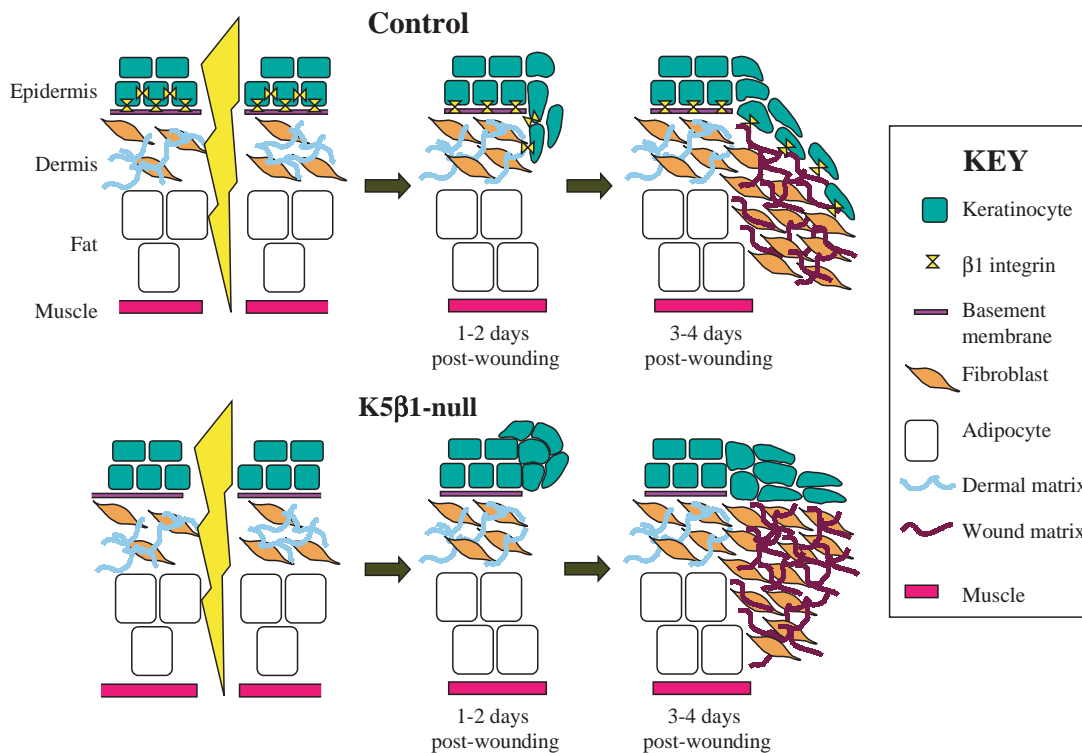
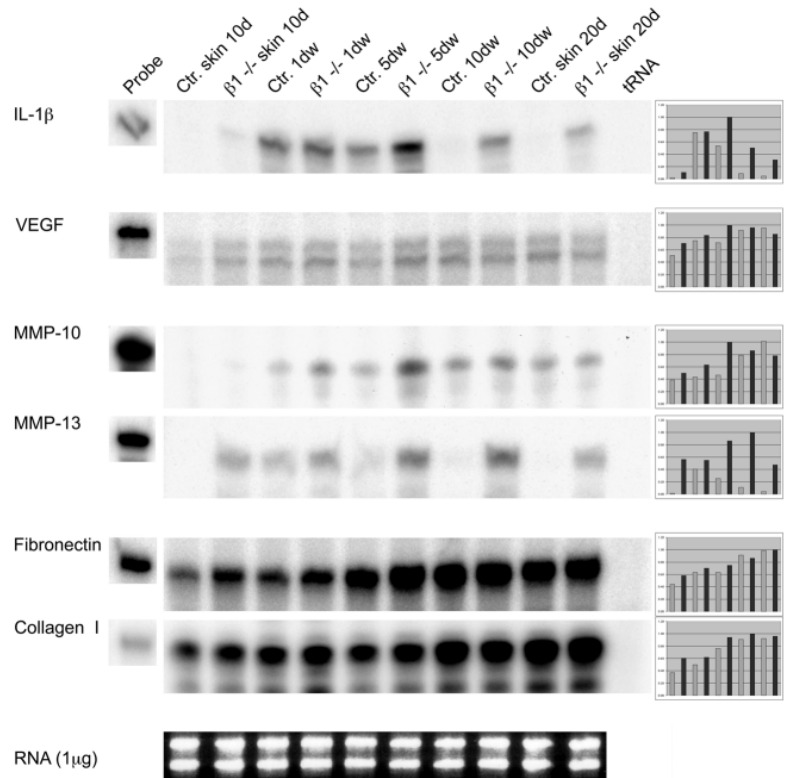
Although the onset of keratinocyte migration was retarded in  $K5\beta 1$ -null mice, a strongly hyperthickened epidermis was observed at the wound edge, most probably due to the continued keratinocyte hyperproliferation in the absence of migration. Several days after wounding, the keratinocytes finally migrated over the wound bed, but the epidermis was strongly hyperplastic, poorly differentiated and poorly attached to the underlying granulation tissue. Most interestingly, the healed epidermis of incisional wounds in control mice had a V-shaped morphology, whereas it was flat in  $K5\beta 1$ -null mice, suggesting that re-epithelialisation in  $K5\beta 1$ -null mice occurs via an alternative, compensatory mechanism (summarised in Fig. 12). Because  $\beta 1$ -deficient keratinocytes lack the principal adhesive receptors used for ligation to dermal matrix, they must wait until compensatory mechanisms are upregulated. Such mechanisms are probably based on the expression of non- $\beta 1$  integrins by keratinocytes and on the presence of the corresponding ligands. The most likely candidates are  $\alpha \nu \beta 5$  or  $\alpha \nu \beta 6$ , receptors for the provisional wound matrix proteins vitronectin, fibronectin and



