β1 Integrin-Mediated Adhesion Signalling Is Essential for Epidermal Progenitor Cell Expansion

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Abstract

Background: There is a major discrepancy between the in vitro and in vivo results regarding the role of β1 integrins in the maintenance of epidermal stem/progenitor cells. Studies of mice with skin-specific ablation of β1 integrins suggested that epidermis can form and be maintained in their absence, while in vitro data have shown a fundamental role for these adhesion receptors in stem/progenitor cell expansion and differentiation.

Methodology/Principal Findings: To elucidate this discrepancy we generated hypomorphic mice expressing reduced β1 integrin levels on keratinocytes that developed similar, but less severe defects than mice with β1-deficient keratinocytes. Surprisingly we found that upon aging these abnormalities attenuated due to a rapid expansion of cells, which escaped or compensated for the down-regulation of β1 integrin expression. A similar phenomenon was observed in aged mice with a complete, skin-specific ablation of the β1 integrin gene, where cells that escaped Cre-mediated recombination repopulated the mutant skin in a very short time period. The expansion of β1 integrin expressing keratinocytes was even further accelerated in situations of increased keratinocyte proliferation such as wound healing.

Conclusions/Significance: These data demonstrate that expression of β1 integrins is critically important for the expansion of epidermal progenitor cells to maintain epidermal homeostasis.

Introduction

Integrins are heterodimeric cell surface receptors consisting of one α and one β subunit. They bind extracellular matrix (ECM) proteins and counter receptors such as VCAM, and play fundamental roles for tissue development and homeostasis [1]. The mammalian genome contains 18 α and 8 β integrin genes whose proteins can give rise to 24 different integrin heterodimers. Integrins are expressed on almost all cells including keratinocytes of the skin.

The epidermis is a multilayered epithelium that protects from environmental assault and damage. The hallmark of the epidermis is its ability to self-renew throughout the entire life span of the organism. This is achieved with epidermal progenitor cells (EPCs) in the basal cell layer of the epidermis, which undergo an unlimited number of symmetric and asymmetric cell divisions, giving rise to daughter cells that either proliferate or exit the cell cycle and move to the suprabasal layer of the epidermis. There they undergo terminal differentiation and are eventually shed from the skin surface [2]. Basal keratinocytes of the murine skin normally express high levels of α2β1, α3β1 and α6β4 integrins and αvβ5 that is weakly expressed. Expression of α3β1, αvβ6 and α9β1 integrins is induced upon wounding or in pathological conditions [3]. Tissue specific or constitutive deletion of these integrin chains revealed their crucial roles in skin development and homeostasis with the most striking phenotypes observed upon deletion of the β1 integrins and the hemidesmosomal α6β4 integrins [4–7].

Mutant mice lacking β1 integrin in skin had multiple blisters due to impaired attachment of basal keratinocytes to the basement membrane (BM), a disorganized and hyperthickened interfollicular epidermis (IFE), impaired proliferation of interfollicular and hair matrix keratinocytes, delayed terminal differentiation in IFE, defects in the hair follicle morphology, progressive hair loss and dermal fibrosis [6]. Moreover, β1-deficient mice showed severely delayed wound healing, which has been associated with impaired migration of β1 integrin-deficient keratinocytes [8]. Despite the severe skin defects and complete hair loss, some of the β1 skin specific conditional knockout mice survived up to 12 months suggesting that loss of β1 integrin is not associated with a stem cell or progenitor cell depletion phenotype. This finding was unexpected, as previous data pointed to an important role for β1 integrins for maintaining skin stem cells [9–11]. It has been shown that keratinocytes with high β1 integrin levels display typical stem cell properties including high colony forming efficiency in vitro. Similar results were also obtained with cells
from adult human palm, sole and breast skin that strongly expressed β1 integrins [12]. Furthermore, it was shown that keratinocytes expressing high levels of β1 integrin are slow cycling cells in vivo and cluster in the basal layer of the epithelium. Interestingly, integrin mediated adhesion to the ECM was shown to negatively regulate terminal differentiation of cultured keratinocytes in vitro, while ectopic expression of β1 integrins in suprabasal cells leads to epidermal hyper-proliferation and perturbed keratinocyte differentiation in vivo [13,14].

In order to clarify the discrepancy between the in vitro and in vivo results on the role of β1 integrin in the maintenance of epidermal stem cells we generated a mouse strain with reduced β1 integrin expression in keratinocytes of the skin. This approach allows to study the function of β1 integrin in skin homeostasis avoiding the gross abnormalities seen in mice with skin specific ablation of β1 integrin. Using this new model we could show that keratinocytes that express normal levels of β1 integrin due to inefficient downregulation of β1 integrin expression are able to quickly expand in the interfollicular epidermis and almost completely replace the mutant cells over time. This rapid expansion of cells ensures nearly normal epidermal homeostasis and strongly indicates that β1 integrins play a very critical role for the proliferation and maintenance of EPC in vivo.

Results

Generation of a β1 integrin hypomorphic allele

To test how reduced expression of β1 integrin affects skin development and maintenance we engineered a β1 hypomorphic mouse strain by diminishing the stability of the β1 integrin mRNA and thus the amount of β1 integrin subunits. The hypomorphic gene mutation was obtained by introducing a 141-bp long wild-type (wt) cDNA fragment of the β1 integrin gene coding for the entire β1 integrin cytoplasmic tail, including the stop codon from exon 16 (called Cyto-cDNA), in frame into exon 15 (see Figure S1A in Supporting Information). In addition, a floxed neo-tk cassette was introduced into the targeting vector (hpmK1pro-neo). The hpmK1pro-neo mutation was transferred into embryonic stem (ES) cells by homologous recombination (see Figure S1B, left panel, in Supporting Information), and the neo-tk selection cassette was subsequently removed by transient expression of Cre recombinase leaving one loxP site in the non-coding region (hpmK1neo). The hpmK1pro/+ ES cells were used to generate germline chimeric mice (see Figure S1B, right panel, in Supporting Information). Successful germline transmission was confirmed by genomic PCR (see Figure S1C in Supporting Information) and by sequence analysis (not shown).