**Fig. 10.** Increased inflammation but normal angiogenesis in the wounds of  $K5\beta 1$ -null mice. F4/80 staining for monocytes/macrophages (brown stain indicated by arrows in A and B) at day 2 post-wounding showed no difference. In 5-day wounds (C-F) there was a clear increase in neutrophil presence (as detected by staining for Ly-6G – brown stain indicated by arrows in C,D) in the wounds of  $K5\beta 1$ -null mice (D) compared with control littermates (C). No difference was observed in angiogenesis as detected by staining for PECAM1 – magenta stain indicated by arrows in E,F). Es, eschar; F, fatty tissue; G, granulation tissue; HE, hyperproliferative epithelium; HF, hair follicle. Scale bars: 200  $\mu\text{m}$  in A-D; 40  $\mu\text{m}$  in E,F.

tenascin C (Gailit et al., 1994; Larjava et al., 1993; Zambruno et al., 1995; Haapasalmi et al., 1996; Huang et al., 1998). Although  $\alpha \nu$  integrins are weakly expressed in normal and  $\beta 1$ -deficient cultured keratinocytes,  $\alpha \nu \beta 5$  and  $\alpha \nu \beta 6$  are upregulated in migrating keratinocytes during cutaneous wound repair (Cavani et al., 1993; Gailit et al., 1994; Haapasalmi et al., 1996).  $\alpha \nu \beta 5$  on keratinocytes is characteristic of a migratory phenotype (Adams and Watt, 1991), and  $\alpha \nu \beta 6$  is important for keratinocyte migration on vitronectin, fibronectin and tenascin C *in vitro* (Huang et al., 1998). The period required for the upregulation of these integrins and for the deposition of their ligands in the wound bed is likely to determine the time frame for the onset of re-epithelialisation in  $K5\beta 1$ -knockout animals.

**Fig. 11.** Gene expression during the repair process. Expression levels of genes encoding key players during wound healing were determined by RNase protection assay on 20  $\mu\text{g}$  RNA samples from non-wounded back skin from 10 and 20 d.p.p. mice plus 1, 5 and 10 day wounds from control and K5 $\beta 1$ -null mice. 1000 cpm of the hybridisation probes were loaded in the lanes labelled 'probe' and used as size markers. tRNA (20  $\mu\text{g}$ ) was used as a negative control. RNA (1  $\mu\text{g}$  of each) was loaded on a 1% agarose gel and stained with Ethidium Bromide to control for sample integrity and concentration (bottom panel). The intensity of the signals as determined by phosphorimaging is shown schematically on the right-hand side. All protection assays were repeated with a separate pool of RNA samples from an independent skin/wound series.



**Fig. 12.** Model for re-epithelialisation of incisional wounds by normal and  $\beta 1$ -null keratinocytes. In skin of control mice,  $\beta 1$  integrins are localised to the basolateral surfaces of basal keratinocytes. After wounding, control keratinocytes use  $\beta 1$  integrins to bind to the newly exposed dermal ligands. Thus, re-epithelialisation occurs rapidly and results in a downward migration of the epidermis. In K5 $\beta 1$ -null mice, keratinocytes are unable to recognise dermal ligands after wounding and remain static at the wound margin, although they still proliferate. Their cell-cell contacts are also tighter than those of control keratinocytes. Once the wound is filled with granulation tissue, which contains matrix molecules recognised by non- $\beta 1$  integrins, K5 $\beta 1$ -null keratinocytes are able to migrate across the surface of the wound to complete re-epithelialisation, but they do not show the V-shaped repair morphology characteristic of control wounds.