The stop codon in exon 15 of the hpmK1pro allele should give rise to a transcript with a premature translation termination signal 200 nucleotides upstream of the exon-junction site generated upon splicing of exons 15 with exon 16. Consequently, this additional nucleotides upstream of the exon-junction site generated upon splicing of exons 15 with exon 16. Consequently, this additional stop codon located 5' relative to the last exon of the β1 integrin gene should significantly downregulate the hpmK1pro mRNA by the nonsense mediated decay (NMD) pathway [15]. To test whether the hpmK1pro mRNA was indeed subject to NMD, we first analyzed the level of hpmK1pro mRNA in primary hpmK1pro/+ keratinocytes treated with the translation inhibitor emetine to abrogate the NMD pathway [16,17]. As expected, incubation of subconfluent hpmK1pro/+ keratinocytes with emetine revealed that the hpmK1pro mRNA levels steadily increased with time of treatment suggesting that the hpmK1pro mRNA is short-lived and can be stabilized by emetine (Figure 1A). To test whether the emetine-mediated β1 mRNA up-regulation is caused by inhibition of NMD or by an emetine triggered stress response [18,19] primary hpmK1pro/+ keratinocytes were treated with the transcriptional inhibitor actinomycin D either in the presence or absence of emetine. Actinomycin D treatment influenced neither the levels of non-stabilized hpmK1pro mRNA nor the levels of emetine-stabilized hpmK1pro mRNA (Figure 1B) indicating that the increase of emetine-triggered hpmK1pro mRNA is caused by the inhibition of the NMD pathway.

Reduction of β1 integrin levels on keratinocytes results in an attenuated β1 integrin null-like phenotype

Mice heterozygous for the hpmK1pro mutation (hpmK1pro+/+) were normal. Heterozygous intercroses revealed that homozygous (hpmK1pro+/hpmK1pro) mice die at around E6.5 (embryonic day 6–6.5) (Table 1), while mice with a complete ablation of the β1 integrin gene showed an empty implantation chamber at this developmental stage, suggesting that homozygous hpmK1pro+/hpmK1pro mice survive slightly longer [20,21]. These data indicate that reduced β1 integrin levels allow the embryonic development to proceed further than in the complete absence of β1 integrin expression.

To test whether the reduced expression of β1 mRNA from the hpmK1pro allele is affecting skin homeostasis, we generated mice solely expressing the hpmK1pro allele in keratinocytes. To this end, we intercrossed the hpmK1pro/+ and floxed β1 integrin strains with transgenic mice expressing the Cre recombinase under the Keratin 5 (K5) promoter [6,22] to generate hpmK1pro+/B1; K5Cre (hpm) mice. The hpm mice lose the β1 floxed allele in Cre expressing cells and thus, express only the hpmK1pro allele in keratinocytes of the IFE and the outer root sheath (ORS) of hair follicles (HFs). To determine the expression of integrins in 3-week-old hpm keratinocytes we measured β1 mRNA levels with quantitative Real-Time (RT) PCR and integrin surface levels with flow cytometry. The β1 mRNA levels were reduced by 93±2% in the hpm mice (Figure 1C), and the cell surface levels of β1 and α2 integrins were reduced by 85.9±3% and 65.1±19%, respectively, compared to control cells (Figure 1D and see Figure S2 in Supporting Information). The expression of the hemidesmosomal integrins α6 and β4 was slightly increased (see Figure S2 in Supporting Information). These data demonstrate that subjecting β1 integrin mRNA to NMD leads to severely reduced β1 integrin levels.

Next we investigated whether ~15% of the normal β1 integrin levels are sufficient to prevent the development of skin abnormalities, which were observed in mice lacking the β1 integrin gene in keratinocytes (K5- β1[6]). In agreement with our previous report, K5-β1 mice had a reduced number of hairs at 2 weeks of age and almost no hair at the age of 4–5 weeks. In contrast, hpm mice had only a slightly thinner fur at the age of 2 weeks and lost their hair coat between 6 and 12 months of age (Figure 1E, and data not shown). Also in sharp contrast to K5-β1 mice, hpm mice never developed wounds, moved unrestrained and were fertile.

Histology revealed that 14-day-old hpm back skin had a normal number of HFs. However, the morphology of hpm HFs was abnormal. About 60% of the hpm HFs were misshapen and arrested in morphogenesis resembling the K5-β1 phenotype, while about 40% reached down to the muscle layer and displayed a severely abnormal and multilayered ORS (Figure 2A). The hpm IFE contained 2–3 layers of nucleated cells (epidermal hyperplasia), occasional small microblisters at the dermal-epidermal junction (Figure 2B, arrows) and laminin-332 (LM-332) deposits reaching into the dermis (Figure 2E). Similar, but more severe defects were observed in K5-β1 skin (Figure 2A,B,E).

To assess whether the reduced β1 integrin levels on hpm keratinocytes are sufficient for in vitro functions we isolated keratinocytes from 2.5-month-old wt and hpm mice and seeded...
them on a surface coated with a mixture of collagen I (Col1) and fibronectin [23]. While the majority of adherent wt cells began spreading one day after plating and grew to confluency 4 days later, hpm cells adhered but failed to spread and proliferate (Figure 3A). To determine the proliferation rates of wt and hpm keratinocytes we performed an ELISA-based BrdU incorporation assay and could not find proliferating hpm cells on Col1/FN surfaces (Figure 3B). To quantify adhesion, we plated wt and hpm keratinocytes on a mixture of Col1/FN or LM-332, respectively, and observed similar adhesion efficiencies in both genotypes (Figure 3C). Next we compared focal contact organization and F-actin distribution between wt and hpm cells after a culture period of 2 days on a Col1/FN mixture. Immunostaining for β1 integrin, vinculin and paxillin, and visualization of F-actin with fluoresceinly labeled phalloidin revealed a complete absence of FA structures and a failure to organize F-actin into stress fibres in hpm keratinocytes (Figure 3D). The immunosignals of FA proteins were observed in the cytoplasm and F-actin appeared as clumps or fine filaments extending from the perinuclear region. Altogether these findings indicate that, 15% of the normal β1 integrin level is not sufficient to support normal epidermal and HF homeostasis in vivo and integrin functions in vitro.

The defects of epidermal hpm keratinocytes ameliorate with age

In agreement with the external appearance (Figure 1E), histology of skin sections from 6.5 months old hpm mice revealed reduced and misshapen HF s, a mild dermal fibrosis, and dermal melanin deposits from perished HF s (Figure 2C). Surprisingly, we observed that while the hyperplasia of the IFE remained similar, the epidermal-dermal blisters almost completely disappeared (Figure 2D). Similarly, the LM-332 deposition appeared either normal or extended to a much lesser extent into the dermis (Figure 2F). Interestingly, the blistering and BM abnormalities ameliorated also in the skin of older K5-β1 mice (Figure 2C,D,F).

Table 1. Genotypes of progeny from heterozygous intercrosses.

| Stage | Total | hpmKIlox/hpmKIlox | hpmKIlox/+ | +/+ |
|-------|-------|-------------------|------------|-----|
| E6.5  | 55    | 13*               | 23.6       | 31  |
|       |       | 56.4              | 11         | 20  |

*a all embryos were extensively degenerated at this developmental stage

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Figure 2. Epidermal and HF phenotype in hpm mice. (A, B) Haematoxylin-eosin stained sections from the back skin of 14-day-old control, hpm and K5-β1 mice. (A) hpm mice displayed stunted HF morphogenesis leading to two HF types (fully developed with multilayered ORS; shortened, malformed and prematurely arrested in morphogenesis), while in K5-β1 mice all HFs were severely distorted. (B) Control epidermis consists of a monolayer of cuboidal cells firmly attached to the dermis, while hpm epidermis consisted of 2–3 keratinocyte layers with few microblisters at the BM zone (arrows). The K5-β1 epidermis consisted of 2–7 layers of roundish, polygonal or flattened keratinocytes that were frequently detached from the dermis forming large blisters (arrows). (C, D) Haematoxylin-eosin stained sections from the back skin of 6.5-month-old control and hpm and of 5-week-old K5-β1 mice. (C) Progressive hair loss in hpm epidermis is accompanied by the development of dermal fibrosis with scattered melanin deposits (arrowheads). The remaining HFs are severely malformed. In K5-β1 mice the dermal fibrosis developed already at 5 weeks of age and was accompanied by an almost complete loss of HFs and large melanin deposition in the dermis (arrowheads). (D) The epidermis of the back skin of 6.5-month-old hpm mice is hyperthickened but has almost no microblisters. The epidermis of 5-week-old K5-β1 mice is severely hyperthickened and shows reduced blistering when compared to 2-week-old skin. (E, F) Immunostaining for LM-332 of back skin sections. (E) At 14 days of age LM-332
When we performed adhesion and spreading assays with freshly isolated keratinocytes isolated from 6.5–7.5 months old animals, we observed that both wt and hpm keratinocytes were adhering, spreading and proliferating with an apparently comparable kinetics (Figure 3E). The similar proliferation rates were confirmed with BrdU incorporation assays (Figure 3F). Furthermore, the hpm keratinocytes formed normal FAs containing β1 integrins, vinculin and paxillin and had a normal F-actin network (Figure 3G).

These findings indicate that the epidermal defects caused by the hpm allele ameliorate with age.

Figure 3. Reduced spreading defect of keratinocytes from old hpm mice. (A) Freshly isolated keratinocytes from 2.5-month-old mice seeded on a mixture of Col1 and FN. In contrast to control cells, hpm cells neither spread nor grow ex vivo. Time points after seeding are indicated. (B) Proliferation rate of keratinocytes from 3-week-old mice cultured for 2 days on Col1/FN and pulse-labelled with BrdU for 10 or 24 hours. Compared to control cells hpm keratinocytes have significantly reduced proliferation rate. Bars represent data from keratinocytes of 3 mice per genotype. Error bars indicate s.d. (C) Cell adhesion of hpm keratinocytes from 2.5-month-old mice plated on Col1/FN or on LM-332 is similar to control cells. 5 and 4 independent experiments were performed on Col1/FN or LM-332, respectively. Error bars indicate s.d. (D) Keratinocytes from 2.5-month-old control and hpm mice were cultured for 2 days on Col1/FN and immunostained for β1 integrin, paxillin and vinculin (red), and F-actin (phalloidin; green). Nuclei were stained with DAPI (blue). hpm cells failed to develop focal adhesions and to organize the actin cytoskeleton into stress fibers. (E) Freshly isolated keratinocytes from 7.5-month-old mice plated on Col1/FN. Both control and hpm keratinocytes were well spread and showed a similar proliferation rate. (F) Control and mutant keratinocytes isolated from 6.5-month-old mice plated on Col1/FN and cultured for 2 days were pulse-labelled with BrdU and harvested at times indicated. No significant difference in BrdU incorporation was observed between control and hpm keratinocytes. Bars represent data from at least 3 mice per genotype. Error bars indicate s.d. (G) Keratinocytes isolated from 7.5-month-old control and hpm mice were cultured for 2 days on Col1/FN and immunostained for β1 integrin, paxillin and vinculin (red) and F-actin (phalloidin; green). Nuclei were stained with DAPI (blue). Size and number of FAs and actin stress fibers is similar in hpm and control keratinocytes.