The dependence on non- $\beta 1$  integrins also provides an explanation for the flat morphology of the healed epidermis. Thus, we propose that K5 $\beta 1$ -null keratinocytes are not able to migrate down the injured dermis, because of the absence of ligands for the dermal substrate, but rather wait until the wound is filled with granulation tissue that contains the ligands for non- $\beta 1$  integrins.

### Wound edge keratinocytes pack together more densely in the absence of $\beta 1$ integrins

An additional difference between the wound margin keratinocytes is that their cell-cell contacts appear tighter, when compared with the loose association in control hyperproliferative epithelium. The normal patterns of E-cadherin and  $\beta$ -catenin staining that we observed suggest that the tight contacts are not due to a difference in the expression or compartmentalisation of adherens junction proteins. Furthermore, electron microscopy revealed that the number of desmosomes was not altered. However, the number of tonofilaments attached to the desmosomes was significantly reduced. It has been reported that the insertion of tonofilaments into the attachment plate is a late event in the formation of desmosomes in the wounded epidermis (Krawczyk and Wilgram, 1973), indicating that our observation reflects a defect in the maturation process of the wound keratinocytes in the absence of  $\beta 1$  integrins.

### Abnormal epidermal architecture in healed wounds of K5 $\beta 1$ null mice

Re-epithelialisation was eventually completed in K5 $\beta 1$ -null epidermis, but the wounds had a thicker epidermal covering, reflecting the prolonged period of keratinocyte hyperproliferation and their delayed differentiation. This is unlikely to be a direct result of a lack of  $\beta 1$  integrins in keratinocytes, but could be due to a prolonged proliferative signal from cells within the granulation tissue, as suggested by the increased expression of epithelial growth factors. In addition, there might be a lack of  $\beta 1$ -dependent contact inhibition when the epithelial fronts meet (Huttenlocher et al., 1998) as supported by the epithelial morphology of some of the wounds where the epidermal edges had not fused and where obviously unlimited growth of the epidermis had occurred.

Finally, K5 $\beta 1$ -null keratinocytes failed to establish a normal basement membrane above the newly formed granulation tissue. This finding is likely to underlie the poor attachment of the new epidermis to the mesenchyme and concurs with the observed blister formation, as well as with the reduced number of hemidesmosomes seen in non-wounded skin of K5 $\beta 1$ -null mice (Brakebusch et al., 2000). Blister formation was also observed in mice that lack the integrin  $\alpha 3$  subunit (DiPersio et al., 1997), suggesting that the lack of  $\alpha 3\beta 1$  in our mice is predominantly responsible for the poor attachment of the epidermis. However, in contrast to K5 $\beta 1$ -null keratinocytes, the exclusive lack of the  $\alpha 3$  integrin subunit in this cell type did not cause an altered expression of  $\alpha 6\beta 4$  in vitro or in vivo (Hodivala-Dilke et al., 1998), suggesting that the additional loss of other  $\beta 1$  integrins is responsible for the reduction in the number of  $\alpha 6\beta 4$  integrins in K5 $\beta 1$ -null keratinocytes.

### Prolonged inflammation in K5 $\beta 1$ -null mice

In K5 $\beta 1$ -null mice, we observed an extended window of

neutrophil presence, most likely as a result of the prolonged exposure of K5 $\beta 1$ -null wounds to the external environment. Because re-epithelialisation is so delayed, their wounds are susceptible to pro-inflammatory stimuli such as desiccation and mechanical stress for a longer period. As neutrophils are a major source of pro-inflammatory cytokines in skin wounds (Hübner et al., 1996), the elevated number of these cells is likely to contribute to the increased expression of IL1 $\alpha$ , IL1 $\beta$  and TNF $\alpha$  in the wounds of K5 $\beta 1$ -null mice. These cytokines induce expression of many epithelial growth factors (reviewed by Werner and Smola, 2001), which could contribute to the increased keratinocyte proliferation rate. Increased expression of pro-inflammatory cytokines was also observed in the unwounded skin of older K5 $\beta 1$ -null mice, but these are thought to be released by macrophages rather than neutrophils (Brakebusch et al., 2000). However, the expression levels associated with the developmental inflammation and fibrosis of unwounded skin were significantly lower than the transient expression levels seen during the repair process. Thus, the developmental fibrosis should have a rather minor effect on the repair process in K5 $\beta 1$ -null mice, as supported by the normal contraction of the healing wound.

Taken together, our results reveal a strongly impaired migratory capacity of  $\beta 1$ -deficient keratinocytes in vitro and a dramatic delay in epithelial migration during wound repair in K5 $\beta 1$ -null mice. We thus present the first in vivo evidence in support of findings from in vitro studies that have shown  $\beta 1$  integrins to be key players in cell migration. However, our results also demonstrate that keratinocytes are not totally dependent on this integrin subunit to heal their wounds. Rather, other integrins appear to compensate at least partially for the lack of  $\beta 1$ , leading to complete, although imperfect, re-epithelialisation.

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