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β1 integrin levels increase on hpm keratinocytes with age and upon skin wounding

We hypothesized that the amelioration of in vivo and in vitro defects in keratinocytes from aging hpm and K5-β1 mice, respectively, could be due to the expansion of keratinocytes that escaped NMD or Cre-mediated deletion of the floxed allele and had elevated β1 integrin levels. To test whether hpm keratinocytes can indeed increase their integrin levels we immunostained sections from the back skin of hpm mice. The immunostaining revealed that even though β1 integrin signals were not detectable in 14-day-old hpm skin, small cell nests with strong β1 integrin expression became apparent in the basal keratinocyte layers of 2.5-month-old hpm skin (Figure 4). Interestingly, the number of cells expressing high levels of β1 integrin increased further in 6.5 months old hpm epidermis (Figure 4).

To determine whether the rise of β1 integrin expressing cells was due to an incomplete loss of β1 floxed alleles, we used adjacent skin sections from both wt and hpm mice to compare β1 integrin and lacZ expression, which is activated upon deletion of the β1 integrin floxed allele in control and hpm mice [6]. Sections from control skin revealed that irrespective of the age all keratinocytes expressed β1 integrins and were positive for lacZ (Figure 4 and not shown). The epidermis from 14 days old hpm mice was lacZ-positive and lacked visible β1 integrin expression (Figure 4). In contrast, the epidermis of 2.5- and 6.5-months-old hpm mice contained lacZ-negative regions of variable size (Figure 4). Several lacZ-negative regions were β1 integrin-positive indicating that the epidermis contains stretches of keratinocytes, which have escaped Cre-mediated deletion of the floxed β1 integrin allele (Figure 4). However, we also observed keratinocytes in the hpm epidermis that were both, lacZ- and β1 integrin-positive, suggesting that they failed to downregulate the hpmKIlox mRNA by the NMD pathway (Figure 4, see arrowheads).

To confirm these findings we determined the expression level of the β1 integrin subunit and lacZ by flow cytometry in keratinocytes from 3-week-, 2.5-month-, 6.5-month- and 20-month-old control and hpm mice. As shown in Figure 5A and B, β1 integrin expression gradually increased to normal levels in hpm keratinocytes from 3-week-old to 20-months-old mice. Interestingly, the majority of β1 integrin-positive hpm keratinocytes expressed high levels of lacZ corroborating that NMD of hpmKIlox mRNA was reverted in ageing hpm mice. To further confirm this finding we isolated RNA from epidermal lysates from 3-week-, 2.5-months-, 6.5-7.5-months- and 20-month-old control and hpm mice and compared their hpmKIlox mRNA levels using quantitative RT-PCR. We observed a significant increase in hpmKIlox mRNA between 2.5- and 20-month-old hpm mice relative to littermate controls (Figure 5C). Finally, the increase in hpmKIlox mRNA concomitantly led to an increase in cell surface levels of β1 integrin-associated α2 subunits on hpm keratinocytes (see Figure S2 in Supporting Information). Flow cytometry of primary keratinocytes from 3-week-, 2.5-months-, 6.5-months- and 20-months-old hpm mice revealed that the surface expression of α2 integrin steadily increased and reached control levels in cells from 20-months-old mice (see Figure S2 in Supporting Information, upper panel). The expression of α6 and β4 integrins slightly exceeded...
that seen in control animals, both in young and old hpm mice (see Figure S2 in Supporting Information, lower two panels).

Finally, we tested whether the expansion of $\beta_1^{\text{high}}$ cells is associated with an increased number of proliferating keratinocytes. At 14 days of age the percentage of BrdU-positive cells was similar in control and hpm mice (10 ± 3.2% in hpm and 10 ± 1.9% in control epidermis), while at 6.5 months of age the percentage of BrdU positive cells was significantly higher in hpm skin (3.9 ± 0.7%) than in control skin (1.5 ± 0.7%). Altogether these findings indicate that keratinocytes escaping NMD expand in the epidermis of aged hpm mice due to their increased proliferative potential.

We next determined if these escape mechanisms are also active in healing skin wounds and if the wound-induced hyperproliferation accelerates this process. To test this possibility we generated full-thickness excision wounds in hpm mice and wild-type littermates at the age of 8–12 weeks. Surprisingly, the rate of wound closure as well as the area of hyperproliferative epidermis was not reduced in hpm mice at day 5 after injury (see Figure S3A in Supporting Information). At day 13 after wounding, wounds in mice of both genotypes were fully reepithelialized. The only difference that we observed was an enlarged width of the healed wound and accordingly, an increased area of wound epidermis (see Figure S3B in Supporting Information). This phenotype most likely results from reduced contraction of the wound due to fibrosis in the dermis. However, the rate of reepithelialization was not affected. When sections from 13-day wounds were stained for $\beta$-galactosidase activity, large areas of the wound epidermis above the healing wound were found to be lacZ-negative (see Figure S3C in Supporting Information), demonstrating that these cells escaped Cre-mediated recombination. This finding suggests that the necessity for rapid keratinocyte proliferation upon skin injury accelerates the selection process that we also observed in ageing skin.

$\beta_1$ integrin-positive keratinocytes increase in K5-$\beta_1$ epidermis with age

To test whether the partial rescue of the skin phenotype in 5-week-old K5-$\beta_1$ mice was caused by the expansion of $\beta_1^{\text{high}}$ cells, we analyzed adjacent back skin sections from 5-day-, 3-week- and 5-week-old control and K5-$\beta_1$ mice for integrin and lacZ expression. At the age of 5 days the K5-$\beta_1$ back skin was positive for lacZ and only single cells or very small cell nests in the IFE also expressed $\beta_1$ integrin (Figure 6, and data not shown). In 3-week-old K5-$\beta_1$ mice an increasing number of $\beta_1$ integrin-positive keratinocytes was observed in lacZ-positive areas. By 5 weeks of age large regions of the K5-$\beta_1$ IFE were $\beta_1$ integrin positive. Interestingly, these areas were either lacZ-positive (Figure 6, upper panel) or negative (Figure 6, lower panel) in the adjacent sections. These results suggest that the $\beta_1$ integrin-positive cells can result from inefficient recombination of either one ($\beta_1$ integrin-positive and lacZ-positive) or both ($\beta_1$ integrin-positive and lacZ-negative) $\beta_1$ floxed alleles.
To confirm and quantify the immunohistochemical analysis we performed flow cytometry on freshly isolated keratinocytes from 3-day, 5-day-, 3-week- and 5-week-old control and K5-β1 mice. We could clearly show that the β1 integrin expressing cell population increased with age in K5-β1 mice from 37.3% ± 2.6 at 3 days after birth to 85.8% ± 3.7 at 5 weeks of age (Figure 7A,B). Remarkably, the vast majority of the β1 integrin-positive cells was lacZ-positive suggesting that in most cells only one β1 floxed allele was deleted.

To define why there was inefficient β1 integrin gene deletion, we measured β1 integrin and Cre levels in primary keratinocytes from 5-week-old control (β1fl/+K5Cre) and K5-β1 mice. We found that high β1 integrin expression levels correlated with low Cre levels in the K5-β1 mice (Figure 7C) suggesting that low Cre transgene expression leads to inefficient recombination of the floxed β1 integrin allele, perpetuation of normal β1 integrin expression on basal keratinocytes, their expansion and finally amelioration of the defects.

**Discussion**

β1 integrins are essential for tissue development and maintenance [1,20,24]. They are highly expressed on stem and progenitor cell populations, which orchestrate organogenesis and represent a cellular reservoir to maintain organ homeostasis [25,26]. Experimental evidence suggests that the high expression level is critically important for supporting stem and progenitor cell properties such as adhesion to their niche, cell cycle quiescence and the appropriate orientation of the mitotic spindle to control asymmetric versus symmetric cell division [27–30]. Although the function of β1 integrins for morphogenesis is undisputed, their role in stem and progenitor cell maintenance and expansion is less clear. Cell type-specific deletions of the β1 integrin gene in the skin, mammary gland and haematopoietic system revealed a differential requirement of the β1 integrin subfamily for stem and progenitor function [6,7,30–33]. While deletion of the β1 integrin gene had no apparent impact on resting hematopoietic stem and progenitor cells, deletion of the β1 integrin gene in keratinocytes reduced the proliferation rate of skin progenitor cells and in the mammary gland epithelium severely affected both, the proliferation and asymmetric division of the stem and progenitor cell population [30].

In the present paper we generated a mouse strain expressing reduced β1 integrin levels to directly investigate the role of β1 integrin on skin formation and homeostasis, avoiding gross damages as seen in complete knockout animals. Since the β integrin subunits are usually expressed in excess, the expression of αβ integrin heterodimers on the cell surface is controlled by the available amount of α integrin subunits. This is the reason why mice heterozygous for the deletion of the β1 integrin gene have normal or only slightly reduced levels of αβ integrins on their cells and are thus phenotypically normal. To significantly reduce the expression level of β1 integrins we decided to impair the stability of the β1 integrin mRNA. To this end we fused the coding sequence of the last exon to the second-last exon of the β1 integrin gene. We thereby introduced a premature translation termination codon into the second-last exon, which in turn induced the NMD
pathway and reduced β1 integrin levels on keratinocytes to ~15%. Mice homozygous for the hypomorphic β1 integrin allele die between E6.0–E6.5, indicating that the remaining integrins allow development to proceed slightly further than the complete β1 gene ablation, as such mice die at the late blastocyst stage [20]. We are currently trying to identify the specific defect(s) underlying the lethality of mice homozygous for the hpmKIlox allele.

Skin development and maintenance depend on the presence of epithelial progenitor or stem cells, which retain their proliferative capacity throughout the entire life span of an animal. They have to replenish the interfollicular epidermis and the hair follicles and are therefore crucial to maintain the skin and the hairs. Genetic studies in mice have shown that the ablation of the β1 integrin gene (K5-β1 mice) or the expression of inactive β1 integrins on keratinocytes leads to severe skin and hair coat phenotypes [6,7,34]. The defects include progressive hair loss, malformation and detached epidermis, defective LM-332 deposition at the dermal-epidermal junction, reduced keratinocyte proliferation and dermal fibrosis. The hpm mice with 15% of normal β1 integrin levels develop similar phenotypes, albeit much less severe and delayed. Their IFE epidermis is less thick, blisters are very small and scarce and almost all HF are malformed. Primary keratinocytes isolated from young hpm mice adhered well to ECM substrates but failed to spread, to proliferate and to survive in vitro, suggesting that the reduced β1 integrin levels are sufficient to promote adhesion but insufficient to appropriately activate signalling pathway(s) required for F-actin reorganisation, cell spreading and proliferation.

Despite the skin abnormalities hpm mice had an apparently normal life span. Old hpm mice continued to lose their hair, but microblisters and irregular LM-332 deposition were significantly ameliorated with age. Moreover, primary keratinocytes from old hpm were able to spread and organize actin into stress fibers, to form robust and normal numbers of focal adhesions and to proliferate. The reason for the ‘rescue’ of the mutant phenotype in vivo and in vitro was the very rapid expansion of β1 integrin expressing keratinocytes in hpm skin. Around 11% of the β1 integrin expressing cells escaped Cre-mediated deletion. Similar findings were obtained in our previous study [6]. Furthermore, ~76% of β1 integrin expressing keratinocytes escaped or compensated for NMD-mediated degradation of β1 integrin mRNA. These results indicate that keratinocytes with high β1 integrin levels have an enormous advantage over cells expressing low β1 integrin levels, leading to their rapid expansion in the mutant skin and partial reversion of the epidermal phenotype. Interestingly, this advantage becomes even more pronounced in situations where the requirement for keratinocyte proliferation and migration is particularly high such as during wound healing.

Concomitant with the restoration of the β1 integrin expression in the mutant animals there was also an increase in cell surface expression of its dimerization partner α2 integrin that was strongly reduced in young animals. On contrary the expression of the
hemidesmosomal integrins α6 and β4 remained relatively stable throughout the entire life span of the hpm mice slightly exceeding that of aged-matched control littermates. These data suggest that the α6β4 integrins do not play a major role in the regulation of the proliferative potential of the keratinocytes, which only increases once cells expressing normal levels of β1 integrin repopulate the skin.

The K5-β1 mice carry disrupted β1 integrin genes in keratinocytes, resulting in loss of β1 integrin expression shortly after birth in around 60–70% of basal keratinocytes. Most K5-β1 mice die within the first three weeks after birth. A few mice survive beyond the age of 5 weeks, despite their severe skin abnormalities. Interestingly, the epidermal phenotype of the 5 weeks old K5-b1 mice also improved indicated by a reduced number of blisters at the epidermal-dermal junction and normal deposition of LM-332. This phenotype improvement is also due to expansion of β1 integrin expressing cells; already 5 days after birth the number of β1 integrin expressing cells increased to ~50%, and at the age of 5 weeks to ~86%. The majority of these cells were still lacZ-positive indicating that in the majority of β1 integrin-positive cells only one β1 integrin floxed allele was excised by the K5-Cre transgene. These data indicate that the proliferative potential of β1 integrin expressing keratinocytes is so superior that they overgrow β1 integrin-null keratinocytes in a few weeks leading to a partial rescue of the interfollicular phenotype.

In contrast to the partial rescue of the interfollicular phenotype, the hair follicle phenotype was progressive in the mutant animals, leading to a complete hair loss in 1 year old hpm and 5-week-old K5-β1 mice. This finding suggests that once a hair follicle is malformed and has lost the connection to the dermal papilla, it is no longer able to fully regenerate independently of the presence of β1 integrin re-expressing EPCs in the skin.

Our findings fully support previous in vitro data showing that high expression of β1 integrins on keratinocytes is important for stem or progenitor cell-like properties [9–12]. Their role for stem/progenitor cells was questioned when mice with a deletion of the β1 integrin gene in keratinocytes maintained an epidermis without premature terminal differentiation [6]. The analysis of the β1 hypomorph mice and of surviving β1 integrin deficient mice revealed that β1 integrins are enormously important for the self-renewal capacity of the proliferating cells of the epidermis [35]. The K5-β1 mice surviving beyond 5 weeks [6] benefit from the enormous proliferative potential conferred by β1 integrin-mediated adhesion and signals. Interestingly, ablation of the floxed β1 integrin allele differs in other cell systems such as the hematopoietic organ. Deletion of the β1 integrins has no severe impact on hematopoietic stem cell/progenitor cell proliferation, lineage commitment and lineage expansion. It will require further studies to explain why keratinocytes so critically depend on the function of this integrin subfamily while blood cells apparently do not.

Materials and Methods

Ethics Statement

All animal studies were approved by the Regierung von Oberbayern (Germany) or the veterinary authorities of Zurich (Switzerland).

Generation of mutant mice

Mouse genomic clones used to generate the targeting construct were described earlier [20]. The loxP flanked neomycin resistance - thymidine kinase gene (neo-tk) selection cassette was inserted together with a 141 bp wt cDNA fragment of the β1 integrin gene coding for the entire cytoplasmic tail of the β1 integrin chain including the stop codon, into exon 15, just behind and in frame with the transmembrane coding region. The targeting construct (see Figure S1A in Supporting Information) was electroporated into K1 embryonic stem (ES) cells (129/Sv) and homologous recombinants were identified by Southern blot analysis of BamHI-digested DNA with a 5’ external probe (see Figure S1B in Supporting Information). Positive ES clones were transiently transfected with Cre recombinase to remove the neo-tk cassette, selected in 2’-fluoro-2’-deoxy-1-β-D-arabinofuranosyl-5-iodouracil supplemented growth medium and identified with a 3’ external probe after BamHI digestion (see Figure S1B in Supporting Information). Cells that lacked the selection cassette were injected into blastocysts to generate germline chimeras. Male chimeras were intercrossed with C57BL/6 females and offspring carrying the hpmK1lox allele were identified by genotyping PCR that distinguishes between the wt and the hpmK1lox allele (5’-GTCCTACTGTCGGCCAC-3’, 5’-TGTTCTCAGTAATGTCTTGATAAC-3’). The hpmK1lox/+ mice were intercrossed with mice carrying a floxed β1 integrin gene [6] and mice carrying a keratin 5 promoter-driven Cre recombinase transgene [22]. For the experiments performed with this mouse line hpmK1lox/+β1i+/ or β1i+/K5Cre age-matched control animals were used, depending on the requirement of the experiment.

For developmental studies, egg cylinders of heterozygous hpmK1lox/+ crosses were collected, the embryos isolated and subjected to PCR-based genotyping using the primers described above.

Isolation of primary keratinocytes, adhesion and proliferation assays

Primary keratinocytes were isolated from mice at indicated ages as described in [36] and either directly applied in a functional assay or seeded on a mixture of 30 μg/ml Vitrogen (bovine collagen type I; Cohesion) and 10 μg/ml fibronectin (In Vitrogen)-coated plastic in keratinocyte growth medium containing 8% chelated FCS (Gibco) and 45 μM Ca2+.

Cell adhesion of freshly isolated keratinocytes to 5 μg/ml LM-332 (kindly provided by Dr. Monique Aumailley, Cologne University, Germany) or a mixture of 10 μg/ml FN and 30 μg/ml Coll was assayed as described previously [36], except that 1×104 keratinocytes were seeded per well and that cells were incubated for 2 hours on the depicted ECM substrata, before the assay was developed. Proliferation of primary keratinocytes seeded on a mixture of 10 μg/ml FN and 30 μg/ml Coll was determined on the second day after plating using a Cell Proliferation ELISA Kit (Roche), according to the manufacturer’s protocol. All experiments have been performed in triplicates.

NMD analysis

The NMD analysis was performed using a modified protocol described by [19]. Subconfluent keratinocytes isolated from adult hpmK1lox/+ mice were treated with 100 μg/ml of emetine dihydrochloride hydrate (emetine, Fluka) or 5 μg/ml actinomycin D (Sigma Aldrich) for up to 6 and 3 hours, respectively, or pretreated for 2 hours with 100 μg/ml emetine and then incubated for up to 3 hours with 5 μg/ml actinomycin D. Cell pellets were lysed using the TRIZOL Reagent (Invitrogen).

RNA isolation and Real-Time PCR

Total RNA was extracted from freshly isolated or cultured keratinocytes using TRIZOL Reagent (Invitrogen) and 1 μg of the total RNA was reverse-transcribed using the iScript Synthesis Kit (BioRad), according to the manufacturer’s protocol. The single
strand cDNA was used as a template for the Real-Time PCR reaction using 2 sets of primers (forward primer hybridizing to exon 14 of the β1 integrin gene (5'-AGGACATTGAT-GACTGCTGG-3') and a reverse primer hybridizing to the loxP site and a linker region of the hpmKIlox allele (5'-TATGATCG-GAAATTCTCGACG-3') to detect the hpmKIlox mRNA and a forward primer hybridizing to the exon 5 - exon 6 border of the β1 integrin gene (5'-AGACCTTCCGATTCGCTGG-3') and a reverse primer binding within exon 6 of the β1 integrin gene (5'-GCCTGTTGAGCTTCTGCAC-3') to detect total β1 mRNA levels and the iQ SYBR Green Supermix (BioRad). The reaction was performed in triplicates in an iCycler (BioRad) and evaluated using the software provided by the manufacturer. In all experiments GAPDH was used as reference gene. Results shown in Figures 1A, 1B are normalized for untreated cells, while results shown in Figures 1G, 5C are normalized for the control expression levels of the β1 or hpmKIlox mRNA at the respective developmental stage.

**Histology and immunofluorescence**

Haematoxylin-eosin and immunofluorescence staining, β-galactosidase activity detection analysis on paraffin embedded or frozen sections, as well as immunofluorescence on fixed cells were performed as described previously [36]. BrdU staining was carried out according to the manufacturer’s instructions (Roche Diagnostics). The following antibodies were used: β1 integrin (Chemicon); LM-332 [laminin γ2 chain; kindly provided by Dr. Rupert Timpl]; BrdU-POD (Roche Diagnostics); paxillin (Transduction Laboratories); 26 integrin-FITC (BD Pharmingen); vinculin (Sigma Aldrich) and phalloidin-Alexa 488 (Molecular Probes). Fluorescence-conjugated secondary antibodies (goat Anti Rat-Cy3, goat Anti Mouse-Cy3 and donkey Anti Rabbit-Cy3) were purchased from Jackson Immunoresearch. Antibodies were diluted according to the recommendation of the manufacturer.

Images were collected by confocal microscopy (DMIRE2; Leica) using the Leica Confocal Software (version 2.5 Build 1227) with 40× oil objective; or fluorescence microscopy (DMRA2; Leica) using the SimplePCI Software (version 5.1.0.0110) with 63× oil objectives; or bright field microscopy (Axiovert; Zeiss), using the IM50 Software with ×10 or ×40 objectives. All images were collected at RT and processed with Photoshop (Adobe).

**Flow cytometry**

Freshly isolated keratinocytes (0.5–1×10⁶ and 2×10⁶ cells per sample for detection of cell surface antigens and for intracellular FACS, respectively) were stained with following antibodies: FITC-labelled anti-β1, anti-α2 and anti-α6 integrin, anti-β4 integrin (all from BD Pharmingen), biotin-labelled anti-Cre recombinase (Covance) or biotin-labelled anti-mouse-IgG1k isotype control (BioLegend) and where required visualized with a FITC-labelled anti-rat-IgG2a, secondary antibody or Cy3-labelled streptavidin (both BD Pharmingen). The intracellular detection of Cre recombinase was performed using LEUCOPERM™ reagents (AbD Serotec) according to manufacturer’s instructions. Stained cells were subjected to fluorescence-activated cell sorting (FACS)-analysis as described previously [36]. To simultaneously analyze lacZ activity and β1 integrin expression in freshly isolated cells, 2×10⁶ keratinocytes per sample were first stained with a PE-labelled anti-β1 integrin antibody (BD Pharmingen) or a PE-labelled anti-hamster-IgG isotype control (BioLegend) and subsequently incubated with a fluorogenic β-galactosidase substrate fluorescein di-β-D-galactopyranoside (Sigma) as previously described for hematopoietic cells [36]. The staining was performed with cells from the hpm mice and age-matched hmpKIlox/+ or β1Δ/+ and β1Δ/K5Cre mice. Cells positive for lacZ activity and β1 integrin were gated according to the staining in keratinocytes from hmpKIlox/+ or β1Δ/+ mice not expressing the lacZ gene.

**Preparation of wound tissue and histomorphometry**

Mice (6–12 weeks old) were anesthetized by intraperitoneal injection of ketamine (75 mg/kg)/xylazine (5 mg/kg). Four full-thickness excisional wounds of 5 mm diameter were generated on the back of mice by excising the skin and the rodent-specific subcutaneous muscle pandanus camous as described previously [37]. Wounds were left uncovered, harvested at different time points after injury and embedded according to standard procedures.

For morphometrical analysis, 7 μm sections from the middle of the PFA-fixed wounds were stained with haematoxylin-eosin and photographed using a Zeiss Axioskop microscope equipped with a HRc camera (Zeiss, Jena, Germany). The area of the hyperproliferative wound epithelium, the wound width, and the percentage of wound closure were determined using the Openlab 3.1.5 software (Improvision Ltd., Basel, Switzerland). Statistical analysis was performed using the unpaired t-test (given the variances were normally distributed) included in the GraphPad Prism4 software package (GraphPad Software Inc., San Diego, CA).

**Supporting Information**

**Figure S1** Targeting strategy for the hpm allele. (A) Partial map of the β1A wild-type (wt) hpmKIlox allele (hpmKIlox+/-) and after Cre-mediated deletion of the floxed neo-tk cassette (hpmKlox). External probes used for Southern blotting after BamHI digest and genotyping PCR primers are indicated. Cyto-cDNA, a wt 141 bp cDNA fragment coding for the complete cytoplasmic domain of the β1A integrin gene including the endogenous stop codon; E15cyto, endogenous sequence of exon 15 encoding for the membrane proximal part of the β1 integrin cytoplasmic tail; filled boxes, exons; D, exon D; neo, neomycin resistance gene; tk, thymidine kinase gene; triangles, loxP sites; pA, polyadenylation signal. (B) Left panel: Southern blot analysis with the 5'external probe demonstrating recombination of the targeting construct (hpmneo-tk) into the wt β1 integrin locus giving rise to a 22.9 kb wt band and a 7 kb recombinant band. Right panel: Southern blot analysis with the 5'external probe demonstrating the deletion of the neo-tk cassette from the knockin hpmKIneo-tk- allele (hpmKlox) with a 22.9 kb wt band and 16 kb hpmKlox band. (C) Representative PCR on genomic DNA from tail snips of offspring from hpmKIlox/+; K5Cre mice. Cells positive for lacZ activity and β1 integrin were gated according to the staining in keratinocytes from hmpKIlox/+ or β1Δ/+ mice not expressing the lacZ gene.

**Figure S2** Integrin profile of hpm keratinocytes. Cell surface expression of integrins in freshly isolated keratinocytes from control and hpm mice at indicated ages were assessed by flow cytometry. Keratinocytes were stained with antibodies against α2, β2 and α6 integrins. Expression of integrin subunits was normalized to the expression level of age-matched controls. Cell surface expression of α2 integrin is significantly reduced in 3-week-old hpm mice and increases to control levels in 20-month-old hpm mice. Expression of α6 and β4 integrin in young and old hpm mutant mice is increased compared to controls (middle and lower
panel, respectively). Mean fluorescence intensities were corrected for background fluorescence. Error bars indicate s.d. At least 2 control and 3 hpm mutant mice per developmental stage were analysed.

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**Figure S3** Wound healing in hpm mice. (A) Morphometrical analysis of wound healing parameters of 5 day wounds. Slightly reduced wound closure in hpm mice (control: n = 19, N = 7; hpm: n = 21, N = 7). The area of the hyperproliferative epithelium (HE) was similar in 5 day wounds of control and hpm mice (control: n = 14, N = 6; hpm: n = 11, N = 6). (B) Morphometrical analysis of wound healing parameters 13 days after wounding. The wound width was significantly increased (p = 0.0005; control: n = 16, N = 3; hpm: n = 16, N = 5) and the epidermis still hyperthickened in 13 day wounds of hpm mice (p = 0.0013; control: n = 19, N = 7; hpm: n = 21, N = 7). (C) 13 day wounds were examined for lacZ expression. Black arrowheads indicate the edges of the wound, white arrowheads mark an area of lacZ negative keratinocytes in the middle of the wound epithelium (control: N = 3; hpm: N = 4). Boxed area is shown at higher magnification. n, number of measurements; N, number of mice.

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**Author Contributions**

Conceived and designed the experiments: APC SW CB RF. Performed the experiments: APC HK HM MB CB. Analyzed the data: APC HK HM MB CB RF. Wrote the paper: APC SW RF.

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